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Señalización química y defensa en las algas pardas en interacción con los herbívoros

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General introduction

I. Chemical communication in plant/herbivore interactions

1. Basic concepts of Chemical Ecology

a. Chemical ecology: definition and main questions

Odors are integral part of our everyday lives. With the senses of view and hearing, olfaction gives lots of information on our surroundings, whether for detection of food sources, our close relatives or even for finding mates and reproducing. To fulfill these vital functions, each living being constantly interact with its abiotic environment like temperature, humidity or nutrient availability, and with its biotic environment by perception of the presence of neighboring living organisms. According to its environment, an organism forms a chemosphere around it, composed of variable volatile blends. As constituted by a wide range of chemical compounds with different relative concentrations, each organism possesses its own scent. The chemical compounds composing the chemosphere of an individual can then be perceived by surrounding living beings, thus influencing the behavior of neighboring organisms, resulting either in connections between organisms or, on the contrary, in repulsion. In the end, chemical communications between organisms shape communities, involving microorganisms like bacteria or viruses, as well as macro-organisms such as animals, plants or fungi.

Investigations of the chemical interactions between living organisms and their environment belong to the domain of Chemical Ecology. At the interface between Ecology and Chemistry, Chemical Ecology could be defined as the art of deciphering the language of the Nature. In other words, this research domain aims at understanding the distribution and abundance of organisms, their complex biotic (mutualism and parasitism, prey-predator relationships, community assembly) and abiotic interactions, through mediation by chemical compounds in different spatial and temporal scales (Amsler, 2012; Raguso et al., 2015). The main questions asked in chemical ecology are relative to either chemistry, or ecology, or even a mix between the two (Heil, 2014): what is the nature of the chemical signals involved in the communication? What are their impacts on the interaction with grazers/pollinators/parasites? What is the evolutionary origin of chemical compounds and how does their emission affect the fitness of emitters and receivers? How does it explain the community structure of the organisms? Are volatile compounds co-evolved signals or simply cues that other organisms use to determine the defensive status of a plant? Are chemical signals just the physiological consequence of internal metabolic resistance-related processes? Does the emitter benefit from warning its neighbors? How do plants release and perceive chemical compounds?

b. The chemical message: emission and perception

Basically, communication is defined as an emission of a message by a sender, which can be perceived by and can modify the behavior of one or more receiver(s) (Fig 1). A distinction was proposed to differentiate two types of information: a simple emitted information, named message, and the perceived and interpreted information named signal (Lindström and Kotiaho, 2002; Soler, 2010). Raguso et al. (2015) added that, "by convention, signals are distinguished from cues in that they confer fitness benefits to the source organism (sender) by altering the behavior of the receiver, and are inferred to have evolved in the context of the sender-receiver interaction". Signals can be used in intraspecific (i.e. between organisms of the same species) as well as in interspecific interactions, thus allowing pollinator attraction, herbivore deterrence or warning conspecifics of a danger (Lindström and Kotiaho, 2002).

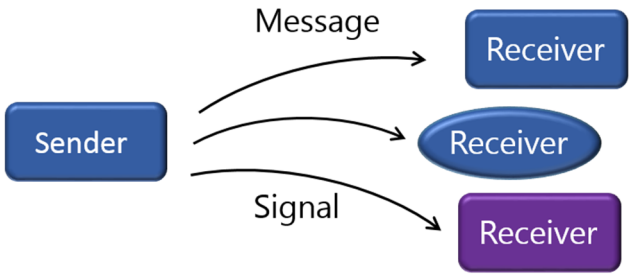


Figure 1. Schematic representation of message transduction between a sender and one or more receiver(s). A simple information can be distinguished from a signal which triggers a modification in the behavior of the receiver. The signal can either be involved in intraspecific or in interspecific interactions.

Living organisms can interact with each other by a wide variety of relationships, involving specific chemical dialogues between partners. For example, species living in symbiosis emit compounds which provide benefits for the other organism, while parasitism, predation or competition involve toxic, repellent or digestion-affecting chemical cues (Raguso et al., 2015). In mutualistic interactions, specific partners are involved and submitted to reciprocal selective pressures, meanwhile avoiding parasites from taking advantages of this chemical communication. This co-adaptation between the sender and the receiver finally shapes the specific interaction. Mutualism thus engages exchanges of chemical compounds specifically deciphered by these two protagonists, but can be detected by any other biological entity. It seems that chemical communications between microorganisms and between them and their environment probably evolved well before terrestrial plants colonized Earth (Mithöfer and Boland, 2016). They likely served first for sensing the environment and finding nutrients and mates. Then, these communication systems may have evolved in plants and in other complex multicellular lineages, from unicellular ancestral Eukaryotes. Thus, for all living organisms, chemical communication is an essential vector of the information, a central and crucial component for interaction between organisms for diverse species, genus or phylum. Among the diverse chemical mediators, Volatile

Organic Compounds (VOCs) are characterized by low molecular weight and low to moderate hydrophilicity (Fink 2007). VOCs produced by marine and land plants are released into the atmosphere, where they are extremely reactive, their life-times varying from minutes to hours (Maffei, 2010). In land plants, they comprise a wide range of chemicals, like terpenes (homo-, mono-, di-, sesquiterpenoids), fatty acid derived C6-volatiles, amino acids and their derivatives, phenylpropanoids such as methyl salicylate (MeSA), as well as certain alkanes, alkenes, alcohols, esters, aldehydes, and ketones (Holopainen and Gershenzon, 2010; Maffei, 2010). The composition of the blends varies according to the plant species, the organ, the developmental stage and environmental conditions (Holopainen and Gershenzon, 2010).

c. Roles of chemical communication in plants

This is now well established that land plants can perceive the abiotic and biotic variations of their environment and respond accordingly. Chemical signals are involved in numerous processes of plant life, such as defense against herbivores and pathogens, as well as attraction of pollinators, seed dispersers and other beneficial animals and microorganisms. VOCs also serve as signals in plant–plant communication (Dudareva and Pichersky, 2008) and sometimes as wound sealers (Maffei, 2010). Chemical communication is well known in attracting pollinators in the mutualistic interaction between the fig tree and its specific pollinators. It has been recognized that one *Ficus* species emits species-specific scents that usually attract a single species of pollinator when fig flowers are ready for pollination, the receptive phase (Grison et al., 1999; Proffit and Johnson, 2009). More recently, the link between the attraction of pollinators and modifications of the bouquet of volatiles has been described in the fig tree *Ficus septica* and its specialist pollinator (Conchou et al., 2014; Appendix 1). Indeed, receptive fig flowers emit a specific ratio of chemical compounds, which attracts pollinators to flowers. After pollination, fig flowers were shown to emit a scent different from the receptivity phase, which would indicate to pollinators that flowers are no longer interesting for them. Moreover, two species of pollinators coexist on the same *Ficus* species, and it appears that, for the activity period of each pollinator species, the scent emitted by receptive figs is different. Thus, the two pollinator species could be exposed to different receptive fig odors, shaping the mutualistic interaction between the fig tree and its pollinators (Conchou et al., 2014). Similarly, it was shown that seed dispersion in fig tree was driven by a specific blend of chemical compounds, attracting bats and other dispersal agents during their activity period (Borges et al., 2011). In *Ficus benghalensis*, scents emitted by ripe fruits are different between day and night, allowing seed dispersion by bats at night and by birds during the day. Therefore, mutualistic interactions between a plant species and beneficial animals are mostly shaped by emission of infochemicals, giving indications on its physiologic state.

As they are sessile, land plants have to deploy defenses against abiotic and biotic stresses. Besides physical barriers like waxy cuticle on the leaf surface, cell wall strengthening (Fu and Dong, 2013) and

callose production (Conrath, 2011), plants produce constitutive and/or inducible chemical defenses. For acclimation to changing environmental conditions such as temperature, light, drought or CO2 level, land plants produce chemical compounds, such as calcium, jasmonic acid (JA), and salicylic acid (SA) (Conrath et al., 2006). Against biotic stresses, plants defenses can act locally against the enemy, like the acacia tree which produces higher concentrations of cyanide at the site of grazing (Fu and Dong, 2013). Plants can also set up defenses remotely either systemically in the whole plant or by allelopathy where chemical compounds act on the neighboring plants by preventing them of a danger or by attracting herbivore enemies (Conrath, 2009). For example, the phytohormones ethylene, JA, SA and abscisic acid (ABA) are involved in distance signaling in plant-insect interactions, from roots to shoots (Soler et al., 2013). As abiotic and biotic stresses trigger degradation and oxidation of plasma membrane components, the release of free fatty acids and oxidized derivatives such as oxylipins is likely to be a key component of defense signaling processes (Mithöfer et al., 2004).

2. Perception of grazing stress and direct chemical defenses against herbivory

a. Chemical recognition of biotic stress

Prerequisite to the induction of effective defenses is the plant perception of an external threat by the recognition of attack-derived molecules (Meng and Zhang, 2013). These molecules are grouped under the term of elicitor, which can be defined as a compound inducing defense responses in plants (Conrath et al., 2002). Elicitors can be of exogenous nature, generally termed Microbe-Associated Molecular Patterns (MAMPs), and defined as a set of molecules coming from the microorganism or the herbivore that are recognized by the plant host immune system. Alternatively, endogenous elicitors or Microbe-Induced Molecular Patterns (MIMPs), are derived from host compounds, such as host cell wall degradation products, through the action of the aggressor (Staal and Dixelius, 2007). MIMPs can be (poly)peptides, glycoproteins, lipids, oligosaccharides, whereas MAMPs are represented by lipopolysaccharides (LPS) from Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, eubacterial flagellin, methylated bacterial DNA fragments and fungal cell wall-derived glucans, chitins, mannans, or proteins (Nürnberger et al., 2004). While MAMPs are not part of the host which qualifies them as ‘non-self’, a MIMP may not contribute to pathogen virulence, but is rather a by-product of the herbivore or microbe’s action, constituting ‘self’ molecules (Mackey and McFall, 2006). These ‘self’ molecules can act as alarm signals, indicating a danger to the plant immune system. Their recognition by receptors thus initiates a signal transduction cascade, like calcium flux, activation of mitogen-activated protein kinases (MAPKs), resulting in early defense responses such as the massive and rapid production of reactive oxygen species (ROS) named the oxidative burst, as well as the induction of

ethylene biosynthesis. Furthermore, recognition of MAMPs and MIMPs is a common trait of innate immunity in animals and in plants (Mackey and McFall, 2006).

b. Local and intra-plant systemic defense signaling

Besides constitutive anatomical and morphological traits, plants also respond to herbivores or pathogens by chemical inducible traits (Heil, 2010). These traits correspond to the production of chemical compounds, and their accumulation in plant tissues, generally in the attacked or infected tissues. Other compounds can affect digestibility of plants like proteinase inhibitors, or repellent compounds which can deter feeding by herbivores (Baldwin et al., 2001; Heil, 2010). For instance, jasmonic acid (JA) was shown to mediate direct defense responses against both chewing and sucking insects, as well as against the necrotic pathogens *Botrytis cinerea* and *Alternaria brassicicola* in *Arabidopsis* (Chehab et al., 2008). Glucosinolates produced in *Arabidopsis* and other plant species in response to herbivore attacks were shown to play a repellent role against generalist herbivores (Baldwin et al., 2001).

As herbivores are mobile, plants respond to attacks not only locally but also systemically in the whole plant, i.e. in healthy undamaged organs, limiting the spread of the attacker to the other parts of the plant (Heil and Ton, 2008; Heil and Karban, 2010). The Systemic Acquired Resistance (SAR) was first highlighted in tobacco, where inoculation of lower leaves with the tobacco mosaic virus (TMV) induced a higher resistance to a second infection in upper leaves (Ross, 1961). Then, SAR was described in many plants and was shown to be triggered by salicylic acid (SA) which acts as a signal transporter through the plant (Heil and Ton, 2008; Conrath, 2009). However, it was demonstrated that SA is not crucial for SAR induction (Heil and Ton, 2008). In addition, SA is known to inhibit the hypersensitive response when innate immunity is activated, to trigger temperature elevation in plants, and to inhibit plant growth, chloroplast development, and photosynthesis (Fu and Dong, 2013). Jasmonic acid (JA) is also involved in wounding-induced responses and is required in distal plant parts for a systemic induction of protein inhibitors, which act negatively on plant tissue digestion by herbivores. At the site of damage, linolenic acid from membranes is released and enzymatically transformed until production of jasmonates (see Part 3) (Feussner and Wasternack, 2002). Establishment of SAR requires the induction of some genes, such as those encoding pathogenesis-related proteins (PR-proteins), which have antimicrobial activity and play a dual role together with SA (Conrath et al., 2002; Conrath et al., 2006).

3. Long distance airborne signaling

a. Priming and plant-plant communication

In some studies, a resistance was observed in distal plant parts which lacked direct vascular connection with damaged parts, as quickly and strongly as in plant parts connected by the vascular system (Heil and Ton, 2008). This external within-plant signaling has now been demonstrated in sagebrush, lima bean, poplar and blueberry, where VOCs released from damaged plant tissues induced resistance in distant undamaged organs of the same plant. In this case, distant plant parts can perceive VOCs as warning signals, more rapidly than internal translocated signals, allowing fast establishment of resistance in the whole plant (Heil and Karban, 2010).

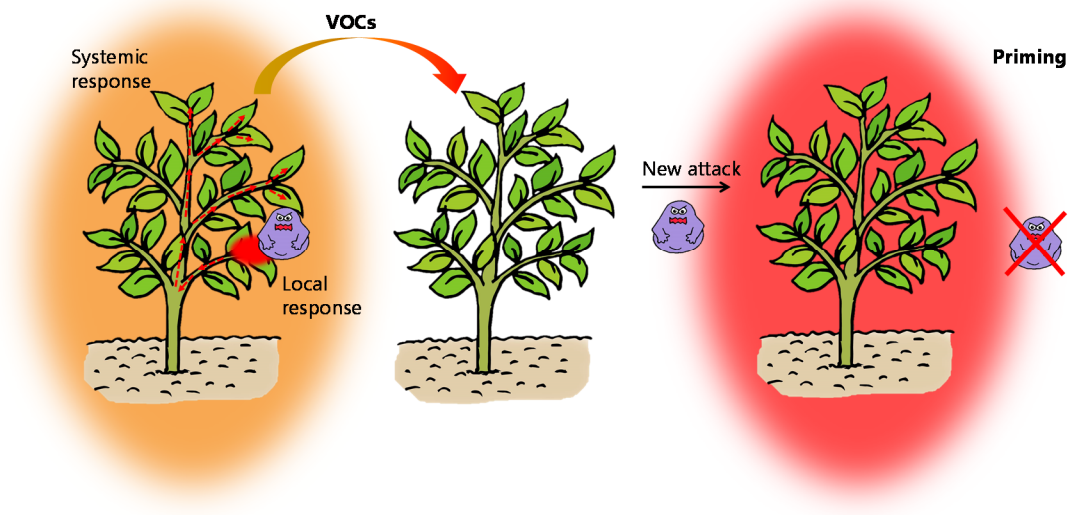


Figure 2. Representation of priming induction. The plant attacked by herbivores releases volatile organic compounds (VOCs) which are perceived by a neighboring plant. Only after a new attack, this second plant is able to defend more efficiently and rapidly to herbivores.

The first evidence of plant-plant communication was proposed by Baldwin and Schultz in 1983. They found that after damage of the poplar *Populus euroamericana* and the sugar maple *Acer saccharum*, damaged plants contained higher amounts of phenolic compounds that undamaged plants. They also demonstrated that their neighboring plants showed similar chemical changes and that these biochemical changes could influence the growth and feeding of herbivores (Baldwin and Schultz, 1983). First performed in laboratory, experiments on plant-plant communication were then made under natural conditions, which provided more evidence of the existence of airborne chemical cues and their impact on the resistance of the receiver organism (Beckers and Conrath, 2007; Heil and Karban, 2010). However, most of these studies were conducted in an ecological context and focused on anti-herbivore defense through plant communication, thus less concerning the volatile-mediated signaling and the perception of the signals by neighboring plants. Studies on plant-plant communication allowed to highlight a defense process in plants, known as ‘priming’ (Fig 2). The VOC-receiver primed plant does not undergo immediate and important phenotypic modifications, but develops a faster and stronger response upon a new herbivore attack (Heil and Karban, 2010). However, it appears that priming does

not induce resistance without confirmation of damage by SAR signaling (Heil and Ton, 2008) and requires modification of chromatin to establish histone memory and information storage for future plant stress responses (Jaskiewicz et al., 2011).

Different chemical volatile compounds were shown to be involved in plant-plant communication upon insect feeding, such as methyl jasmonate (MeJA), methyl salicylate (MeSA), ethylene and green leaf volatiles (GLV), which are C6 volatiles (Fig 3). Each of these compounds acts differently within a few minutes on plant physiology through gene activation and induces the production of various chemical compounds (Heil and Ton, 2008; Baldwin, 2010). After exposure of lima bean *Phaseolus lunatus* to the aldehyde nonanal for 6 h, its resistance to the bacterial pathogen *Pseudomonas suringae* was increased, whereas low concentrations of MeSA for 6 h did not reduce bacterial infections (Girón-Calva et al., 2012). Similarly, β-aminobutyric acid (BABA) emitted during plant stress was shown to induce resistance against a pathogen, in laboratory as well as in field experiments (Beckers and Conrath, 2007). For example, the application of this compound induces resistance in grape against the oomycete *Plasmopora viticola* and suppresses disease symptoms due to *Phytophthora infestans* in potato and tomato plants (Beckers and Conrath, 2007; Ton and Jakab, 2007). Moreover, some signal compounds produced after herbivore attack also induce plant protection against pathogens. For example, methyl jasmonate (MeJA) suppresses the growth of several plant pathogens and limonene stops the growth of the fungus *Fusarium verticillioides* (Heil, 2014). Jasmonate as well as other oxylipins such as epoxy-, hydroxyl-, and divinyl ether derivatives, which are derived from polyunsaturated fatty acids (PUFAs) hydroperoxidation, have also been described to be associated with plant defense responses against pathogens and herbivores (Feussner and Wasternack, 2002; Mithöfer et al., 2004).

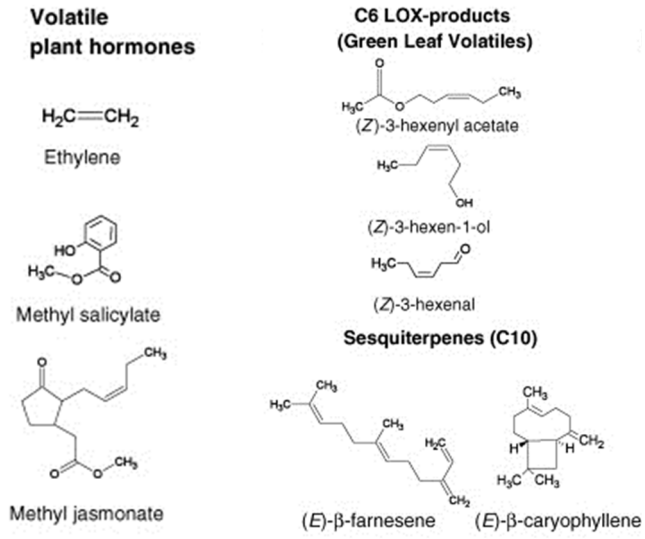


Figure 3. Examples of plant volatiles involved in distance signaling in response to herbivores. Phytohormones are represented by ethylene, methyl salicylate (MeSA) and methyl jasmonate (MeJA). Among C6-LOX products, Green Leaf Volatiles (GLV) are part of the Volatile Organic Compounds, together with sesquiterpenes for instance.

GLVs comprise C6 volatile compounds such as aldehydes, alcohols and their esters, and are produced enzymatically by the LOX pathway (Arimura et al., 2009). They usually come from disruption of galactolipids composing cell membranes, providing free fatty acids such as linolenic and linoleic acids (Matsui, 2006). These fatty acids are transformed by a lipoxygenase (LOX) into hydroperoxides, either on position 13 or 9 of the fatty acid (Fig 4). When the hydroperoxide 13(S)-hydroperoxy-(Z,E,Z)-9,11,15-octadecatrienoic acid (13-HPOT) is formed from linolenic acid, it can then be metabolized by a 13-hydroperoxide lyase (13-HPL) to the aldehyde (Z)-3-hexenal and the oxo acid 12-oxo-(Z)-9-dodecenoic acid. From linoleic acid, the alkane n-hexanal is formed. Then, C6-alcohols can be formed by the action of an alcohol dehydrogenase and an acyltransferase allows the formation of (Z)-3-hexenyl acetate from (Z)-3-hexen-1-ol and acetyl CoA. When the 9-hydroperoxides are produced by 9-LOX, 9/13-HPL catalyze the formation of C9-aldehydes and C9-oxo acids (Matsui, 2006). It has been shown that the aldehyde (Z)-3-hexenal was emitted in the vicinity of wounded sites of *Arabidopsis* immediately after injury, followed by (Z)-3-hexen-1-ol and hexenyl acetate in lower amounts (Arimura et al., 2009). Direct effects of GLVs were observed on microorganisms and it seems that their bactericidal activity is due to their chemical structure: the α,β-unsaturated carbonyl moiety is highly reactive with nucleophiles through Michael-type addition reactions (Matsui, 2006).

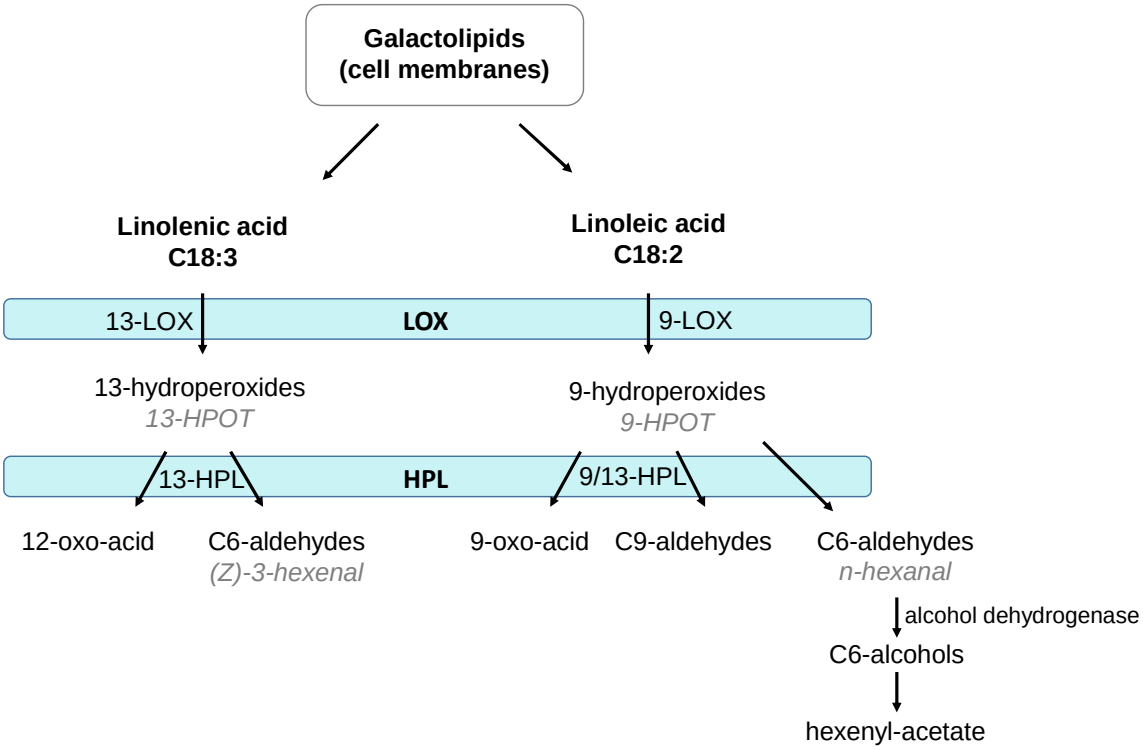


Figure 4. The LOX pathway in plants producing Green Leaf Volatiles. From galactolipids provided by cell membranes, linoleic (C18:2) and linolenic (C18:3) acids give rise to hydroperoxides and then aldehydes through enzymatic transformations. Then, alcohols and hexenyl acetate are derived from aldehydes by the action of an alcohol dehydrogenase and an acyltransferase, respectively. LOX: lipoxygenase; HPL: hydroperoxyde lyase; HPOT: hydroperoxyoctadecatrienoic acid.

A priming effect was also demonstrated in bean plants, when application of nonpathogenic *Pseudomonas putida* reduced disease symptoms induced by pathogenic *Botrytis cinerea*, after higher production of GLVs. Their protective effects were also shown against plant fungal pathogens in *Arabidopsis* and in *Citrus*, and against herbivores in corn through activation of priming (Engelberth and Alborn, 2004). Prior exposure of corn to these compounds induce production of JA and VOCs after elicitation, leading to inducible direct and indirect defense responses. Moreover, it was shown that GLVs emitted by beetle-damaged lima bean plants reduced herbivore damage and increased the growth rate of neighboring uninfested conspecifics (Heil and Silva Bueno, 2007). GLV emitted by lima bean also induce pathogenesis-related protein transcriptional responses, involved in the onset of SAR but they are no longer considered as essential in plant resistance (Baldwin et al., 2002; Conrath et al., 2006; Conrath, 2009). Thus, GLVs can be carried out by air from a plant to another one, even at low concentrations, and can promote defense resistance in receiving plants, acting as priming signals, or inducing SAR-related defense mechanisms when they act as exogenous elicitors (Matsui, 2006).

b. Green leaf volatiles and multitrophic interactions

In natural environment, GLVs are also perceived by other insects in the surroundings of the emitting plant, leading to multitrophic interactions. The VOCs emitted by plants under herbivore attack can have a protective effect as they may provide information representing a 'cry for help' for the plant (Heil, 2008). Indeed, natural enemies of herbivores like carnivorous insects or parasitic wasps, are effectively attracted by these VOCs, thus serving as an additional means of plant resistance. The first study which demonstrated that herbivore-induced volatiles attract herbivore enemies in natural communities was performed by Kessler and Baldwin in 2001. They showed that, upon herbivore attack, *Nicotiana attenuata* released a complex blend which attracted herbivore predators. Among them, cis-3-hexen-1-ol, linalool, and cis-α-bergamotene increased egg predation rates and the complex blend could decrease the oviposition rates (Kessler and Baldwin, 2001). Later, it was found that previous exposure of lima bean plants to VOCs before mechanical damage had a priming effect, leading to secretion of extrafloral nectar (EFN) which plays a role in herbivore predators' attraction (Heil and Kost, 2006; Kost and Heil, 2006). The chemical signature of emitted GLV differs according to the specific interaction between the herbivore and its host. Carnivores can thus distinguish damaged from undamaged plants, as well as different herbivores attacking a plant, according to the blend of compounds emitted by the damaged plant. Moreover, it seems that the type of feeding damage affects the types of VOCs produced. Hence, leaf chewers generally induce jasmonic acid (JA) signaling, while piercing-sucking herbivores (phloem feeders and single-cell feeders) induce resistance via salicylic acid (SA) pathway (Heil, 2008). These precise metabolic regulations allow the plant to finely adjust its defense mechanisms according to the needs, which is also an advantage in terms of energy costs.

c. Costs and benefits of distance airborne signaling

As plant herbivores can easily move from one leaf to the neighboring one, a physiological obvious advantage of airborne signaling compared to SAR is the independency from the plant's vascular system. A fast signaling is crucial for defense against herbivores, within the same plant or among neighboring conspecifics. Nevertheless, both distance signaling, SAR and airborne communication, act synergistically against enemies to achieve on optimized orchestration of the defense response (Heil and Ton, 2008). Furthermore, responding to volatile cues has been shown to increase components of receiver fitness by a higher resistance to pathogens and herbivores (Heil and Karban, 2010). However, receiving a chemical message could be seen as 'eavesdropping' rather than a true communication, and emission of volatile compounds could induce metabolic costs through their production and emission processes. Thus, in this case, neighboring plants fitness is increased without enhancing fitness of the emitting plant and this could lead to an evolutionary disadvantage of the emitter. Evolutionary theory predicts that fitness costs can be compensated by fitness gains of neighbors if these are actually close relatives. In this case, fitness of a genotype providing benefits to its sibs and other close relatives include the fitness of these relatives as they all share a large number of alleles throughout the genome, alleles that are favored thanks to the costly action of the focal genotype (Maynard-Smith, 1964). This theory has been developed and tested mostly in eusocial animals, but may explain how the release of signals for the benefit of neighbor plants has evolved besides the evident cost to the emitting plant.

Other costs include the use of these VOCs by herbivores or parasites for host localization (Heil and Karban, 2010). More than one species of herbivore enemies can also be attracted by these VOCs, or too many individuals of the same species, leading to competition and thus decreasing the positive effects of the intervention of herbivore enemies. The metabolic balance between growth or reproduction and defense can also be affected, as resources used for defense could not be allocated for primary functions (Conrath, 2009; Unsicker et al., 2009). Nevertheless, after perception of the chemical message, neighboring plants can adjust their physiology accordingly, allowing investment of metabolic resources only when defenses are needed. In a population level viewpoint, helping neighbors is considered to be favored and more stable in populations with limited dispersal, as a high proportion of them comes from the direct offspring of the emitting plant. Thus, warning neighbors could increase the fitness of an individual, through a positive effect on the survival of its offspring (Heil and Karban, 2010). Moreover, the intervention of a third trophic level during multitrophic interactions provides an obvious advantage to the plant which attracts predators of herbivores, but also for the predators which rely on VOCs to locate their feeding source (Heil, 2008).

II. Chemical mediation in the marine environment

1. Marine chemical ecology

a. Algal chemical ecology

Research in marine chemical ecology includes investigations on a wide diversity of marine organisms such as microorganisms (bacteria, viruses, fungi), macroalgae, sponges, cnidarians, crustaceans, molluscs, fish or corals, and covers a large range of interactions that organisms have with their biotic and abiotic environments (Paul et al., 2011; Amsler, 2012). This research domain can be divided into sensory and defensive chemical ecology. Sensory chemical ecology concerns chemical communication between organisms, as well as perception and response to the abiotic environment. Chemical communication is essential in processes of brown alga's sexual reproduction, as pheromones released by female gametes attract male gametes and sometimes stimulate the release of male gametes (Maier and Müller, 1986; Pohnert and Boland, 2002). This was demonstrated in the brown alga *Ectocarpus siliculosus* for which the C11 pheromones ectocarpene and pre-ectocarpene secreted by the female gamete induce a modification in the male gametes movements, then trapped in the female gamete area until they are in contact (Müller, 1967). In *Laminaria digitata*, the C11 pheromone lamoxirene released by the female gametes attracts and also stimulates the release of male gametes (Maier and Müller, 1986). Perception of the abiotic environment was shown to induce responses in spores of brown algae, in particular in several species of kelps like *Laminaria japonica*, whose swimming behavior varies according to nutrients, subsequently used for gametophyte development (Fukuhara et al., 2002). Besides sensory chemical ecology, researchers also investigate defensive processes involved in interactions between organisms such as predation, biofouling, competition and defense against pathogens (Amsler, 2012). Algal defenses can be localized in some parts of the thallus and also diffused throughout and outside the seaweed (Jormalainen and Honkanen, 2008). Several studies have focused on defense against predation in brown macroalgae by means of phlorotannins (Pavia and Toth, 2000; Amsler and Fairhead, 2006), while investigations on green algal defense allowed the identification of, for example, caulerpenyne from *Caulerpa sp*. and dimethylsulfoniopropionate (DMSP) from *Ulva* and related genera, as feeding deterrent against some herbivores (Jung and Pohnert, 2001; Van Alstyne, 2008). Caulerpenyne was also found to be converted in highly reactive aldehydes, potentially having a defensive role against herbivores. Algal defense against pathogens include production of chemical compounds against bacteria, fungi and filamentous endophytes (Amsler, 2012). Reactive oxygen species at sufficient concentrations as well as organic compounds were found to inhibit their growth and contain the infection. Against biofoulers, these chemical compounds have to be also present at the algal surface at biologically effective concentrations (Steinberg and De Nys, 2002). This is the case of the red alga *Delisea pulchra* which produces halogenated furanones at sufficient concentrations on its surface to act

against predators and biofoulers (Dworjanyn et al., 1999). Halogenated furanones compete with and inhibit quorum sensing communication among bacteria, thus preventing from biofilm formation (Harder et al., 2012). Finally, defense against competitors in the marine environment is represented by allelopathy, which has already been described between the red alga *Plocamium hamatum* and corals, causing tissue necrosis in soft corals with the monoterpene chloromertensene, or bleaching by several algal species (Amsler, 2012). Moreover, these effects were correlated with the presence of lipophilic chemical compounds in algal extracts, caused by the physiological stress of the microalgal symbionts.

b. Characteristics of the marine environment

Thus, similarly to land plant chemical ecology, algal chemical ecology covers a large range of biological roles in different types of interactions, involving various and numerous chemical compounds. The marine environment, however, presents some unique characteristics in terms of physical and ecological properties which impact the chemical mediation in the marine medium.

- Evolutionary aspects

Marine algae belong to different independent eukaryotic lineages. Chlorophytes (green algae) and Rhodophytes (red algae) are sister groups in the same lineage leading to land plants, called Plantae or Archeaplastida, while Phaeophytes (brown algae) evolved independently into the Stramenopiles or Heterokonts lineage (Fig 5). Brown algae are evolutionarily close to diatoms, which also belong to the Stramenopiles group. The emergence of these different macroalgal groups is linked to the endosymbiosis origins of their plastids. First, a heterotrophic eukaryotic cell has incorporated a photosynthetic ancestral cyanobacteria, giving rise to the first photosynthetic ancestral Eukaryote, at the origin of Chlorophytes and Rhodophytes. Later, during evolution of Eukaryotes, second endosymbiosis events, corresponding to the endocytosis of an autotrophic unicellular eukaryote by a heterotrophic eukaryote would have occurred several times independently, to give rise to the actual diversity of marine photosynthetic organisms. The Stramenopiles, and thus the Phaeophytes, derived from the secondary endosymbiosis of an ancestral unicellular red alga by a heterotrophic protist. Thus, this evolutionary context in Eukaryotes suggests that some traits of the chemical communication, for instance regarding the biosynthetic pathways or identity of chemical compounds, in brown algae could be shared with other photosynthetic Eukaryotes, while other traits could be specific of the brown algal lineage, like for iodine emission or phlorotannins production.

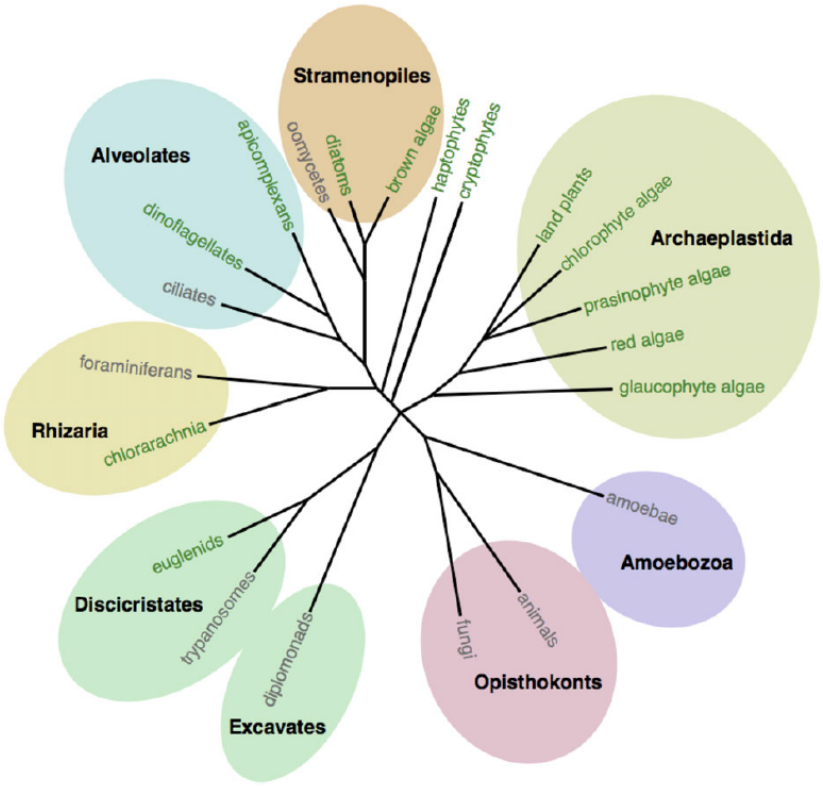


Figure 5. Phylogenetic tree of Eukaryotes and evolution of Brown algae among Eukaryotes.

Photosynthetic organisms such as macroalgae are the unique source of ω-3 and ω-6 essential fatty acids for upper trophic levels, and their fatty acid composition is consistent with these evolutionary processes. Whereas similarities exist between red and brown algae in term of polyunsatured aldehydes (PUAs) composition, the brown algal lineage could have evolved distinct biochemical pathways in fatty acid metabolism, which could possibly have specific defensive effects on their herbivores. Contents in 44 fatty acids were recently investigated in 40 taxa of green, red and brown macroalgae, and it was found that this composition differed significantly according to the phylum, order and family of the taxa (Galloway et al., 2012). The red and brown algae mostly contain the fatty acids arachidonic acid (ARA, C20:4 ω-6) and eicosapentaenoic acid (EPA, C20:5 ω-3), whereas red algae contain more C16:0 and C18:0 than brown algae. However, green algae are mostly composed of linoleic acid (LIN, C18:2 ω-6), linolenic acid (ALA, C18:3 ω-3) and stearidonic acid (SDA, C18:4 ω-3).

- Physical characteristics

The marine environment is defined by physicochemical characteristics which differ from those encountered in the terrestrial environment, mainly because the environment is liquid water, thus modifying some aspects of the chemical communication between organisms. Indeed, the carrier of the information can is mostly seawater and occasionally air, depending on the amplitude of the tide, thus

involving soluble as well as volatile molecules. In addition, these compounds are often composed of halogenated elements such as iodine and bromine (Hay and Fenical, 1988).

- Ecological aspects

Marine ecosystems provide the vast majority of the primary production of the Earth, through the plankton. In coastal environment, macroalgae are the main primary producers. It was demonstrated that the abundance and the growth of the primary producers were strongly affected by grazing by herbivores, even in low rates (Poore et al., 2012; Poore et al., 2014). The marine and terrestrial environments can also be distinguished by a difference in abundance of specialist herbivore communities, barely represented in the marine environment (Hay and Steinberg, 1992). Indeed, most marine herbivores (such as fishes, sea urchins, amphipods, crabs, polychaetes and gastropods) are feeding generalists, capable of feeding on 10 to 20 families of seaweeds. Specialist herbivores exist in marine communities, but they are in limited amounts (Hay and Steinberg, 1992). Grazing by invertebrate mesograzers such as amphipods, polychaetes, small crabs, and gastropods usually removes a small amount of algal production (<10%) (Carpenter, 1986). Thus, defense evolution against marine herbivores is directed primarily by generalist herbivores of low to moderate impact, and it was proposed, according to Hay and Fenical (1988), that the potential for coevolution between a plant and its herbivore is low in marine communities.

Yet, marine algae do not possess a vascular system like in plants that allows transportation of signals towards distant areas from their production site (Cronin and Hay, 1996c). These systems provide a distance communication into the organism and allow the establishment of the systemic acquired resistance (SAR) (Heil and Ton, 2008; Shah, 2009). Multicellular marine algae, however, have evolved analogous systems for intra-plant communications for effective SAR-like defenses against herbivores. Translocation of different soluble compounds by communication between adjacent cells have been demonstrated in very large algae, such as kelps (Floc'h and Penot, 1978) and recent results based on the oligoguluronate-induced defense responses suggested the occurrence of a systemic protection against herbivores in *L. digitata* (Thomas et al., 2014). Seaweeds are also capable of producing a wide range of secondary metabolites, such as terpenes, aromatic compounds, amino acid-derived substances, and phloroglucinol-based polyphenolics, which could play roles in inter-plant defense signaling (Hay and Steinberg, 1992). But if the nature of volatile compounds (e.g. their volatility) depends mostly on molecular weight and temperature in airborne environment, the important factor in water is their diffusion rate (Steinke et al., 2002). Thus, when releasing complex blends of compounds, algae can create a chemical gradient in their surroundings with different physical, chemical and likely signaling potentials. Finally, the efficacy of a chemical signal depends on its recognition by the receiver (Steinke et al., 2002).

2. Chemical mediation in micro- and macroalgae in response to grazing stress

a. Chemical defense in diatoms against copepods

Even if diatoms were previously considered as an optimal food for copepods, the first evidence of inhibitory effects of feeding by diatoms on hatching success of copepods demonstrated that the diatom *Thalassiosira rotula* had a negative effect on *Temora stylifera* egg viability (Ianora and Poulet, 1993) and later, that antimitotic compounds of this diatom species blocked embryogenesis of the copepod *Calanus helgolandicus* (Poulet et al., 1994). Other negative effects were highlighted on generation time and mortality rates of these grazers (Ianora et al., 2003). The diatom-copepod model has been extensively studied and according to (Ianora and Miralto, 2010), it turns out that it has no equivalent in marine plant–herbivore systems. Indeed, most prey–predator chemical defense interactions in the marine environment are based on repellency or poisoning, whereas diatoms are exclusively effective on predator's reproductive failure. D'Ippolito et al. (2002) found that, when fed with the diatom *Skeletonema costatum*, egg production rates and hatching viability of the copepod *Temora stylifera* were strongly affected by food. Hatching success even decreased to 0% within 7 days after feeding with *S. costatum*. This effect was also tested with the diatom species *Thalassiosira rotula*, but it was less intense as it did not modify egg production rates though decreasing hatching success (D'Ippolito et al., 2002b). However, when this diatom was used as food for the copepod *Calanus helgolandicus*, hatching success was strongly reduced. Development of embryos was abnormal, with dispersed chromatin and asymmetrical division of blastomeres, preventing from egg hatching (Ianora et al., 2003). Moreover, inhibitory effects are diatom density-dependent, high diatom densities triggering less hatching success than low densities. In 1999, Miralto et al. observed a significant decrease of hatching success in copepods and a negative effect on cleavage in sea urchin embryos feeding on different diatom species and found that three aldehydes isolated from the diatoms were responsible for this biological activity: 2,4-decadienal and 2,4,7-decatrienals. However, it seems that inhibitory activity was not limited to those aldehydes, but concerned a major part of the poly-unsaturated aldehydes (PUAs), containing α,β,γ,δ-unsaturations, excluding aldehydes without unsaturations which do not have effects on predators (Pohnert et al., 2002; Pohnert, 2004). Active extracts of *S. costatum* against copepods were shown to contain trans,trans-2,4-heptadienal, trans,cis-2,4-octadienal, trans,trans-2,4-octadienal, and 2- trans-4-trans-2,4,7-octatrienal. These authors also found the latter two compounds in the diatom extracts of *Thalassiosira rotula* after sonication (D'Ippolito et al., 2002a). Other studies showed inhibitory effects of different species of diatoms on egg-hatching success and on copepod fecundity, but it appears that not all copepods were equally sensitive to diatom metabolites, and that diatom do not induce the same negative effects on copepods (Ianora et al., 2003). Therefore, investigations increased on the roles and characterization of these PUAs in defense of diatom species against other marine organisms, such as sea

urchin embryos, polychaetes and sea star embryos. Both diatom extracts and the aldehyde decadienal suppress fertilization, embryogenesis and hatching success of these organisms (Pohnert, 2005). Moreover, decadienal was found to significantly reduce hatching success of polychaete at 125 ng.mL^{-1}. However, reactive aldehydes were also shown to be involved in communication between diatoms, as both growth and cell cycle of the diatom *Thalassiosira weissflogii* were influenced by the aldehyde 2-trans,4-trans-decadienal (Ianora et al., 2003). Thus, this is well established that PUAs present in diatoms play a crucial role in regulation of copepod communities, depending on diatom and copepod species, in the laboratory. Laboratory experiments also demonstrated that the PUAs 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal inhibited the growth of two bacterial strains associated with diatom species *Skeletonema marinoi* in the Northern Adriatic Sea (Ribalet et al., 2008). However, attempts of application of these experiments in higher scales to try to come closer to *in situ* conditions were not as conclusive as laboratory experiments. Paul et al. (2012) tested the roles of PUAs in bacteria-diatom interactions as chemical mediators in mesocosm conditions. They found no effect of these PUAs on bacterial and viral abundance, as well as on bacterial community composition, but rather an alternation of bacterial diversity over time and a diatom strain-dependent bacterial diversity. The authors concluded that PUAs did not significantly affect bacteria-diatom interactions, even though they could influence interactions with other marine organisms (Paul et al., 2012).

b. Defensive strategies in macroalgae against herbivory

In response to grazing by herbivores, macroalgae simultaneously employ different strategies in order to defend themselves and conserve high fitness. They use constitutive defenses, that are constant defenses at effective levels, as well as inducible defenses which spread out only in response to a cue indicating a higher risk of grazing (Jormalainen and Honkanen, 2008). The environmental conditions in which macroalgae live dictate the type of activated defenses, without exclusivity. There can be a certain level of constitutive defense and, if needed, inducible defenses can rapidly rise for higher protection of the alga. Though, constitutive and inducible defenses have not the same ratio in costs/benefits balance: since constitutive defenses are constantly produced, they require higher energy costs than inducible defenses which are only activated in response to a warning cue. Marine grazers are considered as feeding over spatial and temporal scales allowing preferentially inducible to constitutive defenses (Jormalainen and Honkanen, 2008). This hypothesis is more and more supported, as observations of inducible defenses with direct anti-grazing effects are accumulating for marine macroalgal species (Cronin and Hay, 1996a; Pavia and Toth, 2000; Sotka and Hay, 2002; Taylor et al., 2002; Toth et al., 2005).

In the red algae *Gracilaria vermiculophylla* and *G. chilensis*, grazing by the sea snail *Echinolittorina peruviana* induces chemical defenses, making them less attractive for a subsequent grazing (Rempt et al., 2012). Moreover, it appears that the oxylipins 8-hydroxy eicosatetraenoic acid (8-

HETE) and 7,8-dihydroxyeicosatetraenoic acid (7,8-di-HETE) are released by these macroalgae and that they have inhibitory effects on the development of other red algae (Lion et al., 2006). 7,8-di-HETE was also found to be active against the herbivore *Echinolittorina peruviana*, while prostaglandins and other oxylipins do not have inhibitory effects (Rempt et al., 2012). In the brown alga *Dictyota menstrualis*, Cronin and Hay (1996) demonstrated that grazing by the generalist amphipod *Ampithoe longimana* increased amounts of defensive compounds in the alga, reducing the susceptibility of the alga to be subsequently grazed by the amphipod. In particular, metabolomics analyses of algal tissues complemented by choice and non-choice bioassays allowed the identification of three diterpenoid secondary metabolites produced in damaged plants - dictyol E, dictyodial and pachydictyol A – which decreased consumption by fish, sea urchins and the amphipod *Ampithoe longimana*. For example, in Dictyotales species, pachydictyol A was fed to the omnivorous fish *Diplodus holbrooki* and it resulted in a significant decrease of their growth rate, compared to control fish that consumed equivalent amounts of food without the compound (Hay et al., 1988). The inhibitory effect of these compounds was different according to the herbivore species and their localization into the algal thallus (Cronin and Hay, 1996b). Similar inducible inhibitory effects were observed in the brown alga *Fucus vesiculosus*, where *Idotea baltica* herbivores preferred control algae over grazed plants (Haavisto et al., 2010; Yun et al., 2010) whereas *L. littorea* did not have preferences for control or grazed *F. vesiculosus* plantlets (Yun et al., 2010). Interestingly, mechanical damage did not induce algal resistance (Rohde et al., 2004) but, on the contrary, increased algal consumption by *I. baltica* (Haavisto et al., 2010). This may come from the way of wounding, allowing better digestibility of algal pieces. In the kelp *Laminaria japonica*, grazing by *Littorina brevicula* reduced the palatability of algae for a subsequent grazing, but this effect was not observed after grazing by the abalone *Haliotis discus* (Molis et al., 2008). This experiment was also made on *Ulva pertusa*, but did not show specificity of grazer species. Direct grazing effects were also observed in two other brown algal species, *Ascophyllum nodosum* (Pavia and Toth, 2000) and *Fucus distichus* (Van Alstyne, 1988). In response to a direct grazing by its specialist herbivore *Littorina obtusata*, *A. nodosum* produces phlorotannins within a few weeks. Interestingly, this defense reaction was not found after grazing by the isopod *Idotea granulosa* or after a mechanical damage. Thus, there is a high degree of specificity in the chemical inducible responses, according to the physical or biotic damage applied to *A. nodosum* (Pavia and Toth, 2000) and *F. vesiculosus* (Rohde et al., 2004). The same was observed in *Fucus distichus*, where grazing by the snail *Littorina sitkana* triggers an increase of phlorotannins in algal tissues, but it appeared that mechanical damage also triggered an increase of ~20% of these defense compounds within two weeks (Van Alstyne, 1988). Highly damaged fronds of *A. nodosum* contained high concentrations of phlorotannins and grazed fronds contained more than 100% more phlorotannins than undamaged fronds. It was also found that damaged algae by *L. obtusata* were less susceptible to a subsequent grazing than undamaged algae, which is not the case after grazing by *I. granulosa*. When herbivores could choose between grazed and ungrazed *A. nodosum*, *L. obtusata* chose preferentially undamaged algae with lower concentrations of phlorotannins, whereas no differences

were found with the grazer *I. granulosa* (Pavia and Toth, 2000). In *F. distichus*, wounded plants were preferred by the snail *L. sitkana* to uninjured plants directly after damage, but herbivore choices changed two weeks after injury, at the time concentrations of phlorotannins increased in damaged algae (Van Alstyne, 1988).

Nevertheless, the roles of phlorotannins in induced defenses of macroalgae do not seem as clear as it was previously supposed. It appears that increases in phlorotannins after algal challenging vary over spatial and temporal scales and that their repellent effects depend on both host and herbivore species (Haavisto et al., 2010). Some studies did not find any induction of phlorotannins production upon natural (Long et al., 2007; Jormalainen and Honkanen, 2008) or mechanical wounding. Other studies found increased amounts of phlorotannin after physical and natural damage (Haavisto et al., 2010), but these authors demonstrated that the sole presence of herbivores induced the same amounts of these compounds without decreasing algal palatability. Thus, they concluded that phlorotannins were not induced by the defense response of the alga to herbivory, or at least that this response was not the only defense response which could explain the repellent effect of *Fucus vesiculosus* against the isopod *Idotea baltica*. Besides inhibitory effects of direct grazing on this alga, these authors found that herbivores consumed less algal pieces when it was first co-incubated with a previously grazed conspecific. Moreover, large females feeding on these treated algae had a lower reproduction success, that is fewer eggs laid and smaller total egg mass (Haavisto et al., 2010). Thus, indirect defenses against grazers seems to be a good strategy for algal defense, through chemical mediation between algae, by means of waterborne compounds which can be perceived by neighboring algae for prevention of a danger. Perception of warning signals trigger epigenetic modifications in the receiver as preparation for future resistance expression (Goellner and Conrath, 2008; Heil, 2010; Pastor et al., 2013). This type of defense was shown earlier in the knotted wrack *Ascophyllum nodosum*, a brown alga, and its specialist grazer flat periwinkle *Littorina obtusata* (Toth and Pavia, 2000). After incubation in seawater containing grazed *A. nodosum*, unharmed neighboring algae contained higher amounts of polyphenolic secondary metabolites, phlorotannins. Thus, grazing on *A. nodosum* induces the production of waterborne compounds which can serve as signals to induce production of secondary metabolites in their undamaged neighboring conspecifics. Moreover, the increasing levels of defense chemicals into algae which received the chemical signals prevent herbivores for a subsequent grazing (Toth and Pavia, 2000). This study proved that chemical signals are emitted by *A. nodosum* during grazing by its specific herbivore, and that these compounds are perceived by neighboring algae. Thereafter, this latter alga produces defense chemicals which enable the deterrence of herbivores for a subsequent grazing. In the species *Fucus vesiculosus*, both direct grazing and co-incubation with a grazed conspecific induce defenses against the same isopod (Rohde et al., 2004). Interestingly, if grazing by the isopod *Idotea baltica* triggers an induction of chemical defenses in the red macroalgae *Furcellaria lumbricalis*, *Delesseria sanguinea*, *Phyllophora pseudoceranoides* and in the brown algae *Fucus serratus* and *Fucus evanescens*, direct grazing on these

algae does not induce defenses in their neighboring conspecifics, as co-incubated algae were as preferred as their control (Rohde and Wahl, 2008a). Thus, it appears that induction of defense chemical compounds depends not only on the grazer species, but also on the algal host.

Indirect defenses include the attraction of other organisms as well, like predators of herbivores, via waterborne cues induced by algal responses. Involving different trophic levels, these interactions are defined as multitrophic. Some cases have been highlighted in the marine environment, like in the brown alga *A. nodosum* (Coleman et al., 2007). When this alga was grazed by its specialist herbivore *L. obtusata*, fishes were more attracted by its odours than by naïve or mechanically damaged plants, and crabs were also more attracted by these grazed algae than by naïve ones. Thus, grazed *A. nodosum* seem to release a specific bouquet which is perceived by predators of herbivores as food source, hence attracting them for herbivore consumption. The fact that specific blends are emitted by grazed algae, then perceived by predators could lead to evolutionary events like coevolution between these carnivores and the host algae. However, this remains to be demonstrated, as reciprocal responses among phenotypically plastic partners is a necessary prerequisite for the evolutionary stabilisation of mutualisms in general and, therefore, of indirect defense strategies.

III. Innate immunity in algal lineages

1. Recognition of MAMPs and MIMPs

Inducible algal defenses against pathogens and herbivores imposes activation of the defensive responses fast enough to limit the spread of the attacker before irreversible damage (Weinberger, 2007). This induction is based on molecular recognition of pathogen infections by innate receptors, located in the plasma membrane, for which some have already been characterized in metazoans and vascular plants (Schwessinger and Ronald, 2012). These innate receptors constitutively present in algae also allow distinction between self (or MIMPs) and non-self (or MAMPs). As previously described, MIMPs and MAMPs perception is followed by defense reactions in land plants. MAMPs are generally highly conserved between organisms and perceived by many eukaryotes (Nürnberger et al., 2004; Weinberger, 2007). In marine algae, MIMPs and MAMPs and their effects on algal resistance against pathogens have already been described in red and in brown algal lineages. For example in *Chondrus crispus*, resistance against its endophytic algal pathogen *Acrochaete operculata* was increased when the seaweed was treated with cell-free extract from this endophyte, considered as MAMPs (Bouarab et al., 2004). Similarly, MIMPs which can be degradation products of algal cell wall, for example fragments of alginates in brown algae or agar in red algae, having biological and ecological effects. In the red alga

Gracilaria chilensis, agar oligosaccharides from algal cell wall increased the resistance of the seaweed against the epiphytic red alga *Acrochaetium sp*. (Weinberger, 2007).

In brown algae, derived products from alginates, the main brown algal cell wall polysaccharide, are one of the most studied MIMPs. They are composed of two types of linear polymers: β-1,4-D-mannuronic acid (oligomannuronates) and its C5 epimer, α-1,4-L-guluronic acid (oligoguluronates) (Küpper et al., 2001). These oligosaccharides are arranged by alternation of homopolymeric blocks of poly-β-1,4-D-mannuronic acid (MM blocks), of homopolymeric blocks of poly-α-1,4-L-guluronic acid (GG blocks), and of heteropolymeric blocks with random arrangements of both monomers (MG blocks) (Fig 6). Oligoguluronates are the functional analog of pectins in plants and agar in red algae and, like pectins, they form intermolecular complexes in the presence of calcium ions (Potin et al., 2002; Weinberger, 2007). The genome of the brown alga *Ectocarpus siliculosus* was mined in order to find homologues of genes encoding ligand-binding and signal transduction domains in plants and animals. It was find that the ROCO and NB-ARC– TPR families could be potential candidates for being involved in recognition/transduction events linked to immunity in the brown algal lineage (Zambounis et al., 2012). Studies on infection of *L. digitata* by its natural endophytic pathogen *Laminariocolax tomentosoides* demonstrated that previous elicitation by oligoguluronates was able to protect the kelp from infection by the endophyte within 7 days, compared to control kelps which were not previously elicited (Küpper et al., 2002). Epiphytic and endophytic filaments were not visible after elicitation treatment. In the kelp *Laminaria japonica*, infection with alginate-degrading bacteria have been shown to induce early responses similar to those following oligoguluronate application, such as the oxidative burst (Weinberger, 2007).

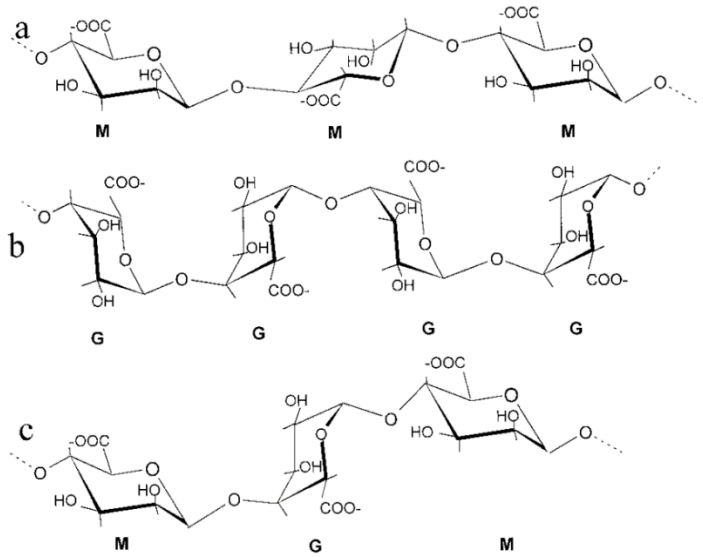


Figure 6. Chemical structure of alginate blocks from brown algae. Oligomannuronates (MM blocks; a) are linear arrangements of β-1,4-D-mannuronic acid, oligoguluronates (GG blocks; b) are formed with homopolymeric blocks of α-1,4-L-guluronic acid and heteropolymeric blocks are composed of both monomers (MG blocks; c). From Küpper et al. (2001)

2. The oxidative burst

The oxidative burst is an important conserved component of innate immunity among all eukaryotes, from red and brown algae to vascular plants, as well as for animals (McDowell et al., 2014). It consists in a transient production of ROS, such as superoxide ions ($O^{2-\bullet}$), hydrogen peroxide (H_2O_2), or hydroxyl radicals ($OH^{\bullet}$), through the activation of plasma membrane-associated NADPH oxidases or apoplastic peroxidases (Küpper et al., 2002; Weinberger, 2007). In the red macroalga *Gracilaria conferta*, an oxidative burst was observed after perception of products of the microbial degradation of its agar cell wall, thus protecting the alga against their epiphytic bacteria (Weinberger et al., 1999; Weinberger and Friedlander, 2000). In brown algae, Küpper et al. (2001) showed that oligoguluronate treatment on *Laminaria digitata* induced a high increase in oxygen consumption during 2 – 3 minutes after their addition. At the same time, the kelp releases a strong and sudden amount of hydrogen peroxide H_2O_2 in the medium, reaching its maximum at 5 to 10 minutes after oligosaccharide addition, then decreasing until its basal level (Küpper et al., 2001). The induction of respiratory and oxidative bursts is dependent on the chemical structure of the oligosaccharides, GG blocks triggering them, while MM and MG blocks were not able to induce significant oxidative bursts. Moreover, H_2O_2 emission rates and amplitude were also dependent on the concentration of alginate oligosaccharides applied to the kelp. An oxidative burst is observed with GG concentration ranging from 2.5 $\mu g.mL^{-1}$ to the saturation of response at 150 $\mu g.mL^{-1}$. However, after an elicitation producing a burst, subsequent elicitation by oligoguluronates were ineffective for at least 3 h, showing a desensitizing effect of the elicitors on kelp responses (Küpper et al., 2001). Interestingly, these responses to alginate oligosaccharide only occur in sporophytes of *L. digitata*, whereas gametophytes show no response to these elicitors. For sporophytes, younger algal parts were more sensitive to elicitation than meristematic and older blade tissues. Subcellular localization of ROS investigated by confocal microscopy was found around epidermal and outer cortical cells, but not in the medulla which does not show sensitivity to elicitation (Küpper et al., 2001). Moreover, it was found that recognition and response to oligoguluronate with induction of an oxidative burst are restricted to the Laminariales (such as *Laminaria digitata*, *L. hyperborea*, *L. ochroleuca*, *Macrocystis pyrifera*, *Lessonia nigrescens*, and *Saccorhiza polyschides*), while Fucales constitutively release high quantities of hydrogen peroxide and Ectocarpales generally do not respond or weakly to elicitor addition (Küpper et al., 2002). The roles of ROS emitted during the oxidative burst were investigated on *L. digitata* pathogens and epiphytes, and it appears that H_2O_2 inhibited their growth and survival at low concentrations (Küpper et al., 2001). Thus, accumulation of ROS following elicitation by oligoguluronate is essential in kelps for defense system and resistance set up against pathogens.

Induction of an oxidative burst in *L. digitata* was also demonstrated upon treatment with exogenous elicitors, lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria (Küpper et

al., 2006). LPS were already described as inducers of defense mechanisms such as oxidative burst in metazoans (Remer et al., 2003) and in land plants (Meyer et al., 2001). The treatment of *L. digitata* thalli with LPS from *Salmonella abortus equi* was shown to induce a strong release of hydrogen peroxide into the surrounding medium, in a similar amount range compared to elicitation by oligoguluronates (Küpper et al., 2006). LPS and lipoteichoic acids (LTA; from the cell envelopes of gram-positive bacteria) both modify patterns of protein expression in the red alga *Chondrus crispus*, strongly suggesting that they are also perceived by this alga (Weinberger, 2007). Thus, LPS can also be considered as exogenous elicitors for marine algae.

The oxidative burst is directly linked to the activation of the plasma membrane-associated flavo-enzyme NADPH oxidase, which seems to be a conserved component of the ancestral immunity system and thus an ancestral trait of innate immunity in eukaryotes (Weinberger, 2007). Indeed, the main subunits of NADPH oxidase in mammalian phagocytes, vascular plants, diatoms, and the red alga *Chondrus crispus* are structurally related (Keller et al., 1998; Torres and Dangl, 2005; Hervé et al., 2006). Studies of the oxidative burst response in *Gracilaria sp*. revealed that it was highly sensitive to diphenylene-iodonium (DPI), a specific inhibitor of NADPH-dependent enzymes, and to other inhibitors of flavo-enzymes. NADPH oxidase was thus supposed to be the source of ROS after elicitation of *Gracilaria sp*. (Weinberger et al., 2005), as already observed in vascular plants and metazoans (Torres and Dangl, 2005).

3. Algal defense and biosynthesis of oxidized fatty acid derivatives

Perception of external molecular signals triggering production of fatty acids and their oxidized derivatives, oxylipins, was shown to be another common feature in land plants, animals as well as in macroalgae (Weinberger, 2007). In plants, the enzymatic processes involved in generation of oxidized fatty acids derivatives and other secondary metabolites, known as oxylipin pathway, proceeds from catalytic oxygenation of free polyunsaturated fatty acids (PUFAs) either by autoxidation or by intervention of lipoxygenases (LOXs), generating hydroperoxides, until production of aldehydes and alcohols by enzymes like allene oxide synthases (AOSs), hydroperoxide lyases (HPLs), and divinyl ether synthases (DESs) (Feussner and Wasternack, 2002). Red and brown macroalgae produce oxylipins from C18 and C20 fatty acids, which could be involved in algal defense (Potin et al., 2002; Pohnert, 2004). After exposure to cell-free extracts of the endophyte *Acrochaete operculata*, two lipoxygenases were upregulated in the red alga *Chondrus crispus*, one day after elicitation (Bouarab et al., 2004), displaying a crucial role of oxylipin signaling in resistance of *C. crispus*. Similarly, treatment of *L. digitata* thalli with LPS from *Salmonella abortus equi* was shown to induce free fatty acids and oxylipins (Küpper et al., 2006). Red algae, such as *Chondrus crispus*, feature both C18- and C20- derived oxylipins, which could also play a role as secondary defense signals in this eukaryotic lineage. For instance, treatment of *C. crispus* by methyl jasmonate activated the metabolism of C20 and C18

polyunsaturated fatty acids , inducing an elevated production hydroperoxides and cyclopentenones such as prostaglandins and jasmonates, a transient resistance to *A. operculata*, as well as an increase of stress gene transcription (Bouarab et al., 2004). Moreover, jasmonic acid was described in vascular plants after derivatization of C18 and C20 oxylipins. In an evolutionary point of view, it is likely that jasmonate signaling have evolved between the primary and the secondary endosymbiotic events and is therefore considered as a putative common trait of photosynthetically eukaryotes, plants, micro- and macroalgae (Bouarab et al., 2004).

Most of recent studies on oxylipin pathways in Stramenopiles originate from investigations on diatoms. In the diatoms *T. rotula* and *S. costatum*, the sources of the fatty acid derivatives are lipids coming from galactolipids of the chloroplast membranes and from phospholipids of the plasma membrane (Fig 7a; Fontana et al., 2007). The fact that unicellular diatoms mobilize these existing resources presents advantages in rapid reactions against herbivores, when transcription and *de novo* biosynthesis of enzymes would be unlikely in wound-activated defense (Pohnert, 2005).

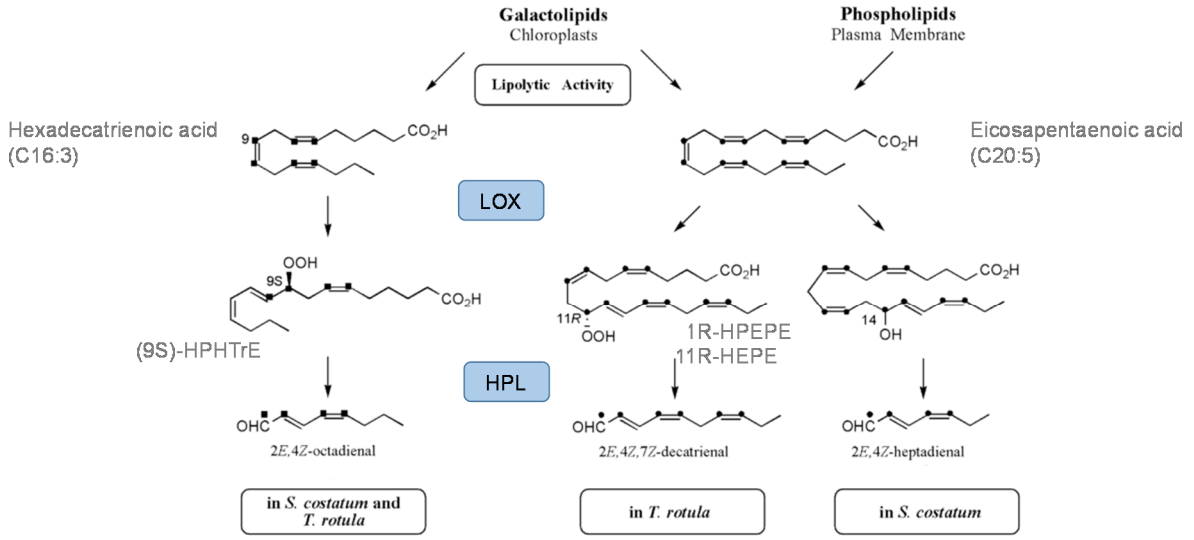
Wounded or mechanically damaged diatoms contain phospholipases A2 which activate and trigger release of free polyunsaturated fatty acids (PUFAs) (Pohnert, 2004). Nevertheless, it has been shown that healthy diatoms do neither contain free mono- nor polyunsaturated fatty acids, but only saturated fatty acids such as C14:0 or C18:0 (Pohnert, 2002). Only wounding of algae can induce a rapid production of fatty acid derivatives as well as a strong increase of free fatty acid amounts, such as eicosapentaenoic acid (C20), C18 and C16 fatty acids, few seconds after cell disruption (Pohnert, 2002; Pohnert, 2005). This leads to high local concentrations of these defensive compounds or of related potentially active aldehydes in the vicinity of diatom cells (Pohnert, 2000). It has been proposed that, following wounding, fatty acids were oxidized either chemically by autoxidation or enzymatically (Blée, 1998). Later on, studies showed that the diatoms *T. rotula* and *S. costatum* possess predominantly enzymatic systems for PUFAs' transformation. They are first oxidized via a lipoxygenase (LOX), producing hydroperoxides, and then a hydroperoxide lyase (HPL) which generates aldehydes (Pohnert, 2004). Generation of fatty acid derivatives by lipoxygenase's activity depends strongly on free polyunsaturated fatty acid availability in cells (Pohnert, 2002; Pohnert, 2005) and works independently from the Phospholipase A2 activity (Pohnert, 2002). Given that healthy diatoms only contain saturated fatty acids and that these fatty acids cannot be transformed by LOX (Pohnert, 2005), intact diatoms do neither contain nor release aldehydes (Pohnert, 2000). In the diatom *S. costatum*, generation of the aldehyde octadienal in wounded cells by oxidation of the fatty acid hexadecatrienoic acid (C16:3 w-4) was shown to involve LOX/HPL activity, thus not spontaneously synthesized by autoxidative process (D'Ippolito et al., 2003). The hydroperoxide intermediate was then described in *T. rotula* as (9S)-hydroperoxyhexadecatrienoic acid ((9S)-HPHTrE) formed by a 9S lipoxygenase (D'Ippolito et al., 2006). Moreover, Pohnert et al. (2002) described the synthesis of decatrienal as originating exclusively from eicosapentaenoic acid (EPA, C20:5 w-3) probably by an 11R-lipoxygenase, via the hydroperoxide

11R-HEPE (Pohnert, 2000), and decadienal coming from arachidonic acid (C20:4). In contrast, the author did not find any of these aldehydes originating from C18 and C22 fatty acids. Thus, *T. rotula* shows a substrate specificity for C20 fatty acids for production of oxylipins and aldehydes. In the same study, it appears that free fatty acids are required as lipoxygenase substrates, as conjugated C20 fatty acids did not induce subsequent production of aldehydes. Moreover, is has been shown that the generation of decatrienal is a rapid reaction, as *T. rotula* cells produce 4.1 fmol of this aldehyde per cell within 1 – 3 min after cell damage (Pohnert, 2000).

In macroalgae, fatty acid derivatives originating from the oxylipin pathway were shown to have attracting roles as sexual pheromones in brown algae (Pohnert and Boland, 2002), as well as defensive roles against epiphytes (Lion et al., 2006), endophytes (Bouarab et al., 2004) and herbivores (Rempt et al., 2012). As previous investigations on diatoms showed major repellent effects of oxylipins on copepods, studies on their ecological effects and their characterization began in macroalgae. Among green algae, studies on *Ulva sp.* composing green tides showed that sea-lettuce like but not tube-like morphotypes produced high amounts of volatile aldehydes upon tissue damage (Alsufyani et al., 2014). 2,4-heptadienal and 2,4-decadienal were the most abundant aldehydes produced by damaged *Ulva sp*., whereas 2,4,7-decatrienal was produced in less quantity. These aldehydes are derived from omega-3 and omega-6 PUFAs mostly with 18 carbon atoms such as stearidonic acid (C18:4 n-3), alpha-linolenic acid (C18:3 n- 6), linoleic acid (C18:2 n-6), and monounsaturated oleic acid (C18:1 n-9) and palmitoleic acid (C16:1 n-7) (Fig 7b). Moreover, they were marginally derived from C20 fatty acids like EPA (C20:5 n-3) and arachidonic acid (C20:4 n-6). Transformation of these PUFAs is performed with LOX, 11-LOX acting on eicosanoids and 9-LOX on octadecanoids (Alsufyani et al., 2014), forming hydroperoxides and finally PUAs. In the red algae *G. vermiculophylla* and *G. chilensis*, Rempt et al. (2012) found that the oxylipin 7,8-di-HETE active against *E. peruviana* was derived from arachidonic acid, through a lipoxygenase-mediated transformation. The release of arachidonic acid and the following hydroxylated compounds come from a phospholipase A, probably directly from galactolipids (Lion et al., 2006). In brown algae, investigations of fatty acids and derivatives and their biosynthetic pathways were already described in *Laminaria angustata* (Boonprab et al., 2003a; Boonprab et al., 2003b). This brown alga forms C6 and C9 aldehydes enzymatically, from two different pathways. C6 aldehydes are derived either from C18 or from C20 fatty acid and C9 aldehydes are formed exclusively from the C20 fatty acid arachidonic acid. The C18 fatty acid linoleic acid is converted to the hydroperoxide 13-hydroperoxyoctadecadienoic acid (13-HPODE), then cleaved to form the aldehyde n-hexanal. Interestingly, the C6-aldehyde-forming branch in the brown alga had almost the same properties as that in higher plants. On the contrary, the C9-aldehyde forming branch uses preferentially arachidonic acid instead of linoleic acid as substrate (Boonprab et al., 2003a). Moreover, it seems that the HPL which catalyzes the formation of C9 aldehydes is highly specific to hydroperoxides from C20 fatty acids (Boonprab et al., 2003b). In the kelp *L. digitata*, Küpper et al. (2006) found that free fatty acids were

produced upon application of LPS from *Salmonella abortus equi* and *Marinobacter hydrocarbonoclasticus*. Hence, linoleic acid (C18:2), linolenic acid (C18:3), arachidonic acid (C20:4), and eicosapentaenoic acid (C20:5) were produced highest amounts after 60 min following the LPS treatment. Other fatty acids such as myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1) were also detectable upon 30 min of LPS treatment. Moreover, oxylipins were found in the kelp after LPS treatment, like 13-hydroxyoctadecatrienoic acid (13-HOTrE) and 15-hydroxyeicosapentaenoic acid (15-HEPE). Investigations on the effect of the endogenous elicitors oligoguluronates were first reported to fail at inducing significant amounts of free fatty acids and oxylipins in *L. digitata* (Küpper et al., 2006). However, Goulitquer et al. (2009) demonstrated that a wide range of aldehydes such as hexenal, hexanal, 4-hydroxyhexenal, nonenal, 4-hydroxynonenal or dodecadienal were emitted by *L. digitata* upon elicitation by oligoguluronate. In addition, it appears that the aldehyde 4-HHE is able to induce significant production of the oxylipin 13-hydroxyoctadecatrienoic acid (13-HOTrE) after 24 hours. Similar results were obtained in this kelp in response to copper stress, which induced production of a wide variety of fatty acids, oxylipins as well as amino acids (Ritter et al., 2014).

a)



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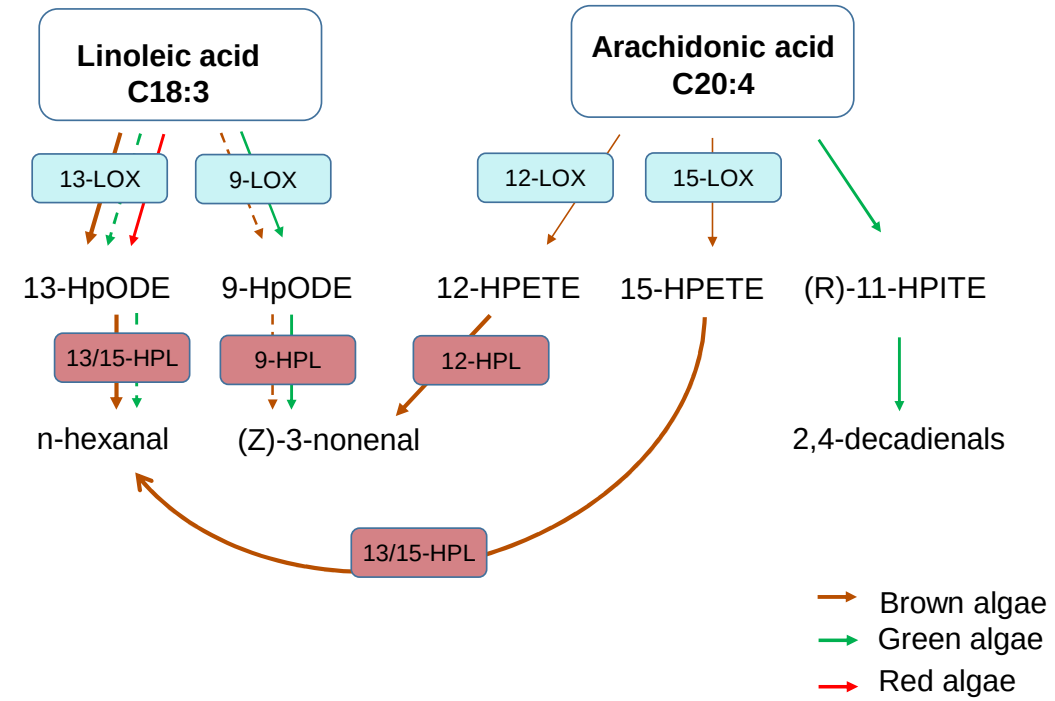

Figure 7. Representation of the oxylipin pathway in diatoms (a; from Fontana et al., 2007) and in brown, green and red algae (b).

IV. Brown algal signaling and defenses against herbivores

1. Interests of studying kelps

a. Evolutionary aspects

Brown algae belong to the Phaeophyceae and constitute complex photosynthetic organisms with a very different evolutionary history from land plants and other multicellular Eukaryotes (Fig 8; Cock et al., 2010). Like red and green algae, land plants, Fungi and Metazoa, brown algae have independently evolved complex multicellularity. Moreover, brown algae share the same evolutionary history as the diatom lineage, and can also feature ancestral traits that are common in other eukaryotic lineages as well as convergent traits that appeared several times independently. As described above, some aspects of defense mechanisms in brown algae can be conserved such as the perception of MIMPs or the oxidative burst response, while others may be specific to the Stramenopiles lineage.

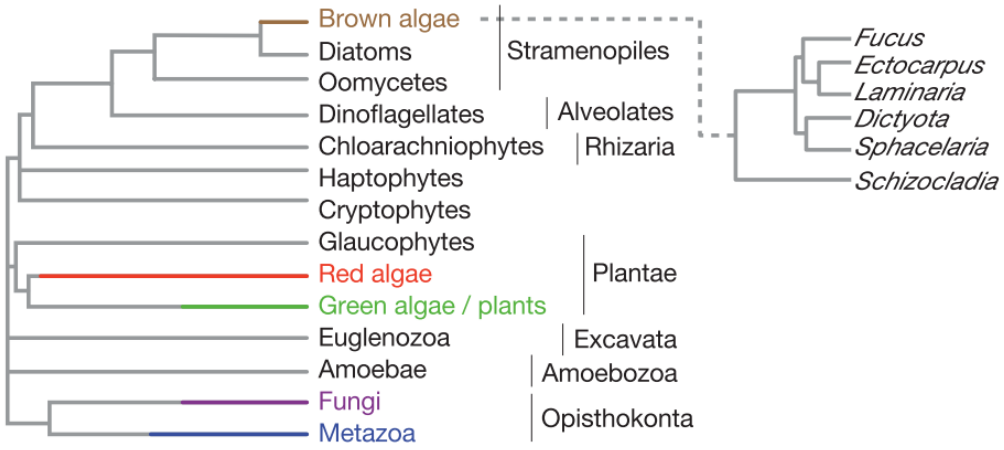

Figure 8. Simplified representation of the evolutionary tree of the eukaryotes showing the five major groups that have evolved complex multicellularity (indicated in color). Kelps are represented by the genus Laminaria. From Cock et al. (2010)

b. Ecological characteristics

The Laminariales order, also known as kelps, constitute ecologically important macroalgae in coastal ecosystems on Earth. Kelps form sub-marine forests dominating coastal rocky shores of cold to temperate zones around the world (Steneck et al., 2002). They are the most important biogenic structures in benthic marine ecosystems, constituting a true habitat providing protection and food source for a wide variety of other organisms such as other macroalgae, molluscs, crustaceans, fish and mammals (Bartsch et al., 2008; Smale et al., 2013). Kelp forests are thus one of the most diverse and productive ecosystem on Earth and can be compared to terrestrial tropical forests. Among the ten families forming the Laminariales, the families Laminariaceae, Lessoniaceae and Alariaceae constitute the most common species in these kelp forests.

Kelp life cycle is characterized by an alternation of generations with heteromorphic free-living life stages (Fig 9). One phase is defined by a large diploid sporophyte and the other is constituted by a microscopic haploid gametophyte generation where males and females differentiate and mate to restore diploidy (Kim and Bhatnagar, 2011). On the distal parts of the kelp, sori which are regions of epidermis along the length of the blade, contain haploid meiospores. Once released in the medium, these meiospores settle on the substrate, germinate and produce a microscopic filamentous gametophyte. The male gametophytes produce the motile, biflagellate sperm, while the female gametophytes transforms the distal part of its filament into an elongated oogonia containing one egg. After fertilization, the zygote germinates to form a flat embryo that subsequently differentiates into the mature sporophyte (Fig 9).

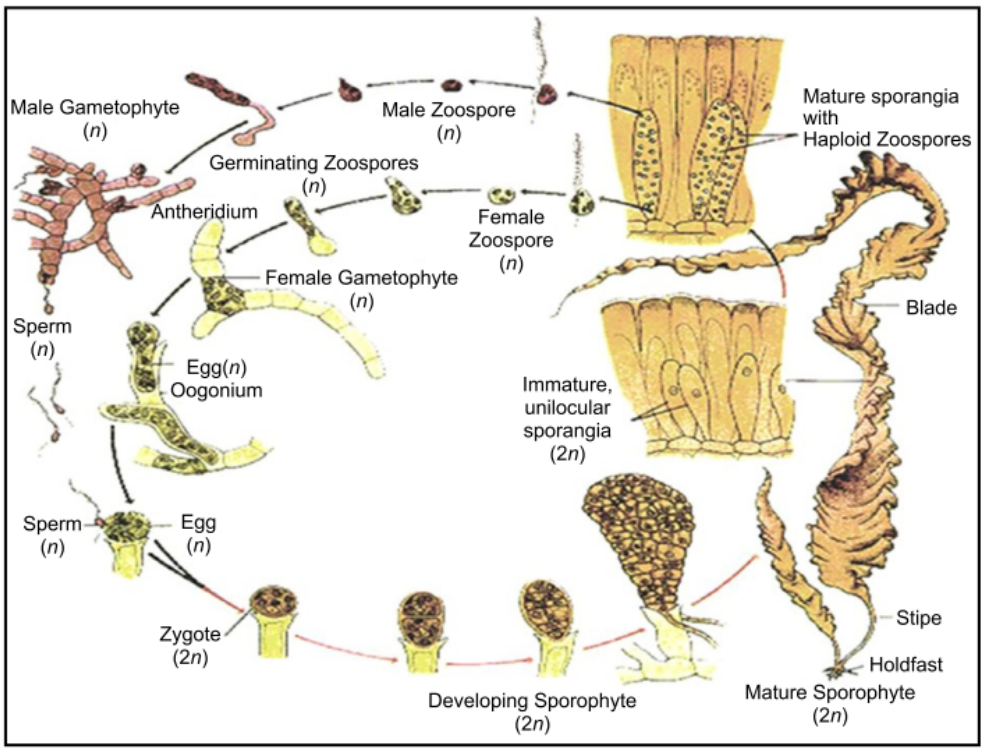

Figure 9. Kelp life cycle showing an alternation of generations with heteromorphic individuals. From Kim and Bhatnagar (2011).

c. Economic interests of kelps

Mainly collected for alginate extraction used in food, pharmaceutical and textile industries for its gellant, emulsifier or texturing properties, kelps provide huge economic interests (Bartsch et al., 2008; Kim and Bhatnagar, 2011). In medicine, *Laminaria* species have important interests for their content in diverse polysaccharides, for the production of wound dressing for the skin or the stomach, and encapsulation of medicines. Kelps are also intensively harvested for abalone feeding, and some specific sources of human diet (e.g. Kombu and Wakame). These seaweeds are also under study for development of aquaculture, as harvesting kelps in their natural environment induces important modifications of ecosystems which imposes to manage finely the algal resources in natural populations (Bartsch et al., 2008).

2. The thesis project

a. Scientific context and problematic of the thesis

In the kelp *Laminaria digitata*, defense elicitation with alginate oligosaccharides triggers a rapid oxidative burst and concomitant release of VOCs, as well as a transcriptional reprogramming before resistance establishment (Küpper et al., 2001; Küpper et al., 2002; Cosse et al., 2009; Goulitquer et al., 2009). In laboratory-controlled experiments, the effects of specialist herbivores have also been shown

to specifically regulate gene transcription in this alga (Leblanc et al., 2011). Moreover, chemical cues present in the kelp bed and/or released from elicited brown algae induced modifications of the physiology of neighboring *L. digitata*, in response to defense elicitors (Thomas et al., 2011). These results suggested the occurrence of water-borne signaling and revealed some physiological responses, similar to the priming responses in land plants. More recently, a systemic reaction was highlighted in *L. digitata* following elicitation on a distant area of the frond. These distant responses included an oxidative burst, an increase in haloperoxidase activities and a stronger resistance against herbivory (Thomas et al., 2014). Pharmacological inhibitors further suggested that the liberation of free fatty acids could play a key role in systemic signaling, reminiscent of what is known in land plants.

In the independent brown algal lineage, we could question the existence of universal mechanisms of defense and distance communication that have evolved in kelps during biotic interactions with herbivores. In the marine environment, most of primary consumers are generalist, but in kelps, few specialist interactions with herbivores have been described. We were especially interested in better understanding the defense responses and signaling processes in two kelp species involved in specialized interactions with their herbivores. The first interaction occurs between *Laminaria digitata* and its specialist herbivore *Patella pellucida* (Fig 10a) on the coastal rocky shore in Brittany. *Laminaria digitata* (Hudson) Lamouroux 1813 has been widely studied in its natural environment, revealing a high trophic specialization of this kelp by only one species, the gastropod *Patella pellucida* (formerly *Helcion pellucidum*), even if other species can also feed on this kelp but only temporally (Schaal et al., 2010; Leblanc et al., 2011). The second specialized interaction implies the kelp *Lessonia spicata* with its specialist and selective herbivore *Scurria scurra* Lesson 1830 (Fig 10b; Meynard, 2014). *L. spicata* (Suhr) Santelices (previously *L. nigrescens*) is one of the most dominant habitat-forming macroalgae in the low intertidal zone in central and southern Chile, producing high amounts of biomass (Santelices et al., 1980; González et al., 2012). The stipes of *L. spicata* are the habitats of the limpet *S. scurra*, digging holes on the stipes and holdfasts of this kelp, where it seems to live and feed, thereby structurally weakening the host (Santelices et al., 1980)

a)

b)

Figure 10. Pictures of *Laminaria digitata* and its specialist herbivore *Patella pellucida* in Brittany (a) and of *Lessonia spicata* and its specialist herbivore *Scurria scurra* in Chile (b).

In the context of defense responses and distant chemical signaling in brown algal kelps during interactions with specialist herbivores (Fig 11), the thesis aims at addressing the following questions: 1) what are the local algal responses following grazing by specialist herbivores? 2) what are the waterborne cues involved in distance signaling? 3) what are the effects of these signals on kelp defenses against herbivores, and are these responses dependent on the degree of specialization of the herbivore with the alga? 4) which modifications does this signaling trigger on metabolic regulations of the receiving kelp?

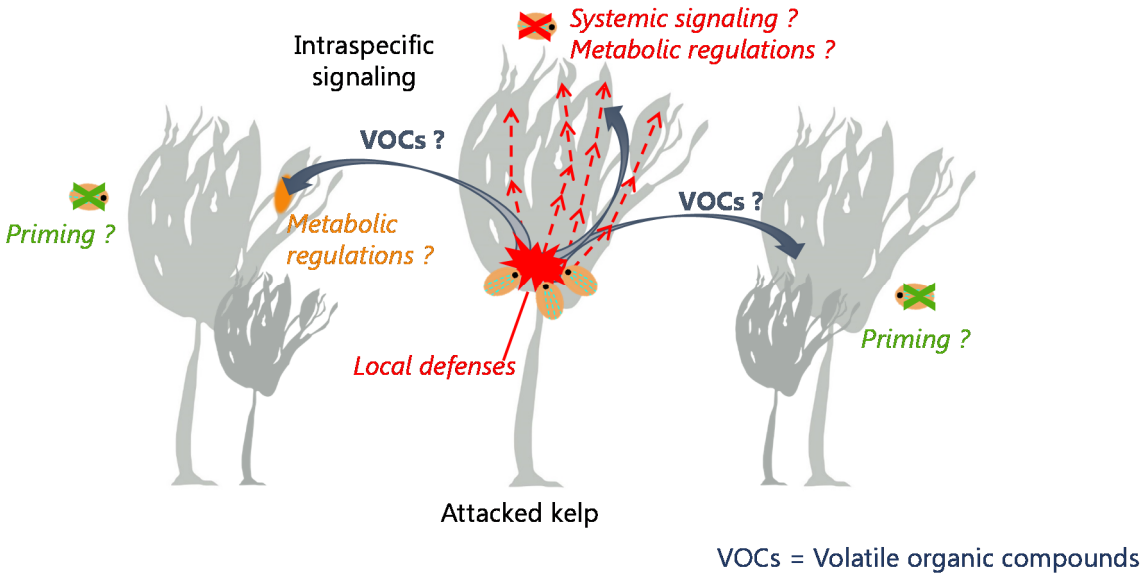

Figure 11. Schematic representation of potential chemical communication in kelps leading to priming. Upon grazing attack, a kelp responses locally directly against herbivores by deterrent or toxic compounds. Grazing can also induce a systemic signaling in the whole kelp, leading to defense in distal algal parts. Finally, a chemical distance signaling could be triggered via Volatile Organic Compounds (VOCs), potentially inducing metabolic regulations in the signal-receiving kelp and resulting in priming.

b. Thesis project and outline

In order to address the above questions in relationship with the chemical ecology research domain, combined approaches of chemistry and ecology were developed. Chemical analyses were performed using metabolomic approaches on algal exo- and endo-metabolome by LC-MS and GC-MS methods, while some ecological questions were investigated through deterrence and preference bio-assays with specialist of generalist herbivores. Bio-assays were preferentially performed in *L. spicata* as experimental conditions in Las Cruces facilitated the set-up of this type of systems, providing current seawater and air flows, while physiological and metabolomic analyses were preferably carried out on *L. digitata* because of the availability of the measuring instruments and chemical reagents in Roscoff.

This manuscript is divided in three chapters followed by conclusions and future prospects. In the first chapter, local mechanisms and potential signaling of defense against grazers are studied in both kelp species *L. digitata* and *L. spicata*. A first part of this chapter is dedicated to the article about herbivore-induced chemical and molecular responses of the kelps *Laminaria digitata* and *Lessonia spicata*. To complete this chapter, results on emitted compounds in the surrounding seawater of *L. digitata* after challenging by elicitors are presented.

The second chapter is devoted to the effects of distance signaling on the interaction between the kelp and herbivores. This chapter starts with the article dealing with ecological and chemical changes in *L. spicata* to grazing by specialist herbivores. In this article, algal deterrence is tested by non-choice experiments after direct or indirect induction of stress, by measuring algal consumption by herbivores. Complementary results then provide precisions regarding preference towards direct or indirect stress induction of algae. Choice tests were performed in the laboratory on *L. digitata* with the generalist herbivore *Haliotis tuberculata*, and on *L. spicata* with both its specialist herbivore *S. scurra* and a generalist herbivore *Tegula atra*. A preliminary experiment was additionally carried out *in situ* to test the attraction of *L. spicata* for different natural or simulated grazing treatments.

The third chapter focuses on studies of aldehyde-based chemical signaling and its effects on algal metabolome and on the interaction between kelps and herbivores. The article related to this chapter reports the algal physiological and metabolic regulations after application of aldehydes on *L. digitata*, as well as their deterrent effect on the grazer *P. pellucida*. Additional results deal with the effects of incubation of *L. spicata* in aldehydes against the generalist herbivore *T. atra*, by measurement of algal consumption by grazers.

Finally, integration of all these results allows to draw a picture of the chemical communication in kelps in response to grazing stress, especially via signaling by aldehydes. This thesis opens new research lines that are discussed in the final part of the manuscript.

Materials and methods

I. Biological material

1. *Laminaria digitata / Patella pellucida*

Experiments on *L. digitata* were conducted alternatively with both wild sporophytes (i.e. born in natural populations) and individuals grown in the laboratory from spores collected in the field. Wild *L. digitata* were collected at Roscoff (48°43'31"N ; 3°58'8"O) at low tide at coefficient 90 or higher. The whole young plantlet of 20 to 30 cm long was delicately taken for the rocks and brought back to the lab within 1 h. There, algae were stored in tanks with 0.45 µm filtered seawater and constant air supply, at 13±1°C. Light was provided by Philips daylight tubes at a photon flux density of 40 $\mu mol.m^{-2}.s^{-1}$ for 12 h per day. Seawater was changed every 3 – 4 days.

To reduce inter-individual variability in kelp responses to biotic stresses, laboratory-born individuals were produced from mature adult wild sporophytes of *L. digitata* collected in the same populations. They were obtained as unialgal cultures grown from random crosses of gametophytes recovered in the laboratory. Developing sporophytes were then transferred in larger flasks in sterilized seawater together with a Provasoli Enriched Seawater (PES) culture media (10 mL of Provasoli per liter of water). They were placed at 13±1°C with constant bubbling in the same light conditions as wild kelps and grown until they reached a size of about 3 to 4 cm. The medium was changed every week. When algae were ~2.5 cm long, PES supply was stopped and filtered seawater was put instead of sterilized seawater.

Patella pellucida were collected at low tide on the same site as *L. digitata*, in Roscoff. Animals from 1 to 5 mm were softly removed from the meristem or the stipes of *L. digitata*. They were acclimated in an aquarium with constant flow of seawater at 16±1°C and air blowing, and fed with *L. digitata* sporophytes tissue while awaiting experiments.

2. *Lessonia spicata* / *Scurria scurra* and *Tegula atra*

Lessonia spicata plantlets and their herbivores *Scurria scurra* and *Tegula atra* were collected at Las Cruces in Chile (33°30'09"S : 71°37'59"O), aside from the marine reserve belonging to ECIM (Estación Costera de Investigaciones Marinas) marine station. Juvenile kelps were harvested entirely, by removing the holdfast from the rocks. Individuals consisted in a holdfast with all the stipes emerging from it, with a total length between 20 and 50 cm. Plantlets were brought to the laboratory within an hour and put together in a tank at ambient temperature, with constant seawater flow and blowing air, without direct sunlight, for acclimation to the lab. *S. scurra* and *T. atra* grazers, between 13 and 28 mm in diameter, were collected in the same place, softly removed from the stipes of *L. spicata*. In the lab, they were fasted for 48h under constant seawater flow before using for experiments.

II. Algal laboratory treatments

The whole thesis is based on a general methodology presented in Fig. 12 and the different treatments and experiments applied to each kelp species are detailed in Table 1. The distribution of experiments in *L. digitata* and *L. spicata* shows a clear complementarity between chemical analyses and bio-assays carried out in each model species, based on different expertise and facilities available in ECIM and Roscoff's laboratories.

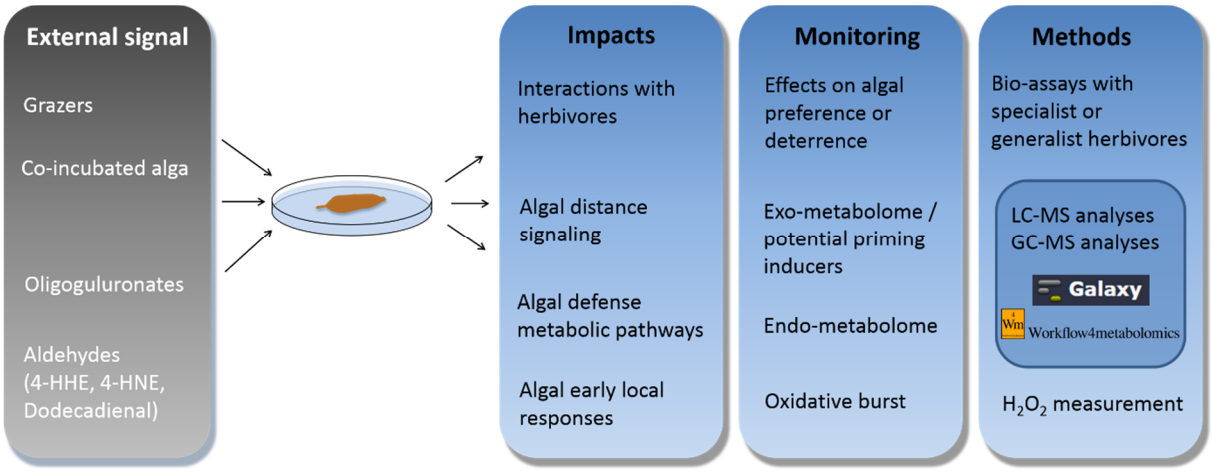

Figure 12. General methodology used in the whole thesis project. After application of biological or chemical external signals, impacts on local to distant levels were investigated via methods of bio-assays, metabolomic analyses and hydrogen peroxide measurement.

Table 1. Details of treatments and experiments applied to each kelp species *L. digitata* (X) and *L. spicata* (X).

	Algal local early responses		**Algal distance signaling and defense metabolic pathways**		**Interactions with herbivores**	
	Oxidative Burst		Exo- and endometabolome		Effects on algal preference and deterrence	
	H_2O_2 quantity measurement		Metabolomic analyses		Bio-assays : choice and non-choice tests	
Grazing				X		X
Co-incubation	X		X	X		X
Elicitation by GG	X		X		X	X
Aldehydes	X		X		X	X

In order to evaluate impacts of biological and chemical external signals on algal responses in terms of early local responses, in terms of metabolism modifications and interactions with herbivores, different treatments were tested on both kelp species *L. digitata* and *L. spicata*.

1. Control

The kelp was put in seawater, without any treatment, in the same conditions as the compared treated kelps.

2. Grazing by herbivores

To determine the effects of a natural grazing on kelp defense and chemical communications, including other effects than mechanical wounding on algal responses such as effects of herbivore saliva, exposure of plantlets to grazers was performed in each kelp-herbivore pair. Herbivores were first starved during 24 h to 30 h before grazing and they were then placed directly on the frond for 24 to 48 h, according to the experiment. This grazing treatment was exclusively performed with *L. spicata*'s grazers *S. scurra* and *T. atra*.

3. Elicitation by oligoguluronate

Using oligoalginate as endogenous elicitors constitutes a simple and standardized way to mimic an herbivore attack, assuming that herbivore consumption is accompanied by a partial digestion of cell wall polysaccharides that releases oligoalginate in the environment. The standardized quantity of elicitor used in these experiments avoids the inter-individual variability of herbivore appetite or consumption rate, influencing stress applied to the kelp. Elicitation was performed with poly-alpha-1,4-L-guluronic acid blocks (GG blocks) prepared by acid hydrolysis of sodium alginate from *L. digitata* stipes (Danisco, Landerneau, France), following Haug et al. (1974). A solution of 0.5% alginates was hydrolyzed at 100°C for ~4 h with 0.3 M hydrochloric acid. The three types of oligosaccharides obtained, oligoguluronates (GG), oligomannuronates (MM) and hybrid oligosaccharides (MG), were separated by alternating centrifugation and selective precipitation steps. The oligoguluronate (GG) fraction was dialyzed, lyophilized and analyzed by gel filtration for testing for purity.

The homopolymeric GG blocks were used as an elicitor at a final concentration of 150 $\mu g.mL^{-1}$, as described in Küpper et al. (2001). Treatments were performed differently according to the kelp species. Indeed, *L. digitata* plantlets, from 2.5 to 4 cm long, were elicited in 30 mL of filtered seawater, allowing a treatment of the whole thallus (Fig 13a). For *L. spicata*, individuals were bigger and only one isolated frond (10 to 15 cm long) per individual was treated in 50 mL Falcon tubes filled with GG solution (Fig 13b) and later refereed as elicited alga. If the total amount of elicited tissue was different between the two species, the external elicitation was applied in similar conditions.

a)

b)

Figure 13. Pictures of elicitation set up in *L. digitata* (a) and in *L. spicata* (b). Laboratory-grown *L. digitata* were small enough to be entirely immersed in GG solution, while fronds of *L. spicata* were partly incubated in a Falcon tube containing the GG solution at 150 µg.mL^{-1}.

4. Co-incubation of two kelps

Determination of waterborne signals effects on algal defense from a neighboring kelp naturally involves a contact of seawater between the two kelps. Thus, and in order to stay close to conditions in nature, co-incubation of two kelps was performed, allowing chemical emission and potentially simultaneous perception by the neighboring kelp.

5. Incubation in aldehyde solutions

As aldehydes were shown to be emitted after GG elicitation of *L. digitata* (Goulitquer et al., 2009), it is supposed that they could play a role in chemical communication between kelps, then perhaps having an effect on their interactions with grazers. Thus, some aldehydes from Thomas et al. (2011) were chosen and applied to *L. digitata* or *L. spicata* fronds at different concentrations. We chose the 4-hydroxy-2-hexenal (4-HHE; 10 mg.mL^{-1}), 4-hydroxy-2-nonenal (4-HNE; 10 mg.mL^{-1}) both obtained from Cayman Chemical, and 2,4-Dodecadienal (20 mg.mL^{-1}) from Sigma-Aldrich. For application in experiments, they were diluted in EtOH 70%.

III. Experimental setups

1. Exo-metabolome of an elicited alga

Wild *L. digitata* were either elicited with GG solution at 150 µg.mL^{-1} or let as controls in filtered seawater for 30 min at ambient temperature. Four replicates were made, each containing 4 algae put

together in 100 mL filtered seawater. Algae were rinsed three times for 5 min with filtered seawater and placed individually in 50 mL filtered seawater. After 1 h, 4 h, 12 h and 24 h, the surrounding seawater was recovered, and algae were blotted dry and weighted. Both algae and seawater were freezed in liquid nitrogen and conserved at -80°C until metabolomic analyses.

2. Co-incubation of *L. spicata*

a. Development of general laboratory experimental set up

The experimental unit consisted in two *L. spicata* plantlets placed in a 40 liters aquarium with constant seawater and air supplies. One of them was submitted to a stressful treatment, either chemical elicitation by oligoguluronates or direct grazing by *S. scurra*, and the other kelp was co-incubated with the treated one. In parallel, two control plantlets were manipulated in the same way, but without any elicitor or grazer. The co-incubation of the treated individual with its neighbor (or the respective controls) lasted 24 or 48 hours, according to the type of treatment (see below for details). Independently, the autogenic changes of algal mass was assessed during the whole period of the experiment and checked for the different treatments of the *L. spicata* plantlets. For this, a separate set of experimental units with the same elicitation treatments was dedicated to estimate biomass changes for the duration of the grazing experiment, but kept without any further grazing.

b. Grazing experiment

Two individuals of *S. scurra* were first deposited on one frond of 10 *L. spicata* plantlets kept in a net, to avoid herbivore escape. The kelp in the net was named DG (direct grazed), whereas the other was named IG (indirect grazed, e.g. not directly in contact with the grazer, but co-incubated with the DG alga). The controls used for each treatment were named C_{DG} (Control direct grazed) and C_{IG} (Control indirect grazed) respectively. After 48 hours of co-incubation, the most grazed frond of DG and one frond of IG, C_{DG} and C_{IG} plantlets were selected for the bio-assay. The Grazing experiment was independently repeated three times.

c. Elicitation experiment

Chemical elicitation was done by placing, during 1 hour, one frond of the directly elicited individual (E) in a 50 mL-Falcon tube filled with 150 $\mu g.mL^{-1}$ GG dissolved in seawater. The Falcon tube was adjacent to the aquarium so the integrity of the individual was kept, and no more than 5 cm of frond tissue was exposed to air, between the aquarium and the tube. The control treatment was identical, except for the Falcon tube that was filled with seawater only. The separately-treated fronds (E and C_E) were then rinsed with seawater and restored in the aquarium, with the co-incubated *L. spicata*. In August, seawater was renewed one hour after elicitation and let in the aquarium for 24 hours. In November,

plantlets were placed in aquarium with constant water supply. Fronds elicited in GG solution (E ; Elicited) or immersed in seawater (C_E ; Control elicited), and fronds from the co-incubated kelps (IE and C_{IE} respectively for Indirectly Elicited and Control indirectly elicited) were cut at the stipe, blot-dried and weighted for the bio-assay. The Elicitation experiment was done three times.

3. Elicitation treatment of *L. digitata* for food preference test of *H. tuberculata*

Wild adult *L. digitata* from 60 cm to 1.20 m were treated either by elicitation with GG or as control and were then exposed to a feeding choice tests on abalones. Treatments were done 3 days before the bio-assay. Elicitation was performed in 150 $\mu g.mL^{-1}$ GG for 1 h with bubbling, while control algae were incubated in seawater. They were then placed separately in two tanks until the beginning of the choice test.

4. Grazing and co-incubation of *L. spicata* for food preference test of *T. atra*

L. spicata plantlets were exposed to different treatments, then presented as food choices for *T. atra* in Y tubes bio-assay. Algae were either treated as controls (without treatment), directly grazed by *T. atra* or by *S. scurra*, co-incubated with a kelp grazed by *T. atra* or by *S. scurra*. For grazing and co-incubation treatment with *T. atra*, one treatment (treatment $^{(A)}$) was performed with grazing by 17 herbivores placed on 14 fronds of *L. spicata* for 36 h and the co-incubation lasted 36 h, and the other treatment (treatment $^{(B)}$) involved 30 herbivores grazing on 12 fronds for 24 h and the co-incubation lasted 24 h. For *S. scurra* treatments, 17 herbivores were placed on 3 *L. spicata* fronds for 36 h and the co-incubation lasted 36 h. Grazed fronds were put individually in 6 L boxes with constant seawater flow, each upstream to an alga receiving the seawater flow from the grazed kelp and thus treated by co-incubation with a grazed kelp. Control fronds were put individually in boxes with seawater flow.

5. Attraction of natural or artificial grazing of *L. spicata* for *S. scurra in situ*

In situ experiments were carried out at Las Cruces on *L. spicata* individuals and their herbivores *S. scurra* in order to investigate the attraction of natural and artificial wounding on *S. scurra*'s behavior. Different treatments (Table 2) were applied to kelps and presence or absence of *S. scurra* on *L. spicata* individuals was noted after 1 and 5 days.

Table 2. *In situ* treatments of *L. spicata* tested on attraction of *S. scurra*.

A	**Attraction of an artificial wounding 24h after wounding generation**
	5 *L. spicata* with artificial wounding made at D0
	1 *S. scurra* added on the wounding at D1
B	**Control artificial wounding**
	5 *L. spicata* with artificial wounding made at D0
	No addition of *S. scurra*
C	**Control of grubbing-up effect**
	5 *L. spicata* with natural wounding and 1 *S. scurra* into the wounding
	Removing and putting back the herbivore in the wounding
D	**Attraction of the natural wounding**
	5 *L. spicata* with natural wounding and 1 *S. scurra* into the wounding
	Removing and putting the herbivore outside the wounding, on the same stipe
E	**Natural recolonization**
	5 *L. spicata* with natural wounding and 1 *S. scurra* into the wounding
	Removing and putting the herbivore on a neighboring stipe on the same *L. spicata* individual
F	**Observation of S. scurra in its wounding**
	5 *L. spicata* without treatment

6. Aldehyde incubation and elicitation treatments of *L. digitata*

Laboratory-grown *L. digitata* plantlets were submitted either to an elicitation, a control or an aldehyde treatment, at 13°C under agitation. They were incubated in filtered seawater and in GG solution at 150 µg.mL^{-1} for 1 h, for control and elicitation treatment, respectively. Aldehyde treatment lasted 4 h and consisted in incubation of algae in 4-HHE, 4-HNE or in dodecadienal. 4-HHE was tested at 1 µg.mL^{-1}, 500 ng.mL^{-1}, 100 ng.mL^{-1}, 50 ng.mL^{-1} and 10 ng.mL^{-1}; 4-HHE was tested at 1 µg.mL^{-1} and 100 ng.mL^{-1}; dodecadienal was only tested at 100 ng.mL^{-1}. H_2O_2 emission was measured before and during 1 h incubation in all solutions. Then, algae were either used for metabolomic analyses in LC-MS, or in non-choice feeding bio-assay with *P. pellucida*.

7. Aldehyde treatment for *L. spicata* consumption studies by generalist grazers

L. spicata fronds were treated either with the 2 aldehydes 4-HHE and 4-HNE, both at 100 ng.mL^{-1} and 10 ng.mL^{-1} or as controls, or co-incubated with a grazed kelp by *T. atra*. Treatment by aldehydes lasted 4 h, like for *L. digitata*, at room temperature. The co-incubation treatment was performed with a grazed kelp by 2 *T. atra*, placed upstream to a kelp receiving the seawater flow from the grazed kelp, thus treated by co-incubation with a grazed kelp. This co-incubation treatment lasted 24 h at room temperature.

IV. Hydrogen peroxide measurement by chemoluminescence

Kelps rapidly respond to grazing mimicry via application of exogenous or endogenous elicitors by emitting hydrogen peroxide (Küpper et al., 2001; Küpper et al., 2006), then providing higher algal resistance against pathogens (Küpper et al., 2002). Thus, we used the accumulation of H_2O_2 contained in the surrounding seawater as an estimation of algal stress measurement. The concentration of hydrogen peroxide in the medium around algae was determined using the luminol chemiluminescence method (Glazener et al., 1991) with a Tristar luminometer (EG&G Berthold, Bad Wild-bach, Germany). This device is equipped with two injectors, bathed in two reagents: the luminol dissolved in DMSO solution (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) diluted at a final concentration of 0.3 M in distilled water and a horse-radish peroxidase (HRP; Sigma) in solution with 10 mM pH 7.8 phosphate buffer. The reaction between these two reagents and the hydrogen peroxide triggers emission of light, whose quantity is proportional to the concentration of H_2O_2 in the sample: luminol + H_2O_2 $\xrightarrow{HRP}$ aminophtalate + N_2 + hν.

An optimization of the luminometry measurement method was developed on the Tristar luminometer. For each measurement, 150 µL of surrounding seawater were collected and put in 96-well microplates (Lumitrac 200, White, Greiner Bio-One). In the luminometer, after a first injection of 50 µL of 20 $U.mL^{-1}$ horse-radish peroxidase, the microplate was shaken for 1.0 s and 100 µL of luminol solution were added to the sample. Then, chemoluminescence was recorded immediately after the last injection with a signal integration time of 0.55 s. Finally, after several tests of delays, a time delay of 10 s was introduced for device stabilization before the next measurement. This sequence was repeated for each well of the microplate. At the end, for calculating the concentration of H_2O_2 present in the samples, calibration with a standard curve was carried out once during any series of measurements.

V. Global metabolomic analyses

1. Metabolite extraction

Metabolome analyses were performed both on seawater surrounding algae (exo-metabolome) and on algal tissues (endo-metabolome). This allowed to investigate the potential waterborne signals emitted by kelps in the medium, together with the metabolites produced into the alga in response to a stimulus. Thus, different types of compounds were targeted: small compounds of molecular weight <1 kDa and bigger compounds. This implied different extraction methods and analysis techniques.

a. Solid-liquid extraction

Metabolite extraction was performed with 100 mg of frozen algal powder mixed with 1 mL MeOH:H_2O (8:2), and placed under agitation during 1 h at 4°C. In each sample, 1.25 µg.mL^{-1} 12-OH-lauric acid was added as an internal standard for the negative ionization mode, and 10 µg.mL^{-1} of reserpine for the positive mode. The MeOH phase was recovered and dried under N_2 at 40°C. For metabolomic analyses, 90 µL EtOH were added to each sample and aliquots of 50 µL of each extract were used for analyses.

For the analysis of the endo-metabolome of algae after application of aldehydes, an additional extraction step was performed after the first extraction with MeOH/H_2O. We chose to focus on regulation of the fatty acid pathways, thus targeting fatty acids and their oxidative derivatives such as oxylipins. To separate free fatty acids and their derivatives from polar compounds, samples were first dried under N_2 at 40°C and a second metabolic extraction was made with 200 µL Ethyl acetate and 200 µL H_2O. The ethyl acetate phase was recovered and dried under N_2 at 40°C. 90 µL EtOH were added to each sample and aliquots of 50 µL of each extract were used for metabolomic analyses in LC-MS.

b. Solid Phase Extraction (SPE)

At the end of the treatment step, 50 mL of the surrounding seawater were collected from each sample and diluted in 200 mL distilled water, in which 50 µg of the internal standard 12-OH lauric acid were added. SPE instrument (Dionex Autotrace) is equipped with 6 injection systems designed for 6 SPE cartridges. C18 SPE cartridges (REC18 1 g/6 mL or 500 mg/6 mL) were first conditioned twice with 10 mL MeOH, then equilibrated twice with 10 mL H_2O. Samples were then entirely loaded on the cartridges. Washing the tubes twice with 10 mL H_2O removed the most polar compounds from the cartridge and drying them with liquid nitrogen for 15 min permitted to eliminate H_2O traces in samples. Finally, elution of compounds was performed by 3 injections of 10 mL MeOH on the cartridges. The 8 mL eluates were dried with N_2 and heated at 60°C. They finally were recovered in 200 µL MeOH/H_2O 80:20 (v:v) and placed in vials for LC-MS analyses.

c. Stir-Bar Sorptive Extraction (SBSE)

The extraction by sorption on a stir bar is based on the equilibrium of analytes between the water and a polymeric phase immersed in this matrix, the polydimethylsiloxane (PDMS), supported by a stir bar. Extraction of compounds was carried out during stirring, by sorption of small compounds into the PDMS matrix. Therefore, a SBSE stir bar (GERSTEL Twister) was introduced in the 50 mL of collected surrounding seawater containing a plantlet in the experiment, with 20 ng of the internal standard

adonitol, then placed under stirring for 12 h. After extraction, stir bars were blotted dry and placed in GC-MS tubes (1 stir bar per tube).

2. Metabolomic analyses methods

a. Gas-Chromatography coupled with Mass Spectrometry (GC-MS)

GC-MS is an analytic method permitting to detect and identify small volatile compounds present in samples, by thermal desorption. This technique may require a previous derivatization of compounds, in order to make them volatile or to stabilize them. Thus, samples were dried under N_2 at 60°C, acetonitrile and Sylon (BSTFA/TMCS 99:1) were added to dry extracts and they were let for 30 – 60 min at 60°C. Solvents were evaporated under N_2 and hexane was added to take the dry extract and analyze in GC-MS.

Metabolites were analyzed by GC-MS using an Agilent GC 7890+ coupled to a 5975 quadrupole mass detector (Agilent, Les Ulis, France) and equipped with a DB/HP-5MS column (methyl phenyl siloxane: 30 m × 0.25 mm I.D. × 0.25 µm film thickness; J&W Scientific, Agilent) in the Electronic Impact (EI) mode at 70 eV. A sample (volume of 2 µL) was injected into the capillary column at 1 $mL.min^{-1}$, with a temperature of injection of 230°C. Then, the temperature gradient used was 60°C for 2 min, 60 to 290°C at $10°C.min^{-1}$ and stabilization at 290°C for 5 min. The range of masses analyzed were from 50 to 800 m/z.

b. Liquid-Chromatography coupled with Mass Spectrometry (LC-MS)

LC-MS is used to analyze and identify small compounds in a liquid phase, driven by solvents of different polarity. Chemical compounds were first separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an electrospray ionization source (ESI). Scans were collected in both positive and negative ionization modes over a range of m/z 50–1000. Ionization parameters were set as follows: sheath gas 5 psi, auxiliary gas 5 (arbitrary units), sweep gas 0 (arbitrary units), spray voltage 2.7 kV, capillary temperature 300°C, capillary voltage 60 V, tube lens voltage 127 V and heater temperature 300°C. Metabolic samples were analyzed using a 1.9 µm Thermo Hypersil Gold C18 column (100 x 2.1 mm) maintained at 20°C using 5 µl injection volume and a flow-rate of 250 $µl.min^{-1}$. Mobile phase A was composed of 0.1% acetic acid in H_2O, and mobile phase B was 0.1% acetic acid in acetonitrile. The gradient, starting at 0% B, consisted of an initial hold at 60% mobile phase B from 1 to 3 min, followed by a gradient to 95% B in 12 min and a hold for 4 min, followed by re-equilibration for 4 min at 0% B, for a total run time of 23 min. Xcalibur 2.1 software was used for instrument control and data acquisition.

A specific LC-MS method was developed for analysis of the endo-metabolome of *L. digitata* after exposition to aldehydes. The gradient, starting at 0% B, consisted of an initial hold at 60% mobile phase B from 1 to 3 min, followed by a gradient to 95% B in 12 min and a hold for 4 min, followed by re-equilibration for 4 min at 0% B, for a total run time of 23 min.

c. LC-MS/MS

Complementary analyses by LC-MS/MS were performed on samples of the endo-metabolome of *L. digitata* after exposition to aldehydes and on chemical standards, in order to determine the absence or presence of these compounds in the algal samples. These analyses were performed by Yann Guitton in LABERCA (Laboratoire d'étude des résidus et contaminants dans les aliments) in Nantes.

- Chemicals

All solvents and reagents were of analytical LC-MS grade quality. Acetonitrile (ACN), water (H2O) and acetic acid were purchased from Sigma-Aldrich. Methanol (MeOH) was purchased from VWR. Standards were leucine-5,5,5-d3, L-tryptophan-2,3,3-d3, Indol 2,4,5,6,7-d5-3-acetic acid and tetradecanedioic-d24 acid (Sigma Aldrich). The standard mixtures used for the external calibration of the Q Exactive™ were Pierce™ LTQ Velos ESI Positive, for the positive ionization mode, (caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate and Ultramark 1621) and Pierce ™ Negative (consisting of SDS, sodium taurocholate and Ultramark 1621) (Thermo Fisher Scientific). Chemical standards tested for their presence in the samples came from Interchim: jasmonic acid, 9(S)-hydroperoxyoctadecatrienoic acid (9(S)-HpOTrE), 13(S)-hydroperoxyoctadecatrienoic acid (13(S)-HpOTrE), 20-hydroxy-leukotriene B4 and stearidonic acid.

- Instrumentation

A high performance liquid chromatography (HPLC) system (Ultimate® 3000 Dionex, Thermo Fisher Scientific) was used. The chromatographic separation was performed on Hypersil Gold C18 column (2.1 mm × 100 mm, 1.9 µm particle size, Themo Fisher Scientific). Mobile phase consisted in water (A) and acetonitrile (B) both containing 0.1 % acetic acid (A). The used elution gradient (A:B, v/v) was as follows: 100:0 from 0 to 1.0 min; 40:60 at 3 min; 5:95 at 15 min for 4 min then 100:0 for 4 min. The injected volume was 5 µL, the flow rate was 350 µL min^{-1} and the temperature of the column was maintained at 35 °C.

The LC system was coupled to a Hybrid Quadrupole-Orbitrap Mass Spectrometer (Q Exactive™, Thermo Fisher Scientific) with a heated electrospray ionization source (HESI). Nitrogen was used as sheath gas and auxiliary gas at flow-rates of 55 and 10 a.u (arbitrary units), respectively. The ion transfer tube temperature was set at 350°C, the vaporizer temperature at 300°C and the electrospray voltage was set at 3.0 kV in positive mode and -3.0 kV in negative with a s-lens RF level of 50%. For fullMS

acquisition a polarity switching ion mode (positive/negative) with a mass range of *m*/z 65-975 at a mass resolving power of 70 000 Full Width Half Maximum (FWHM at 200 m/z) are used. The automatic gain control target and maximum injection time were 1e6 counts and 100 ms, respectively. The normalized collision energy (NCE) used for targeted MS/MS and data dependent MS/MS modes was 35. Each target m/z was monitored with a 40 seconds window (retention time ± 20 seconds), 2-amu isolation window (target m/z ± 1 amu).

For injection, the extract was diluted by 2 in ACN:H_2O (50:50) and 5 µL were injected the same day on the LC-HRMS system.

VI. Bioassays: preference or algal consumption by herbivores

Algal responses to grazing by herbivores were evaluated by the proxy of the consumption of algal tissues by herbivores, following different treatments applied to the alga. Both kelp/herbivore couples were used for bio-assays. Herbivory pressure was regulated according to the size and the number of herbivores on the frond and depending on the size of the frond. *P. pellucida*, much smaller than *S. scurra* or *T. atra*, were put by 5 or 6 on *L. digitata* laboratory-grown fronds, whereas maximum 2 *S. scurra* or *T. atra* were placed on a 20-cm *L. spicata* frond. Two approaches were tested to determine either food preference of the herbivores by choice tests or deterrence by non-choice tests.

1. Multiple choice feeding assays

Food preference of herbivores was estimated by attraction to different types of food offered to grazers. Thus, the movement of the herbivore towards one fixed proposition of food allowed to predict potential effects of chemical compounds on herbivore behavior. In each experiment, two food choices were proposed to herbivores.

In the *L. digitata* experiment, food preference of the kelp generalist herbivores abalones (*Haliotis tuberculata*) was tested. Prior to the experiment, abalones of the same age were fasted for 3 days in the experimental system for acclimation. Five tanks, each containing 8 abalones and 4 feeders were installed, with constant seawater and air supply. The photoperiod was 10h30 of light, from 6:30 am to 8:00 pm. As described below, the first food proposition was elicited algae with GG, and the second was healthy algae (without treatment). The first day of bio-assay, in each tank, two feeders were filled with control kelps and the two others with elicited *L. digitata*, 20 g of algae per feeder. The order of elicited and control feeders in each tank was chosen randomly. Consumption of treated algae were measured after 2 and 4 days. Algae were blotted dry and weighted, then replaced in the tank until the end of the experiment. Moreover, during the whole experiment, one infrared camera over each tank filmed 24/24h abalone movements, thanks to animal tagging with phosphorescent paper on their shell.

In the *L. spicata* experiment, choice test was performed with *T. atra* and *S. scurra*, through a Y tube. Food propositions were situated at the end of the two upper branches of the Y tube, and the herbivore was placed in the lower branch (Fig 14). A seawater flow was created, from each food source to the lower branch, in order to permit the herbivore to detect chemical signals coming from the sources and thus going until this emitting source. At the source, water comes from 6 L boxes which can contain algae. The food preference of *T. atra* was tested between different treatments (Table 3). The experiment lasted 3 min and repeated 15 times for each couple of choices.

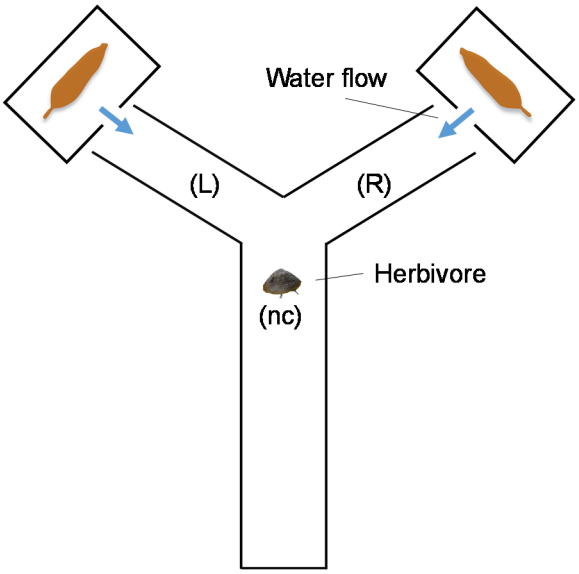

Figure 14. Schema of the Y tube experiment. Food choices are situated at the end of each branch of the Y tube and a seawater flow at the food sources is created. The herbivore is placed in the lower branch and can choose between the left side, the right side or the non-choice zone.

Table 3. Choices tested in the Y-tube experiment on *T. atra*. In each experiment, 30 *T. atra* individuals were submitted to the choice test. Their choice (choice A, choice B or non choice) and the time of choice were recorded from the moment they show the first movement. C_{DG}: Control Direct Grazing, DG: Direct Grazing, IG: Indirect Grazing.

Exp. number	Choice A	Choice B
1	None	None
2	None	C_{DG} (Control)
3	C_{DG} (Control)	IG (Co-incubated with grazed by *T. atra*) [(A)]
4	None	DG (Grazed by *T. atra*) [(A)]
5	C_{DG} (Control)	DG (Grazed by *T. atra*) [(A)]
6	C_{DG} (Control)	DG (Grazed by *T. atra*) [(B)]
7	DG (Grazed by *T. atra*) [(B)]	IG (Co-incubated with grazed by *T. atra*) [(B)]
8	C_{DG} (Control)	IG (Co-incubated with grazed by *S. scurra*)
9	DG (Grazed by *S. scurra*)	IG (Co-incubated with grazed by *S. scurra*)

Laboratory choice tests were also performed in *L. spicata* to assess the preference of *S. scurra* for natural wounds on stipes. Stipes containing wounds naturally made by *S. scurra* on the field were collected by cutting 15 cm long piece of stipes with a wound on it. In the laboratory, the stipes were individually placed in tanks together with one *S. scurra* individual at a distance from the wound. In parallel, control stipes without grazing marks were cut and placed individually in tanks, with one *S. scurra* put in the middle of the stipe. Herbivore's movement was evaluated, either choosing the wound or another direction. The experiment was repeated 10 times for control stipes and 9 times for wounded stipes. Herbivore's position was noted after 1 h and 16 h in the first experiment, and after 30 min and 1 h in the second experiment.

2. Non-choice feeding assays

In non-choice bio-assay, herbivores had only one source of food, directly available for consumption. According to the algal and herbivore species, the number of herbivores on the frond was variable.

For *L. digitata*, laboratory-grown algae were used with its specialized herbivore *P. pellucida*. Bio-assay made after incubation in aldehydes or in GG consisted in placing one young plantlet in 50 mL filtered seawater, exposed to 6 herbivores for 6 days at 13°C. Algal mass was measured before the bio-assay, 3 days and 6 days after exposition to grazers. Five replicates were made for each treatment, while 3 plantlets were used to control autogenic changes of mass during bio-assay.

For *L. spicata*, one frond was cut after treatment and placed in a 6 L box with constant seawater supply and 2 *S. scurra* or *T. atra*. After Grazing or Elicitation treatment, the frond exposed to one of these treatments was cut and used for the bio-assay. Algae were blotted dry and weighted before the experiment, and 2 and 4 days (end of the experiment) after exposition to grazers. Ten replicates were made for each treatment.

VII. Data processing and statistical analyses

Analyses of the oxidative burst, metabolome modifications and herbivore consumptions led to different types of data, involving specific statistical analyses for each of these types of data. Measurement of H_2O_2 at different sampling times during 60 min allowed to calculate the total amount of H_2O_2 accumulated over 60 min. Then, after checking normality with the Shapiro-Wilk test, a 1-way ANOVA was performed, followed by a Student-Newman-Keuls (SNK) post-hoc test to determine the significant differences between each treatment.

Metabolomic data obtained with Xcalibur software corresponded to a chromatogram and mass spectra for each sample. Mass spectra data were processed by Galaxy-Workflow4Metabolomics (W4M) on the online version (Giacomoni et al., 2014), after conversion of raw spectra to mzXML format for LC-MS data and in CDF format for GC-MS data. In W4M, default settings were used for GC-MS data. For LC-MS analyses, data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples necessary at 0.7. For correction of retention time, the obiwarp method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 10. Fillpeaks step was used with the chrom filling method. Finally, annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. The final table contained ions abundances in samples, which corresponded to peak areas. Principal Component Analysis (PCA) and Partial Least Squared – Discriminant Analysis (PLS-DA) were performed with W4M in order to check the quality of the analysis and the distribution of the samples. Then, normalization was done by dividing integrated areas either by the area of the internal standard in the sample when it was detectable, or first by the highest one of the same sample, and then by dividing the areas of one ion by the highest in all the samples. This would allow to compare the relative amount of one ion in different samples. Manual elimination of background and redundant (isotope) ions led to a selection of the most relevant ions which could play a role in metabolomic modifications of algae. Subsequent analyses were performed using the free software R. After installation of the packages vegan, pls and RVAideMemoire, PCA and PLS-DA were performed and Number of MisClassifications (NMC) and p-value of PLS-DA were obtained. Then, using W4M, extraction of the 10 most discriminant ions using VIPs and annotation of some of those ions were made, also from previous studies (Ritter et al., 2008).

Bio-assay results were analyzed differently according to the type of experiment. For non-choice tests, data were often non-normal due to inter-individual variability from variation between algae, between the appetite of herbivores, and also because of the variation in the interaction between the kelp and its herbivore. Thus, to compare mean algal consumption after exposition to different treatments (usually more than 2 treatments per experiment), Kruskal-Wallis tests were performed. For choice tests, multinomial tests were applied for the Y-tube experiment because herbivores had three choices. Food preferences of abalones were analyzed in two ways: Wilcoxon test was applied for comparison between consumption between elicited and control algae.

Chapter I

Herbivore-induced chemical and molecular responses of the kelps *Laminaria digitata* and *Lessonia spicata*

Introduction

Kelp forests constitute complex ecosystems that play habitat and food source roles for the diverse associated species. As they are sessile, these brown macroalgae have to cope with biotic stresses, such as grazing by herbivores. During herbivore attack, oligoguluronates are likely to be released at the surface of the kelp and this is well known that kelps respond directly to these MIMPs at different spatio-temporal levels like a transient oxidative burst, metabolic and transcriptomic regulations, and later resistance against some pathogens (Küpper et al., 2002; Bouarab et al., 2004; Weinberger, 2007). However, nothing was known about the nature and the timing of the chemical and molecular responses of kelps upon grazing by herbivores. Therefore, we investigated direct inducible responses of two kelps *Laminaria digitata* from north Atlantic coasts and *Lessonia spicata* from Chile. They were studied at two levels, inside and outside the kelp tissues, in order to assess both compound production and accumulation in tissues and emitted compounds which could be perceived by herbivores or by other kelps. Later on, a part of the emitted compounds could represent a potential signaling for the neighboring kelps to prevent a danger.

Inner molecular and chemical responses in algae were discussed in the article. Indeed, we already know that, upon challenging with oligoguluronates, the kelp *L. digitata* recognizes this external signal as an elicitor signal, leading to activation of signaling pathways and molecular responses (Küpper et al., 2001; Cosse et al., 2009; Goulitquer et al., 2009). Elicitation generates an oxidative burst as well as a release of polyunsaturated free fatty acids coming from biological membranes, then oxidized and liberated in the medium. Animals and land plants utilize these compounds as stress signaling molecules and brown algae contain fatty acids from both lineages, allowing synthesis of a wide variety of these oxidized derivatives in the brown algae. Simultaneously, defense responses are activated at the transcriptional level, through regulation of genes related to grazing stress, such as in Fucales in which a massive gene regulation is combined with defense responses against herbivores (Flöthe et al., 2014). The objective of this article was to carry out comparative metabolome and transcriptome analyses of the South Pacific kelp *Lessonia spicata* and the North Atlantic kelp *L. digitata* submitted to grazing stress.

My contribution to this article concerned the metabolomic component of this work, with the objective of investigating the metabolic regulations occurring into algal tissues in response to direct grazing stress. It consisted in analyzing the modifications of the endo-metabolome of *L. spicata* and *L. digitata* after exposition to a direct grazing over two days, by comparison to control algae, with metabolomic tools like Liquid Chromatography – Mass Spectrometry (LC-MS) followed by data processing with the developing device Galaxy-Workflow4metabolomics. The experimental design is described in the article and data analysis was processed by two approaches, the first approach consisted in a global analysis of the algal endo-metabolome in order to determine if this metabolome globally changed after a grazing stress. The chemical profiles of some compounds were then investigated in a

second approach, for which we chose to focus on amino acids, fatty acids and oxylipins. Indeed, as previously described, these chemical compounds are involved in algal response to abiotic and biotic stress, and application of the aldehyde 4-HHE which is derived from the fatty acid oxidation activates this pathway (Ritter et al., 2008; Goulitquer et al., 2009). Moreover, as fatty acids in brown algae are related to those in plants and in animals (Fig 15), we can rely on this information to reconstruct the biosynthetic pathways induced in kelps.

In addition to chemical modifications into the alga, we also know that kelps emit chemical compounds in the surrounding medium in response to oxidative stress such as low tide or in response to elicitation by oligoguluronates (Goulitquer et al., 2009). Indeed, C6- and C9-aldehydes were found to be strongly induced and emitted during and 1h after low tide, and their concentrations decreased when the sea rose. However, the quantity changes of the emitted compounds were not monitored over time. We thus investigated chemicals emitted in the surrounding seawater of stressed laboratory-grown *L. digitata* after 1h, 4h, 12h and 24h of elicitation by oligoguluronates, supposed to reproduce a grazing effect. The seawater was collected and extracted both with Solid Phase Extraction (SPE) and with Stir Bar Sorptive Extraction (SBSE) methods, then respectively analyzed in LC-MS in negative and positive ionization modes, and in GC-MS. This complementary approach would allow to have a global vision of the emitted compounds during defense responses and grazing stress.

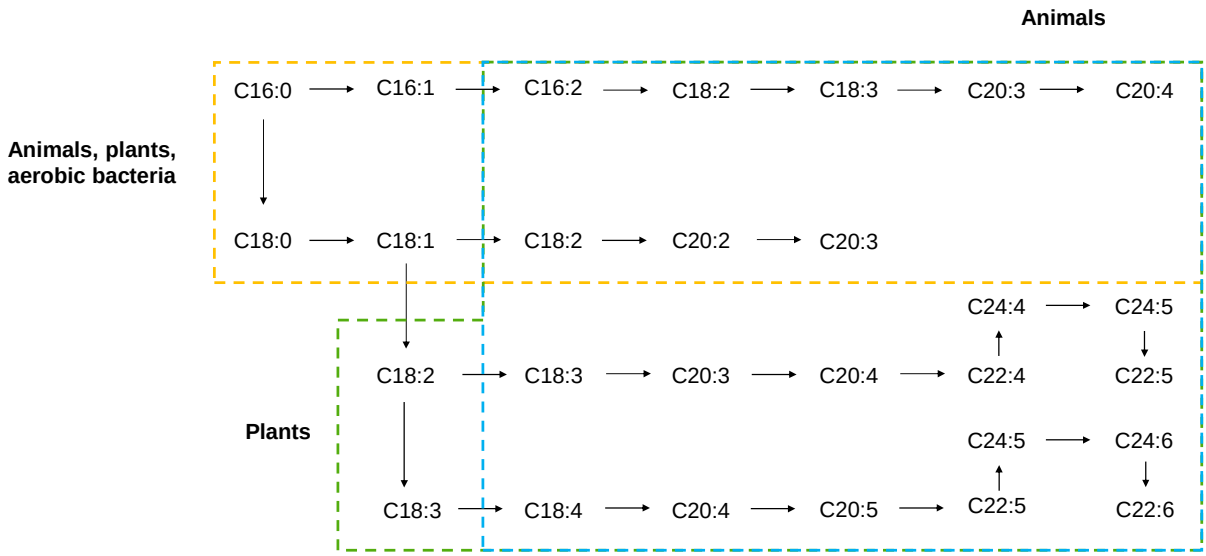

Figure 15. Free fatty acids biosynthetic pathways in plants, animals and aerobic bacteria. From Goulitquer (2008).

I. Kelp endo-metabolome in response to grazing

General outline

Global metabolomic analyses resulted in acquisition of 211 ions for *L. digitata* and 818 ions for *L. spicata*, which were considered for statistical analyses. In both kelp species, PLS-DA allowed to discriminate between grazing and control treatments. However, they did not show differences of the metabolome according to time. This could suggest that produced compounds into algal tissues after 6h undergo a concentration stabilization, at least during 48h. Moreover, annotation of 9 free fatty acids (FFA), 3 oxylipins, and 9 amino acids (AAs) was achievable, and the patterns of these targeted compounds showed a clear difference between samples collected after 6 and 12h and those collected after 24 and 48h of grazing. Amounts of amino acids strongly increased at 12h, then reaching control levels at 24h. However, free fatty acids and their oxidized derivatives started to accumulate at 12h, reaching higher levels later than amino acids, at 24h, then decreasing at 48h. Fatty acids released during grazing stress were eicosanoids and octadecanoids, both direct precursors of oxylipins such as prostaglandins, also detected in samples mostly at 24h after grazing. Thus, this work on metabolic regulations in kelp tissues during grazing stress allowed to highlight the activation of metabolic pathways related to accumulation and oxidation of free fatty acids, as well as a release of amino acids in algal tissues.

Article

Herbivore-induced chemical and molecular responses of the kelps *Laminaria digitata* and *Lessonia spicata*

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Abstract

Kelps are founding species of temperate marine ecosystems, they evolve in the intertidal coastal areas where they are often challenged by generalist and specialist herbivores. As most of sessile organisms, kelps develop defensive strategies to restrain grazing damage and preserve their own fitness during interactions with herbivores. To decipher some inducible defense and signaling mechanisms, we carried out metabolome and transcriptome analyses in two emblematic kelp species, *Lessonia spicata* from South Pacific coasts and *Laminaria digitata* from north Atlantic, when challenged with their main specialist herbivores. Mass spectrometry based metabolomics allowed to observe large metabolic changes induced in these two brown algae following challenges with their own specialist herbivores. Targeted metabolic profiling also showed that free fatty acid (FFA) and amino acid (AA) metabolisms were particularly regulated under grazing in *L. spicata*. An early stress response was illustrated by the accumulation of Sulphur containing amino acids in the first twelve hours of herbivory pressure. At latter time periods (after 24 hours), we observed FFA liberation and eicosanoid oxylipins synthesis likely representing metabolites related to stress. Transcriptomic analysis identified sets of genes specifically induced by grazing in both kelps. Most of these genes were particularly activated by herbivore stress after 24 hours, suggesting that transcriptional reprogramming could be operated at this time period. We demonstrated the potential utility of these genes as molecular markers for herbivory by measuring their inductions in grazed individuals of field harvested *L. digitata* and *L. spicata*. By unravelling the regulation of some metabolites and genes following grazing stress in two kelps representative of the two hemispheres, this work contributes to provide a set of herbivore-induced chemical and molecular responses in kelp species, showing similar inducible responses upon specialist herbivores in their respective ecosystems.

Introduction

Brown algae are photosynthetic sessile macro-organisms of high ecological relevance in coastal ecosystems of temperate and polar regions. They belong to the Stramenopile lineage, which diverged early in the evolution from Opistokonts (including animals, fungi) and Plantae (land plants, green and red algae) and constitute one of the rare eukaryotic lineages to reach complex multicellularity [1]. Little is known about their biology, as they were mostly studied for their taxonomic and ecological diversity. The recent genome sequencing of the filamentous brown alga *Ectocarpus siliculosus* underlined particular molecular features in these organisms to cope with highly variable tidal environment such as an extended set of light-harvesting and pigment biosynthesis genes, and original metabolic processes such as halide metabolism [2]. Brown algae include mostly marine species ranging from small filamentous organisms to large kelps measuring several meters in size. Kelps (order of Laminariales) have primordial roles in rocky coastal ecosystems of temperate regions, from North and South hemispheres, as they form true forests of considerable biomass, hosting highly diversified ecosystems [3]. These marine organisms are also exploited for nutritional or industrial purposes and therefore constitute a significant economical resource. One particularly important question is what makes the ecological and evolutionary success of these giant marine primary producers. Indeed, kelps are continuously submitted to biotic attacks including viruses, bacteria, fungi brown algal endophytes or grazing herbivores. Yet, they are the only taxon that evolved large sizes (i.e. few m to tens of m), somehow escaping or overriding the effect of these attacks. The question is relevant also for the future of the rapidly growing field of kelp aquaculture. Indeed, as it is not possible to spread pesticides in the ocean (i.e. the currents would wash away the products before they actually have any effect in a marine culture), it is necessary to explore methods of protection against herbivore, pests and pathogens that rely on the activation of defense mechanisms by the seaweed itself.

Among biotic pressures, herbivore grazing has a major impact in marine benthic ecosystems [4]. To respond to these challenges, kelps are likely to develop adaptive strategies based on activated and induced metabolic responses [5,6]. Most of the knowledge comes from studies on the north Atlantic kelp *Laminaria digitata*. This organism recognizes biotic threats by the perception of molecular cues such as oligoalginates, recognized as cell wall degradation fragments or lipopolysaccharides related to bacterial pathogens [7,8]. These molecules elicit a signaling cascade resulting in molecular defense responses [8,9]. Elicitor perception generates an early oxidative burst followed by the release of polyunsaturated free fatty acids that are subsequently oxidized into lipophilic signaling molecules grouped under the term oxylipins. In an evolutionary context, this innate immune response in kelps is reminiscent to animals and land plants mechanisms, where oxylipins play pivotal roles as stress-related signaling molecules. Animals predominantly synthesize eicosanoid (C20) derived oxylipins such as prostaglandins, thromboxanes, prostacyclins, leukotrienes or hydro(pero)xy-FA whereas land plants synthesize octadecanoid (C18) derived oxylipins including hydro(pero)xy-FA, divinyl ethers, volatile

aldehydes or jasmonic acid and its derivatives (JAs) [10,11]. In contrast to plants, brown algae retained the capability of synthesizing eicosanoid fatty acids. As a consequence, these organisms synthesize a wide variety of oxylipins such as “animal-like” eicosanoid derived hydro(peroxy)des, epoxides and prostaglandins or “land plant-like” octadecanoid derived aldehydes and the JA precursors 13-HPOTrE or the 12-OPDA [12-14]. The synthesis of oxylipins in brown algae is concomitant to the activation of defense responses at the transcriptional and metabolite level [6, 9, 10]. Moreover, previous results show that the kelp *L. digitata* can recognize the land plant oxylipin, MeJA that triggers defense reactions resulting in the establishment of resistance against invasion by its brown algal endophyte *Laminariocolax tomentosoides* [15]. Furthermore recent analyses demonstrated the existence of elicitor triggered systemic signals in *L. digitata* [16]. Localized oligoguluronate treatment in this kelp generates a systemic signal that induces a distant defense response against the limpet *Patella pellucida*. Interestingly, Chlorpromazine treatment (phospholipase inhibitor) could impair systemic signaling in *L. digitata*, suggesting the implication of FFA oxidation cascade in this process [16].

During interaction with herbivores, induced anti-grazing defenses have already been reported for most of marine macroalgae, including Laminariales [17,18]. However, up to recently, these field or laboratory-controlled experiments were mainly based on herbivore feeding behavior, and few studies have yet explored the molecular and chemical bases of these inducible defense responses in brown algae, at the contrary of land plants. For kelps, although previous studies setup the basis for the study of kelp elicitor-induced defense responses, very few is known about metabolic regulation occurring during grazer attacks. In this context, we carried out comparative metabolome and transcriptome analyses of the South Pacific kelp *Lessonia spicata* and the North Atlantic kelp *Laminaria digitata* challenged with their limpet specialist herbivores to infer about common and specific responses in two kelp families (Lessoniaceae and Laminariaceae).

Materials and Methods

Algal material

Juvenile sporophytes of *Laminaria digitata* (Hudson) J.V. Lamouroux (i.e. 5 cm long) and *Lessonia spicata* (Suhr) Santelices (i.e. 20-40 cm long) were collected from the surrounding shores near the Station Biologique of Roscoff (SBR) – France, at the Bloscon site (48°43'31"N ; 3°58'8"O), and at the Estación Costera de Investigaciones Marinas (ECIM), Las Cruces – Chile (33°30'09"S : 71°37'59"O), respectively, and maintained at 14°C in running or filtered seawater (FSW) for 1-3 days before use. Their associated specialized herbivores, *Patella pellucida* Linnaeus in SBR and *Scurria scurra* in ECIM were collected at the same time under kelp beds and kept separately under running seawater until the experiment.

Controlled grazing experiments

Grazing experiments consisted in co-incubating kelp individuals with their respective grazers: 3 individuals of *P. pellucida* for each *L. digitata* in 50 mL FSW Petri dish and five individuals of *S. scurra* for each *L. spicata* in 10 L of running seawater aquarium at 17°C. Grazing pressure lasted from 6, 12, 24 and 48 hours (3-4 replicates per incubation time, per species). Three to four replicates of sporophytes maintained in absence of limpets in similar experimental conditions were considered as controls for each incubation time. Kelp tissues were then frozen in liquid nitrogen and stored at -80°C.

Metabolic profiling analyses

Metabolic extracts were obtained from 100 mg of frozen algal powder of *L. digitata* (n=4 except for grazed-48H n=2) or *L. spicata* (n=3 except for control-48H n=2) samples collected after 6H, 12H, 24H and 48H, with 1 mL MeOH:H_2O (8:2), as already described [19]. For untargeted metabolomic analyses, 1.25 µg of 12-OH-lauric acid was added in each sample, as internal standard. After extraction, aliquots of 50 µL were separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an Electro Spray Ionization (ESI) source at running on the negative mode, as described [19]. Samples were separated using an Acclaim RSLC 120 C18 1.9 µm (2.1 x 100 mm) column 545 (Dionex; Thermo Fisher Scientific, Courtaboeuf, France) maintained at 20 °C using 5 µL injection volume and a flow-rate of 250 µL min^{-1} and the mobile phase (0.2% acetic acid in water–acetonitrile) was programmed from 95 : 5 to 5 : 95 acetonitrile–water (v : v).

Mass spectra data were processed by XCMS using the online version of Galaxy-Workflow4metabolomics [20], after conversion of raw spectra to mzXML format. Data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples

necessary at 0.7. For correction of retention time, the obiwarp method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 10. Fillpeaks step was used with the chrom filling method. Finally, annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. Areas for all peaks were normalized using that of the internal standard in the same sample, and normalized areas were used as ion abundances. The multivariate data analysis of relative ions abundances using Partial Least Squared – Discriminant Analysis (PLS-DA) was performed to test for differences in metabolite profiles among grazed and control samples. Further statistical multiple testing analyses were performed to identify the different ions, based on Student T-test and FDR-adjusted p-values. The analysis was carried out on Pareto scaling and log10-transformed data using the software SIMCA (13.0, Umetrics, Umeå, Sweden).

For amino acid, free fatty acids and oxylipin targeted profiling in *L. spicata* samples (n=3 except for control-48H n=2), the same extraction and quantification process was applied than for untargeted analyses (see above), adding 1.25 µg of 12-OH-lauric acid and 10 µg of atropine in each sample, as internal standards for negative or positive mode analyses, respectively. Free fatty acid and oxylipin profiles were obtained through negative mode LCMS analyses, and amino acid measurements in positive ion mode. The Hierarchical Clustering analyses of relative abundances changes was carried using Pearson Correlation values in Multi Experiment Viewer 4.9 [19]. Statistical Kruskal-Wallis tests were then performed for each metabolite with grazing stress and time as factors, using the free software R version 3.2.5.

cDNA library sequencing and data analysis

Total RNA was extracted from 100 mg of frozen algal materials according to Apt et al. [21], treated with RNAse-free DNAse I (Stratagene, La Jolla, CA, USA) to eliminate genomic DNA contamination, and quantified using a Nanodrop ND 1000 spectrophotometer (Labtech International Ltd, Lewes, UK). For each species, two pools of RNA, corresponding to Control or Grazed treatments, were prepared by pooling the same amount of total RNA, extracted from three replicates of control or grazed algal samples, collected after 6H, 12H, 24H and 48H for *L. digitata* (Ld) and after 6H, 12H and 24H for *L. spicata* (Ls). The four non-normalized shot-gun cDNA libraries, namely Ld-Control, Ld-Grazed, Ls-Control and Ls-Grazed, were constructed using the SMART cDNA library kit from Clontech (CA, USA), differentially-labelled and sequenced using 454 sequencing technology (Roche, Branford, CT, USA) at the Max Planck Institute of Berlin-Dahlem.

Raw sequencing data were deposited in the SRA database at NCBI (accessions SRR4149283 for Ld-Control, SRR4159455 for Ld-Grazed, SRR4240918 for Ld-Control, SRR4240919 for Ls-Grazed sequences). Raw reads were cleaned by removing adaptor sequences, empty reads and filtering reads with poor quality using SeqClean (https://sourceforge.net/projects/seqclean/files/). For each species, the

cleaned 454-generated reads for all sequences (derived from Control and Grazed libraries) were assembled into non-redundant unigenes using Newbler v2.0.01.14 v2.5 (http://www.my454.com), by including cDNA sequences of the two species available in public databases, to increase the quality of clustering. The unigenes sequences are available upon request. Candidates of differentially-expressed unigenes were identified according to their respective number of reads into the two libraries, using IDEG6 web tool [22], based on statistical tests [23]. The putative function of these putative differentially-expressed sequences was analyzed using Blastx and Blastn against the nr database (March 2016).

Validation of differentially-expressed genes by qPCR analysis

To validate the molecular markers of grazing stress, total RNA was extracted from 100 mg of frozen algal material of *L. digitata* or *L. spicata* sampled during laboratory-controlled grazing kinetics or in natural populations. For *L. digitata* grazing laboratory kinetics, the RNA samples were those used in EST analyses, whereas for *L. spicata*, a new experimental grazing experiment was conducted up to 48 hours, corresponding to a new set of RNA samples for both control and grazed algae. For *in situ* survey, grazed and un-grazed fronds from adult sporophytes of *L. digitata* and *L. spicata* (n = 7-8) were sampled at the same locations at Roscoff and Las Cruces (see above and Fig S1). Field samples were immediately flash frozen in liquid nitrogen and kept at -80°C before RNA extraction. Gene expression was then analyzed by qPCR as described by Cosse et al. [9], using the primers sequences provided in S1 Table. Gene transcript level was normalized to the geometrical mean of two reference gene transcript levels in the same sample (tubulin and EF1a genes for *L. digitata*; EF1a and RPL36 genes for *L. spicata*). The Hierarchical Clustering analyses of gene expression changes was carried out on Pearson Correlation values in Multi Experiment Viewer 4.9 [19]. A two-way analysis of variance (ANOVA) with grazing stress and time as categorical predictors was applied to qPCR expression data. All tests were performed using Statistica 7 (Statsoft, Tulsa, OK, USA).

Results

Specialist herbivores induced large metabolome alterations in both Chilean and European kelp species.

Metabolomic profiling by LCMS in the negative mode of grazed algae provided 211 ion peaks in *L. digitata* and 818 in *L. spicata*, that were considered for multivariate analysis (S2-S3 Tables). Partial least squares discriminant analysis (PLS-DA) of these datasets for *L. digitata* explained ~50% of the observed variation. In particular, the axis t[2] discriminated two groups of grazed and control individuals (Fig 1A). For *L. spicata* datasets, PLS-DA analysis explained 80% of the observed variation. Grazed and controlled individuals were distinctively grouped along t[1] axis that explained 54% of the observed

variation (Fig 1B). Although multivariate metabolome analysis succeeded to discriminate between control and grazed kelps, PLS-DA analyses failed to distinguish a general pattern according to time in both species. This result suggests the establishment of a metabolic response from 6h of grazing exposure, which would persist over time of the experiment until 48h. Moreover only a small proportion of ions showed a significant change between the two treatments (T-test and FDR tests, p-value<0.05, 12 ions over 211 ions for *L. digitata* and 15 over 818 ions for *L. spicata*; see S4-S5 Tables).

Metabolic profiling of grazed *L. spicata* highlighted distinctive accumulation patterns of Amino Acids and Free Fatty Acids.

We previously demonstrated the regulation of free fatty acid (FFA), oxylipin and amino acid (AA) metabolisms by biotic and abiotic stress in brown algae [7,12,19]. Accordingly, we profiled these metabolites in *L. spicata* to evaluate their possible functions in grazing stress defense. LC-MS analysis of extracts obtained from grazed and controls individuals of *L. spicata* identified 9 FFA, 3 oxylipins, and 9 AAs. Hierarchical Clustering Analysis of metabolite accumulation in grazed algae compared to controls made a clear distinction between early harvested samples at 6h and 12h opposed to late harvested samples at 24h and 48h (Fig 2A). Moreover, statistical analysis showed a significant induction effect of grazing stress (Kruskal-Wallis test; $P< 0.05$) for 12 of the 21 profiled metabolites (Fig 2A; S6 Table).

Free AAs accumulated early upon grazing, with a 17-fold increase in total detected AA content after 12h of grazing compared to control. This was followed by a drastic drop in AA content, reaching control levels on longer stress periods from 24h onwards (Fig 2A-B). Specifically, 12h of grazing pressure increased concentration of methionine (34-fold), and its precursors, cysteine (16-fold) and aspartate (16-fold). The AA proline (14-fold) and its precursor glutamate (16-fold), but also valine (14-fold), leucine (15-fold) and isoleucine (9-fold) showed a similar pattern of induction with a peak after 12 hours of grazing stress compared to controls (Fig 2B).

In contrast to AAs, FFA and FA-derivatives accumulated at latter stress periods, and 10 out of the 12 profiles presented a significant induction by grazing stress (Fig 2A; S6 Table). This difference in timing was clearly observed in the Hierarchical Clustering Analysis of metabolite accumulation (Fig 2A). Most of the detected FFA and FA-derivatives accumulated from 12 hours of grazing to peak at 24h and then dropped at 48h (Fig 2C-D). Remarkably, grazing stress induced a strong liberation of FFAs from both octadecanoid and eicosanoid pathways. Octadecanoic acid (C18:0) was the most highly accumulated FFA upon grazing stress showing 10-fold increase at 24h (Fig 2A;C). Linoleic (C18:2), linolenic (C18:3) and arachidonic (C20:4) acids were the most highly accumulated desaturated FFAs and showed increases of 5-6 folds at 24h of grazing stress compared to control conditions (Fig 2A;C). These FFAs are direct precursors of oxylipins. Accordingly, we detected significant accumulations of the cyclopentenone prostaglandins J2, A2 and B2 under grazing stress. These oxylipins co-accumulated

with their precursor arachidonic acid, increasing after 12H of grazing stress, reaching a peak at 24H and then decreasing up to control levels at 48H (Fig 2A;D).

EST analysis identified differentially expressed genes under grazing challenge in kelps

We next examined transcriptome variations of grazed *L. digitata* and *L. spicata* to identify particular genes or eventual processes related to herbivory in kelps. In this purpose, juveniles of *L. digitata* and *L. spicata* were challenged or not with their respective specialist grazers up to 48 or 24 hours, respectively, and an EST approach was developed using alga sampled along the experimental time-courses. A total of 444,474 reads were produced with an average length of 184 nucleotides (Table 1). After assembly, 15,454 and 16,511 unigenes (singletons + contigs) were retrieved for *L. digitata* and *L. spicata*, respectively. Among these, only a small fraction (0.8%) showed significant ($P<0.01$) differential expression between grazing and control treatments. (Fig 3A; S7-S8 Tables). Namely, 64 unigenes showed significant up-regulation and 58 unigenes were down-regulated in grazed *L. digitata* (S7 Table). *L. spicata* analysis resulted in 64 up-regulated and 70 repressed unigenes upon grazing (S8 Table).

Blastx and Blastn analysis of differentially expressed sequences against the nr database retrieved few homology with gene databases, underlying their probable specificity to brown algal sequences (S7-S8 Tables). In many cases (i.e. 97/122 for *L. digitata* and 84/132 for *L. spicata*) Blast results retrieved non coding sequences, likely corresponding to 3'UTR sequences of the cDNA library. Indeed the brown algal genes have been shown to feature very long the 3'UTR [2], leading to their over-prevalence in cDNA libraries.

To confirm results obtained from *in silico* analyses of EST libraries, we selected 15 unigenes in *L. digitata* and 13 in *L. spicata* among the top up-regulated expressions under grazing stress (S7-S8 Tables). Expression patterns of these unigene candidates were followed in juvenile individuals of both species challenged with their specific limpet grazers *P. pellucida* and *S. scurra*. Samples of both species were harvested after 6, 12, 24 and 48 hours of grazing challenge for RNA extraction.

The qPCR expression analysis in *L. digiata* showed significant induction effect (2-way ANOVA; $P< 0.05$) of grazing stress in 14 out of the 15 tested unigenes (Fig 3B; S9 Table). The 2-way ANOVA test also found some significant effects ($P< 0.05$) of time, meaning that expression of 12 of them varied significantly over time. Furthermore most of tested unigenes presented significant interactions between time and grazing factors ($P< 0.05$), meaning that the up-regulation of 10 genes was dependent on a particular time (S9 Table).The relation between grazing and time was represented by a hierarchical clustering analysis of unigene fold-change expression of grazed compared to control algae in Fig 3B. This analysis identified a large group of 10 unigenes showing an expression pattern of induction at 24-48H hours of grazing stress. The remaining 5 unigenes showed rather rapid induction, or significant downregulation.

We used the same validation approach in *L. spicata* by testing 13 unigenes that showed strong induction by grazing stress in the EST library. The qPCR results of unigene expression were then analyzed though 2-way ANOVA test using as factors grazing treatment and time. This analysis identified 8 out of 13 unigenes as significantly different ($P< 0.05$) between control and grazed groups (Fig 3C; S10 Table). In less cases than for *L. digitata*, 2-way ANOVA test also found some significant effects ($P< 0.05$) of time and its interaction with grazing stress, suggesting that the induction of one gene is dependent on a particular timing. Hierarchical clustering analysis grouped together 6 out of the 13 genes being significantly affected by grazing, time and the interaction between these factors (namely Ls_contig02470, Ls_contig00344, Ls_contig01374, Ls_contig02225, Ls_contig00467, Ls_contig00239, Fig 3C). These genes showed expression patterns increasing from 6H to peak at 24H of grazing stress following a drop of induction at 48H (Fig 3C).

Field validation of grazing stress markers

We further evaluated whether the identified up-regulated sequences could serve as markers of grazing exposure in the field. With this purpose, *L. digitata* adult fronds grazed by *Patella pellucida* (Fig. S1) were harvested at the shore of Roscoff – France together with ungrazed individuals as negative controls. RNA was then extracted from individuals to quantify expression of 8 unigenes (*i.e.* Ld_contig02543; Ld_contig02608; Ld_contig02668; Ld_contig02406; Ld_contig02587; Ld_contig02511; Ld_contig00126; Ld_contig00071) that were previously validated in laboratory grazed conditions (Fig 3B). The results showed significant transcript accumulation of only three unigenes, Ld_contig02543, Ld_contig02587 and Ld_contig00071, in grazed adult individuals compared to un-grazed algae collected in the field (Fig 4A).

Similarly, adult fronds of *L. spicata* grazed by *S. scurra* were harvested at the shore of Las Cruces – Chili (Fig. S1) together with ungrazed individuals as negative controls. RNA was then extracted from individuals to quantify expression of 7 unigene sequences (Ls_contig01083; Ls_contig00239; Ls_contig01374; Ls_contig00467; Ls_contig02225; Ls_contig02470 and Ls_00344) presenting high confidence up-regulation in laboratory conditions (Fig 3C). All tested unigenes showed significant up-regulation in grazed individuals compared to controls. Altogether, these results have validated a number of grazing molecular markers in *L. digitata* and *L. spicata*, demonstrating their strong activation by grazing regardless of developmental stages or laboratory set-up conditions.

Discussion

Our metabolome analysis succeeded to make global distinctions between grazed and control metabolomes of *L. digitata* and *L. spicata* (Fig 1). This result suggests that these two kelps are able to recognize their specialist herbivores, and regulate their metabolism, according to grazing challenge. Moreover, metabolite profiling (Fig 2) revealed that grazing induces in *L. spicata* early and late responses, implicating an early accumulation of aminoacids, followed by the later liberation of FFA and the synthesis of prostaglandins.

In *L. spicata*, significant accumulations of AAs were detected in grazed individuals during the first six hours of stress and peaked after 12H. Sulphur containing AAs (SAAs) such as Methionine and its direct precursors Cysteine and Aspartate were rapidly and highly accumulated under herbivore attack, suggesting their functions in grazing stress. Indeed SAAs play key roles in protein synthesis and function, and these Sulfated groups AA are also particularly sensitive to oxidation caused by stress. In this context high accumulation of SAAs could be explained by oxidative stress conditions generated by grazer attack. This hypothesis is supported by previous transcriptome analysis of *L. digitata* elicited with oligoguluronates (GGs) [9]. GGs is a kelp cell wall digestion product constituting a microbe-associated molecular pattern. *L. digitata* associates the presence of GG with a biotic attack, thereafter generating an oxidative burst cascade resulting in the transcriptional activation of defense responses [6,9]. Results of this analysis identified as induced a Methionine Sulfoxide Reductase (MSR) gene. MSRs are enzymes that act to reduce sulfoxide groups generated in Methionine residues by oxidative stress, therefore restoring protein function. In relation with antioxidant components, Glutamate contents were also highly increased by grazing in *L. spicata*. This AA constitute building block of glutathione that plays a key role as anti-oxidant in both biotic and abiotic stress mechanisms in eukaryotes [24]. This tripeptide is used by land plants and brown algae as one of the main antioxidant component in the Ascorbate-Glutathione (ASC-GSH) cycle [25,26]. Beside its function as compatible osmolyte, proline contributes to stabilizing sub-cellular structures (e.g., membranes and proteins), scavenging free radicals thus buffering cellular redox potential under stress conditions in land plants [27]. Moreover, exogenous proline application upregulates the activities of enzymes in the ASC-GSH cycle in plants [28]. It has also been proposed that its accumulation may be part of plant stress signal influencing adaptive responses. Previous studies in *E. siliculosus* determined this AA as particularly accumulated during short-term response to oxidative and osmotic stress [29]. Altogether, the observed increased contents of these AAs is likely to respond to the alteration in the redox cellular status following herbivore attack.

FFA and oxylipins contents were also differentially accumulated by grazing stress in *L. spicata*. In contrast to AAs, the accumulation dynamics of these compounds increased from 12H to peak at 24H then decreased at 48H of grazing. This timing is in accordance to our previous observations of FFA and oxylipin biosynthesis in *L. digitata* following abiotic stress conditions [12]. Grazing stress induced in *L.*

spicata significant accumulations of both octadecanoid and eicosanoid FFAs together with eicosanoid derived oxylipins. Similar results of FFA and oxylipin accumulations were observed in *L. digitata* following bacterial lipopolysaccharide induction, which was recognized by the kelp as a PAMP [7]. Animals, plants or red algae react to biotic attacks by the activation of signaling reactions which include oxidative bursts and oxylipin formation from either octadecanoid (land plants), eicosanoid (animals) or both (red algae) pathways such as jasmonic acid or prostaglandins [30,31]. In agreement with the functions of FFAs and oxylipins, it was previously shown that arachidonic acid or methyl-jasmonate could trigger an oxidative burst in *L. digitata*, which induced resistance against the infection of the endophyte pathogen *Laminariocolax tomentosoides* [15]. In this context, the observed accumulations of FFAs and prostaglandins in *L. spicata* in response to grazing stress likely constitute molecular cues triggering defense reactions against *S. scurra*. Grazing in *L. spicata* stress induced the synthesis of PGA2, PGB2 and PGJ2 oxylipins that derived from arachidonic acid. In agreement, previous observations in *L. digitata* showed that only arachidonic acid but not linoleic acid could trigger defense response against pathogen infection in this kelp [15]. Altogether, these results suggest the preponderant importance of eicosanoid derived metabolites to trigger defense responses during seaweed-herbivores interactions.

Environmental abiotic and biotic stresses (such as wounding or pathogen attack) in plants can trigger the stress defensive mechanisms through massive reprogramming of gene expression. Our transcriptome analysis attempted to evaluate if a 2 days-long grazing challenge could induce large gene expression changes in kelps and succeeded to identify as differentially expressed only a reduced number of unigenes (122 and 132 for *L. digitata* and for *L. spicata*, respectively; Fig 3), representing 0.8% of the total identified unigenes. It's likely that our results don't accurately reflect whole transcriptome changes operating in kelps under grazing stress, over this short term period. However, recent transcriptomic analysis of *F. vesiculosus* grazed by the adapted specialist herbivore, *Littorina obtusata*, for 3 days revealed 61 up- and 124 down-regulated genes, the stronger gene regulation occurring between 2 and 3 weeks of grazing (241 and 270 genes differentially expressed at day 15 and 21, respectively) [32]. Moreover, we found very few homologies between our unigene library and known gene functions. This results could reflect the reduce sequence length coverage of our unigene libraries [2], but also partly underlines the paucity of reliable gene annotations for brown algal sequences. Most of the identified differentially expressed genes in both kelp species showed a strong peak of induction after 24 and 48 hours of grazing stress, suggesting their implication in stress responses. Remarkably, this result is in co-occurrence with our observed oxylipin synthesis in *L. spicata*, suggesting a possible link between oxylipin accumulation and the defense responses. Nevertheless further experiments are needed to confirm their direct activation by FFAs or prostaglandins. Future gain of genomic information will allow to identify functions of these interesting genes, for which strong relatively early inductions observed under grazing support their implication in activation of anti-herbivory adaptive responses.

An additional line of evidence supporting the implication of the identified unigenes in grazing stress was obtained from our field experiments. This approach was particularly successful in *L. spicata*, but not in *L. digitata*. In this later kelp, the up-regulated genes, validated in laboratory conditions but not *in situ*, might correspond to very early responsive genes (up to 2 days of grazing) or be rather related to the experimental setup and to early developmental stages, as young sporophytes were used. Indeed the timing of grazing pressure in the field samples was unknown and could therefore exceed 2 days. Interestingly, in *L. spicata*, the herbivore *S. scurra* generated large wound zones where our identified unigenes showed high expression. Moreover the high induction of these genes in recurrently grazed fronds further suggests the association of these sequences with the setup of persistent responses to grazing. Recent studies demonstrated the ability of *L. digitata* to vehicle systemic signals inducing defense reactions distantly of elicited zones [16]. GG elicitation generated an oxidative burst signal that was transmitted systemically through the kelp tissue, activating defense genes in a process that is likely dependent on oxylipins signaling. Moreover, authors observed reduced grazing activity of *P. pellucida* distantly from elicited areas. These studies are reminiscent to recent foundlings in land plants showing distant propagation jasmonate signaling triggering defense distantly of wounded areas [33]. In line with these results it would be interesting to asses if the identified unigenes can be activated by oxylipins signaling pathways distantly to grazed areas.

Conclusion

Using laboratory-controlled bioassays we have shown that grazing by specialist herbivores induces both significant modification of the kelp metabolome and specific gene regulation. In addition, our metabolite profiling results show that *L. spicata* responds to herbivore attack by a rapid response following the first hours of stress consisting in the accumulation of AAs likely constituting an antioxidant response. This response is followed by a response consisting in the synthesis of FFAs and oxylipins which may act as signaling cues to trigger latter immune responses against herbivores. Finally our results determined a set of genes related to grazing stress in field populations of marine kelps. These genes could be used as markers to study the functional dynamics of seaweed-herbivore interactions in natural populations or as selection markers *i.e.* in the context of kelp breeding programs. Recent developments in next generation sequencing technologies open exiting perspective of this work. Genome Wide Association Studies of grazed kelp populations might provide more precise traits of evolution related to herbivore defense in these organisms.

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Figure legends

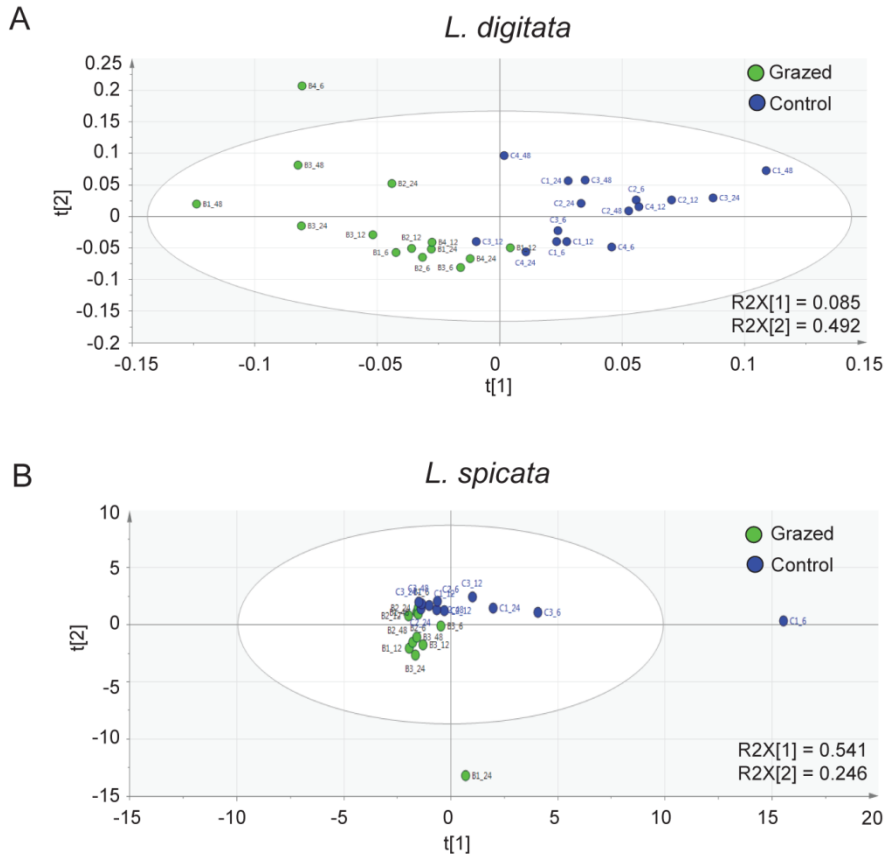

Fig 1. **Specialist herbivores induced large metabolite variations in kelps.** PLS-DA score plots of metabolite ions detected by LC-MS/MS in *L. digitata* (A) and *L. spicata* (B) juveniles under controlled conditions (211 and 818 monoisotopic peaks respectively). Blue spots represent control algae and green spots represent grazed algae.

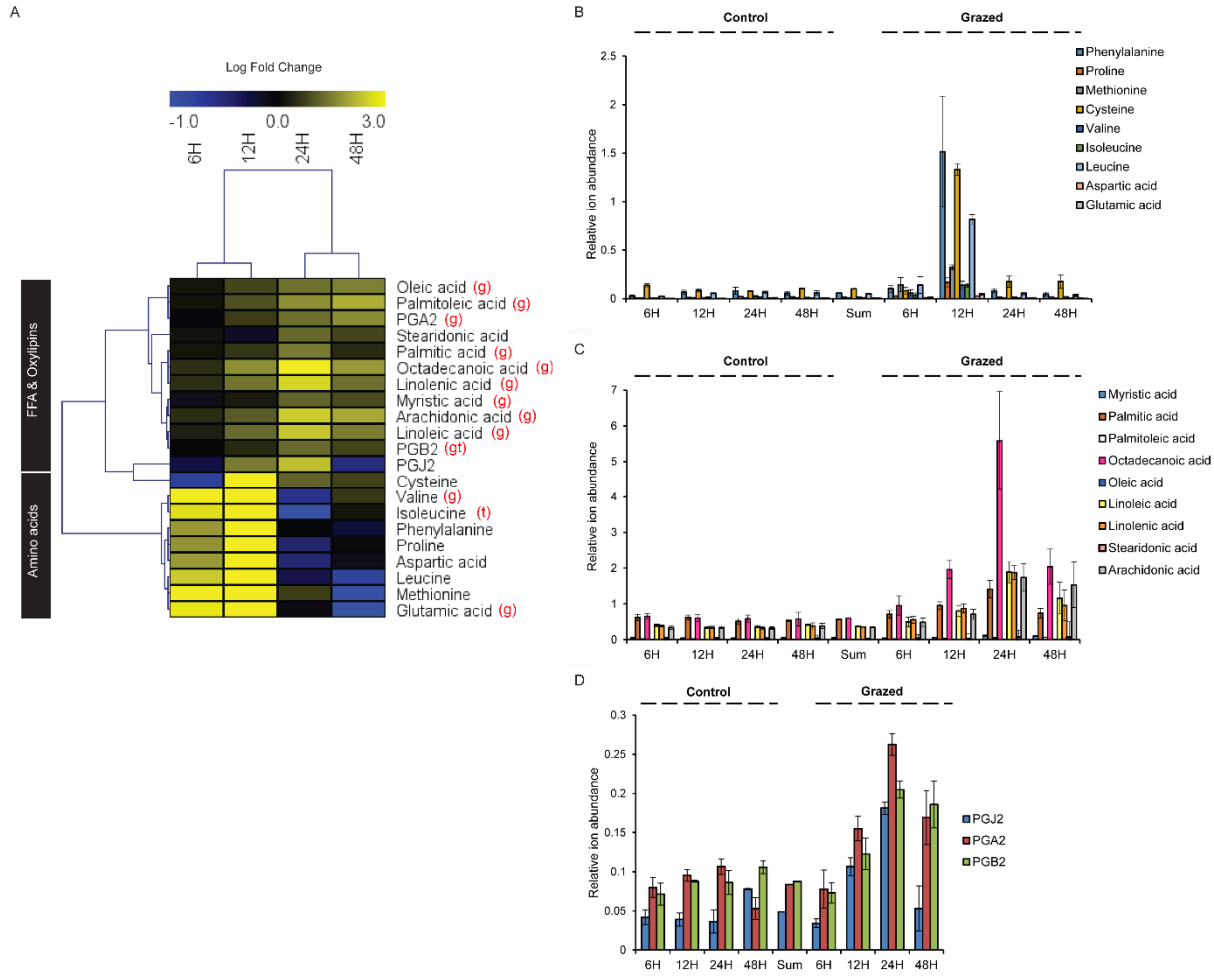

Fig 2. Metabolic profiling in grazed *L. spicata* highlighted distinctive accumulation dynamics of Amino Acids and Free Fatty Acids. (A) Heat map of the amino acids, free fatty acids and oxylipins detected in *L. spicata* in laboratory controlled conditions. Values represent the log fold-change of control conditions (n = 3). Time points and compounds were clustered according the Pearson Correlation. The letter (g) indicates a significant difference according to grazed condition, and (t) indicate significant differences for time (P<0.05, Kruskal-Wallis test). (B-D) Time course of relative abundances of free amino acid (B), free fatty acids (C) and oxylipin contents (D) in control and grazed *L. spicata* (mean of three replicates ± SE).

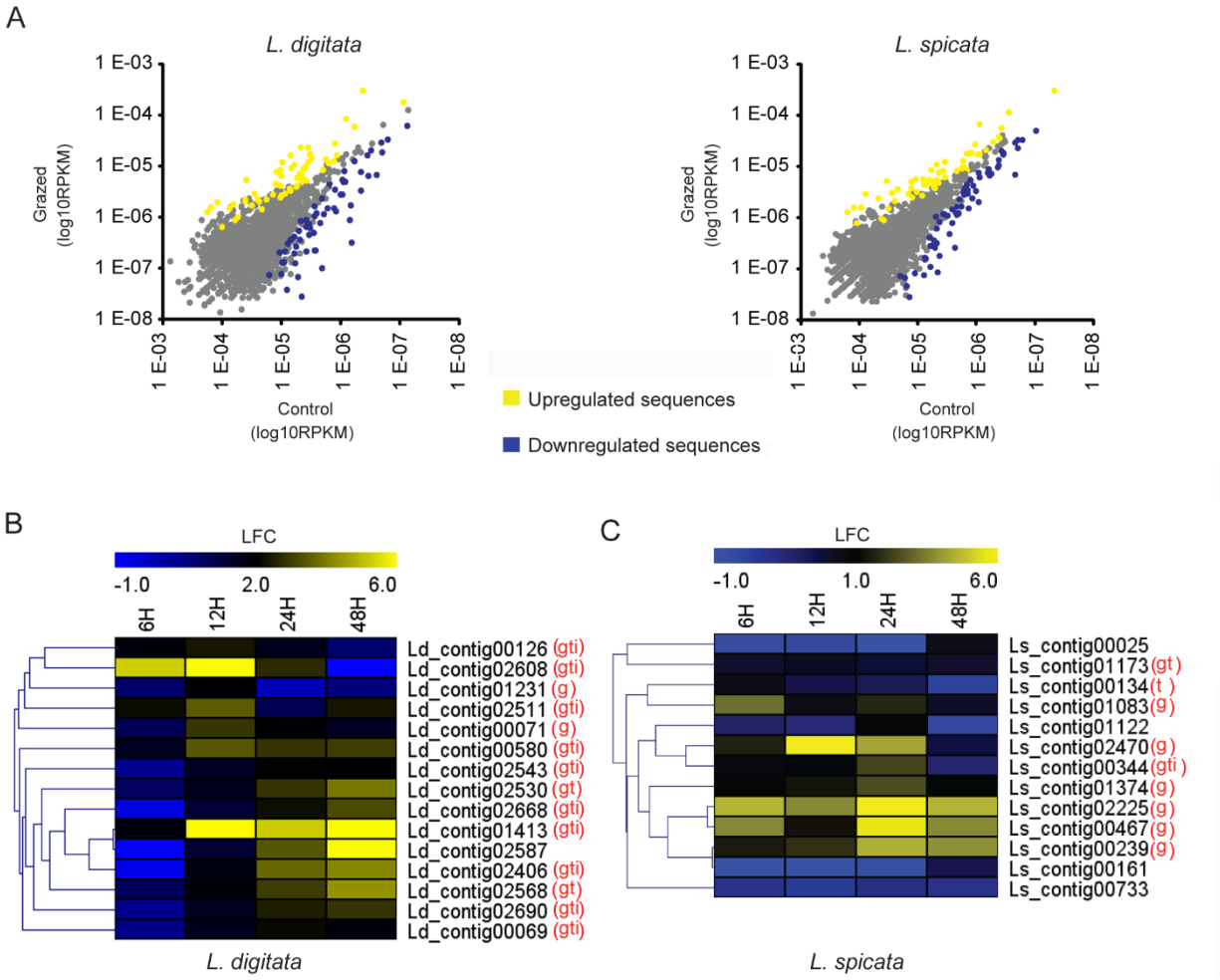

Fig 3. Unigene libraries of grazed *L. digitata* and *L. spicata* revealed differentially expressed transcripts. (A) Scatter-plot representations comparing Grazed and Control unigene libraries of *L. digitata* and *L. spicata*. Values are expressed as Reads Per Kilobase of transcript per Million of total reads (RPKM). Yellow dots represent significantly up-regulated unigenes whereas blue dots significantly down-regulated unigenes. (B-C) Heatmaps showing the qPCR validation of grazing markers in laboratory controlled conditions. Unigenes are presented according to a hierarchical clustering analysis. Values are represented as log fold-change (LFC) of control conditions (n=3). The letter "g" indicates a significant difference according to grazed condition, "t" indicate significant differences for time and “i” indicates the significant interaction between grazing and time ($P<0.05$, 2-way ANOVA).

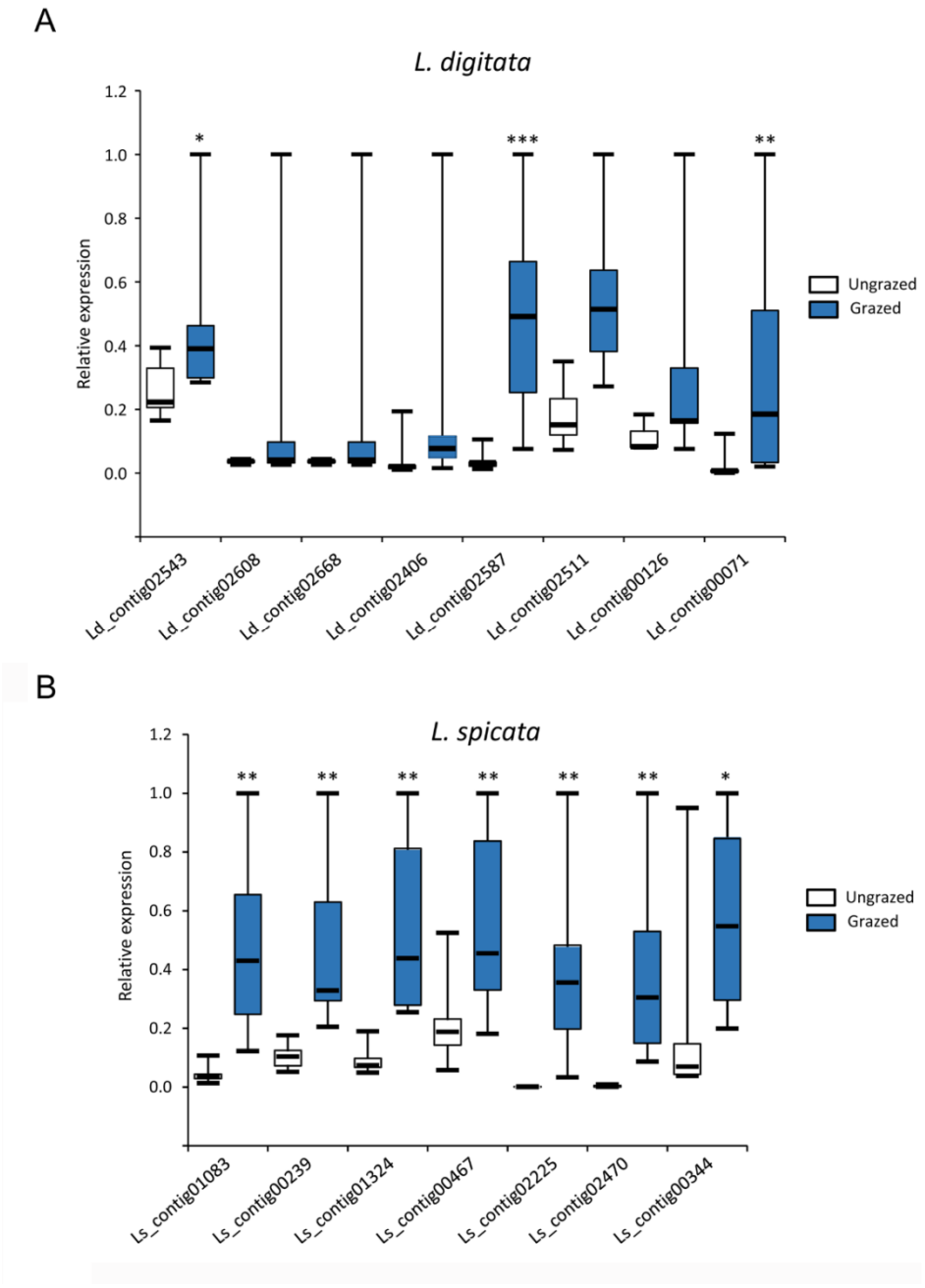

Fig 4. Validation of grazing marker genes in natural kelp populations of *L. digitata* and *L. spicata.* (A-B) Whisker-plot representations of qPCR expression values from candidate grazing marker sequences in ungrazed (white) or grazed (blue) natural populations of *L. digitata* and *L. spicata* (n= 7-8). Expression values were normalized to a maximum of 1. Continous horizontal bars along boxes represent the median values. For each marker, the effect of grazing was tested using U-test (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 1. General features of 454-generated ESTs libraries

	Laminaria digitata	***Lessonia spicata***
# Total reads	201664	242810
# Total ESTs (after cleaning)	104479	126002
# Unigenes (after assembling)	15454	16511
Size range (nt)	50-2817	50-1335
Average length (nt)	187	181
# Contigs	2676	2716
Average length (nt)	358	361
# Singletons	12778	13795
Average length (nt)	151	146

Supporting Information

S1 Fig. Representative pictures of *Laminaria digitata* (A) and *Lessonia spicata* (B) with their respective specialist herbivores *Patella pellucida* Linnaeus (A) and *Scurria scurra* (B), showing typical grazing dammages on algal tissues. Photos by LC, FT, CF, SF and CL.

S1 Table. Primers used in the qPCR analysis study.

S2 Table. List of ions identified in *L. digitata* and relative abundance peaks in control and grazed individuals at 6, 12, 24 and 48h of stress.

S3 Table. List of ions identified in *L. spicata* and relative abundance peaks in control and grazed individuals at 6, 12, 24 and 48h of stress.

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S4 Table. Statistical analysis comparing metabolite ions of grazed vs. control individuals of *L. digitata.*

S5 Table. Statistical analysis comparing metabolite ions of grazed vs. control individuals of *L. spicata.*

S6 Table. Statistical analysis comparing amino acid, free fatty acids and oxylipin relative profiles of grazed vs. control individuals in *L. spicata*.

S7 Table. Differentially expressed unigenes under grazing stress identified by sequencing analysis of the *L. digitata* cDNA libraries.

S8 Table. Differentially expressed unigenes under grazing stress identified by sequencing analysis of the *L. spicata* cDNA libraries.

S9 Table. Validation of differentially expressed unigenes under grazing stress by qPCR analysis in *L. digitata*.

S10 Table. Validation of differentially expressed unigenes under grazing stress by qPCR analysis in *L. spicata.*

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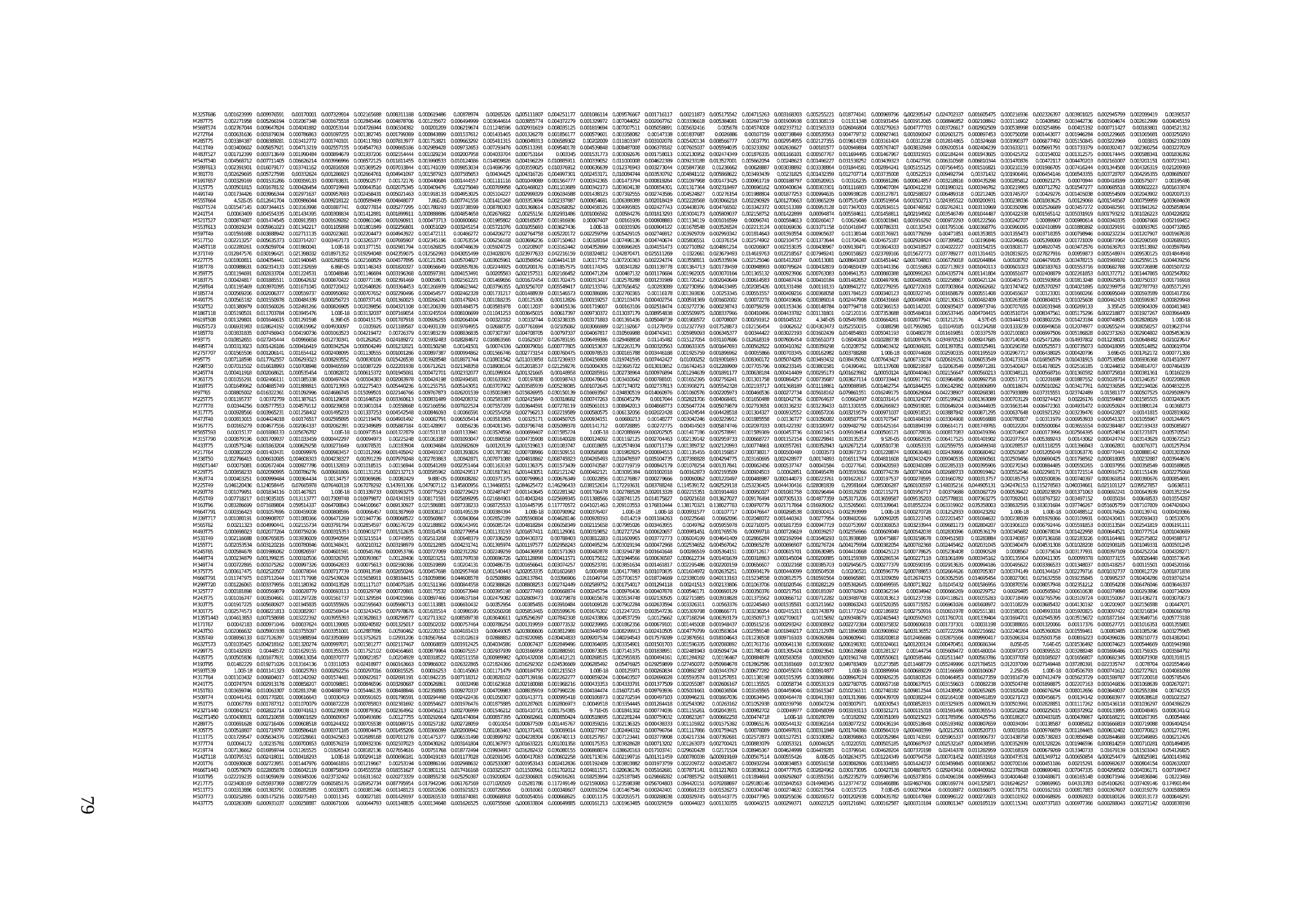
Fig. S1

S1 Fig. Representative pictures of *Laminaria digitata* (A) and *Lessonia spicata* (B) with their respective specialist herbivores *Patella pellucida* Linnaeus (A) and *Scurria scurra* (B), showing typical grazing dammages on algal tissues. Photos by LC, FT, CF, SF and CL.

S1 Table. Primers used in the qPCR analysis study.

Primer name	Sequence	Tm	Product Length
Ld_EF1a_fw1q	GGCCGCTTGTTGCTGATC	60	71
Ld_EF1a_rev1q	GGAACAGAGGAGAGAAGGAACGA	60	
Ld_Tuba_fw1q	TCGTGCAGTTGGGTTTCGA	60	62
Ld_Tuba_rev1q	CAAGACGCCATGCGATACG	60	
Ld_Act_fw3q	GGATGTCGCGCACGATCT	59	87
Ld_Act_rev3q	ACCTGACGGACAACCTGATGA	59	
Ld_contig02587_PCRQ_F	CGCCGTGCTCCCTGTTCC	59.8	120
Ld_contig02587_PCRQ_R	GCCCAATCCCGTGCTGTAATAAAG	60.2	
Ld_contig02406_PCRQ_F	TGCCATAGGGGTAAAGTGCTGTAG	60	80
Ld_contig02406_PCRQ_R	AGTAGTTCCTCCGTTGAGAAGCC	59.6	
Ld_contig02608_PCRQ_F	GGATGATGATGCTGCTGCTTGTG	60.3	142
Ld_contig02608_PCRQ_R	ACCCTTAGAGGTATGTGTGAGTGC	59.7	
Ld_contig02511_PCRQ_F	GGGGACGCAGCAGTACGC	59.9	132
Ld_contig02511_PCRQ_R	GCCGCAGCATCAAGCCAAC	59.7	
Ld_contig00580_PCRQ_F	TGTGAAGTGTCGGCGAGAACC	60.1	149
Ld_contig00580_PCRQ_R	ATACCTGCCTGGCTTATCATCCTG	59.9	
Ld_contig02543_PCRQ_F	GCAGTAGTACCCTTCACTAAATCGC	59.5	93
Ld_contig02543_PCRQ_R	AGGTGATGCTTGTGTTGAATGAGG	59.4	
Ld_contig01231_PCRQ_F	AGCGGAAGATGGAACTGAGATTCG	60.6	63
Ld_contig01231_PCRQ_R	GTTCAGGGAAATTCCTCGTCGTATG	59.8	
Ld_contig00069_PCRQ_F	GCAGTAGTACCCTTCACTAAATCGC	59.5	93
Ld_contig00069_PCRQ_R	AGGTGATGCTTGTGTTGAATGAGG	59.4	
Ld_contig02568_PCRQ_F	GTGGGCAACAAGAAAGAGAAGAGAG	59.8	119
Ld_contig02568_PCRQ_R	CGGGAACCCTAAGGACGATATGG	59.8	
Ld_contig00071_PCRQ_F	TGGTCAACTAACGCTCCCTTCC	59.7	136
Ld_contig00071_PCRQ_R	CTCGCCACAACAGCACTACATTC	60	
Ld_contig02690_PCRQ_F	ACTACAACTTAAACGAGCACCAACG	60.1	82
Ld_contig02690_PCRQ_R	GCGGTATTACCAAGACCAGTCAATC	59.8	
Ld_contig02530_PCRQ_F	GTGGGCAACAAGAAAGAGAAGAGAG	59.8	119
Ld_contig02530_PCRQ_R	CGGGAACCCTAAGGACGATATGG	59.8	
Ld_contig02668_PCRQ_F	AGGTGCTACCAGTACAGTAGTTGAG	59.6	100
Ld_contig02668_PCRQ_R	GGTGCTCGTTTAAGTTGTAGTCGTG	60.4	
Ld_contig00126_PCRQ_F	GGTCTTCGCAAGCAAGGTATGTC	59.7	109
Ld_contig00126_PCRQ_R	GCTGGAGTATATGTCGTGGTTTGTC	59.8	
Ld_contig01413_PCRQ_F	GCGTGTAGTAAGGCTTGGGTTTC	59.7	108
Ld_contig01413_PCRQ_R	ATGTGGTGTAGTTGTCATGCTTGC	60	
Ls_EF1a_F	TTTGCGTGCGGGCTCTGG	60.1	144
Ls_EF1a_R	TGGAGGGTAGCGAACGACATATC	59.5	
Ls_RPL36_F	CGTGAAGATGGTGAGGGAGGTC	59.8	125
Ls_RPL36_R	GCTTGGCGAACTTGTAGATGCG	60.2	
Ls_contig01122_F	AGCGGATGGATCAGGCAAAGG	60	126
Ls_contig01122_R	GGCAACCGTTGTTCGCACTG	60	
Ls_contig00733_F	GGCGAGGTTTGTTAAGGACTGAG	59.1	109
Ls_contig00733_R	GGCACCACCGACTGCTCTG	59.9	
Ls_contig00239_F	CGGAGTCTGCCTGCGGTAC	59.4	86
Ls_contig00239_R	TTCGCTATCCCTGACGGTGATG	59.6	
Ls_contig02470_F	AGGAAGTTGTGCGACGAGGTG	60.1	81
Ls_contig02470_R	TACGAGGTGTGCGGCAATAGC	60.3	
Ls_contig00467_F	CATCCGCCCGAGTATGAACAAAG	59.5	129
Ls_contig00467_R	GCCACAAATCCCGCATCCAAAC	60.6	
Ls_contig01173_F	ACAGCACGACAATGGAGCAGAC	60.8	127
Ls_contig01173_R	TCATCCGCCGCACTGATTATCC	60.3	
Ls_contig00344_F	CCAGCAGCACGGACAGAAATG	59.4	91
Ls_contig00344_R	TTCCTCATCGGCGGCTAACG	59.6	
Ls_contig00161_F	CGGGTTCGGCTTTGAGTGTAAC	59.5	99
Ls_contig00161_R	CGTGGCGAAACCTTCCGTAAC	59.4	
Ls_contig00025_F	TGGAAAGTTGCCGTTGTAGG	55.4	103
Ls_contig00025_R	GTCGTCAACATTAACCCTTGC	55.1	
Ls_contig02225_F	ACGCATAAATCGCCAATGTTG	55.5	66
Ls_contig02225_R	GGCAGACCACTGGAGACC	55.7	
Ls_contig01083_F	GCTCGCTGGGTTAGATGC	54.7	70
Ls_contig01083_R	GGCACGAACTACGAACGG	55.1	
Ls_contig01374_F	GTCGTTCTTGGTCGTCTGG	55	111
Ls_contig01374_R	GATTATTCGTCCCTTTCACAGC	55	
Ls_contig00134_F	CTCCTTCAGACTTGTTCGTTATG	55	75
Ls_contig00134_R	AGCCTCTTCACACTTACATCC	54.6	

S2 Table. List of analyzed proteins and relative abundance in control and grazed individuals at 6, 12, 24 and 48h of stress.



S3 Table. List of ions identified in *L. spicata* and relative abundance peaks in control and grazed individuals at 6, 12, 24 and 48h of stress.

	Contro											Grazed											
	6H			12H			24H			48H		6H			12H			24H			48H		
Ion	C1_6	C2_6	C3_6	C1_12	C2_12	C3_12	C1_24	C2_24	C3_24	C2_48	C3_48	B1_6	B2_6	B3_6	B1_12	B2_12	B3_12	B1_24	B2_24	B3_24	B1_48	B2_48	B3_48
M124T85	0.91608333	0.56851511	0.67438262	0.28587172	0.13677432	0.3146054	0.30708639	0.02927159	0.05826523	0.72459362	0.73767904	0.05832163	1.10124078	0.33052005	0.04467171	0.04226492	0.17444078	1.44195603	0.070537	0.23306169	0.14367784	0.08692043	0.17098783
M178T93	0.71006189	0.05820817	0.28075475	1E-18	0.10068392	0.0855445	0.07973509	1E-18	1E-18	1E-18	1E-18	1E-18	0.01423157	1E-18	0.11350608	1E-18	0.195324	1E-18	1E-18	1E-18	0.03538003	0.03651758	0.02846719
M83T561	64.661621	1.80695406	11.7407414	0.88556198	1.84990755	1.78091013	9.14712692	0.88038237	2.16023084	1.02753001	1.12932124	0.22234137	0.89039359	6.27341757	1.32876992	1.42189295	6.21733197	4.92083659	1.65162776	1.19891813	3.20146333	2.92491766	2.6607409
M388T600	0.27991823	1E-18	1E-18	1E-18	1E-18	0.21134929	1E-18	1E-18	1E-18	0.01642447	0.02290081	0.03806908	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.03850515	1E-18	1E-18	1E-18	1E-18
M219T597	13.9112646	0.58260254	30.5323255	1.14682915	3.24446205	0.32517174	12.8343638	1.86266747	0.5443856	2.42866093	0.27552206	0.13737282	1.37221973	0.10389892	50.4041451	1.20166767	3.20424764	74.8933204	0.04807646	7.10213705	8.89550113	7.41997583	8.55388612
M352T602	1E-18	0.02449303	1E-18	0.01287974	1E-18	0.12353057	0.04674113	1E-18	1E-18	0.03817654	0.057563	0.02063199	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.11050793	0.04995771	1E-18	1E-18	1E-18
M291T496	22.1386144	0.11398115	0.72351173	0.01408355	0.17441823	0.05139141	0.07295974	1E-18	1E-18	0.007764	0.01259109	0.00525786	1E-18	0.02156351	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18
M129T299	2.05559043	0.02171442	0.13926186	0.02925735	1E-18	0.01673872	1E-18	1E-18	0.00922578	0.00767635	0.01019475	0.00974848	1E-18	0.03130196	0.05324169	0.00398631	0.01884426	0.13337362	1E-18	0.01265983	0.00337755	1E-18	1E-18
M131T88	1E-18	1E-18	0.52491849	1E-18	0.01473085	0.36536913	0.08719129	0.18104272	0.01632414	0.2462084	0.18406776	0.02086297	0.01559814	0.13375673	0.0801279	0.00745701	0.09671915	0.45218594	0.03308446	0.13445102	0.01609413	0.10246933	0.04766836
M269T787	1E-18	0.02802598	0.40390367	0.01339749	0.0136619	0.01894244	0.14965461	0.00501434	0.0318	0.04134692	0.02835594	0.03357535	0.02943848	1E-18	0.01027854	0.00719527	0.03372569	0.15536803	1E-18	1E-18	0.00319136	0.01426995	1E-18
M383T738	1E-18	1E-18	1.0452724	0.04532744	0.194	1E-18	0.571	1E-18	0.11821152	0.127	0.0615	1E-18	1E-18	1E-18	0.03878953	0.02762432	1E-18	2.37183813	0.151	0.57475398	1E-18	1E-18	1E-18
M135T86	9.30559976	0.14486818	1.66678776	0.02599075	0.0646275	0.17268048	0.18905427	0.01846438	0.02305783	0.02035259	0.01574111	0.0547657	0.07074407	0.09990696	0.0427187	0.019438	0.12257906	0.50581419	0.05537941	0.07277034	0.19389368	0.09256251	0.17096953
M312T705	0.75651953	1E-18	1E-18	0.01364917	1E-18	0.10430106	0.19470925	1E-18	1E-18	1E-18	0.00279687	0.0126613	1E-18	0.01896196	0.15426024	1E-18	0.01648467	1E-18	0.01047784	0.07716654	0.01117073	0.02445015	0.02232178
M706T1573	1E-18	0.03445524	0.51876996	0.10879915	0.15440118	0.14895049	0.97387668	0.01929162	0.20490186	0.18318943	0.07460967	0.09916308	1E-18	0.44787404	1E-18	0.02590756	0.19779227	1.67446722	0.01329941	1E-18	1E-18	1E-18	1E-18
M359T498	1E-18	0.010767	1E-18	1E-18	1E-18	1.08	0.02811983	1E-18	1E-18	0.00985247	0.0246	0.06854506	0.00617949	1E-18	1E-18	1E-18	1E-18	1E-18	0.08623044	1E-18	1E-18	1E-18	1E-18
M231T89	3.4598289	0.12132733	0.43217836	0.10810775	0.05553052	0.82031951	0.19074977	0.04872321	0.04083442	0.02952627	0.0184374	0.02785668	0.04974418	0.49872932	0.03988349	0.0620651	0.77316169	1.00182937	0.37247088	0.1242448	0.05571184	0.08968842	0.05785423
M359T724	0.55710104	0.22209565	1.35	0.12235606	0.118	1E-18	0.09508794	0.00483936	0.02715121	0.0179062	1E-18	0.118	0.01856622	1E-18	0.0979	0.04407276	1E-18	0.6063639	0.07315818	0.54773714	0.04807753	0.0342	0.02911068
M155T88	2.10979447	0.07966447	0.81433222	0.06956471	0.90629749	0.09022596	0.93734506	0.01624291	0.02501516	0.13656767	0.14759896	0.07511693	0.03390377	1.56047166	1.74932157	0.01819391	0.09139268	0.33505775	0.03987557	0.06234089	0.08310664	0.08930762	0.02144221
M705T1573	1E-18	0.68860481	2.6381499	0.21323449	0.07421263	0.07135415	1.05375187	1E-18	0.53711558	1E-18	0.01130451	0.03441651	1E-18	1E-18	1E-18	0.04379409	0.2109221	3.21393218	0.11807964	0.38220277	0.05635025	0.16391726	0.12718199
M223T602	5.96110812	0.14546138	2.17135307	0.05425101	2.99815893	0.53109612	3.29195696	0.96353042	0.08633036	0.04972283	0.12321226	0.07306653	0.58418115	0.10551107	11.3681375	0.29424425	0.05506502	31.0371856	0.06304333	0.2142339	0.71097943	1.881605	2.05197198
M217T92	3.19827925	0.02424306	0.64884785	0.09806435	0.20225627	22.5302679	16.9761328	0.02234704	0.72239864	19.7071245	6.59466998	0.00011197	3.22029333	6.12842623	46.7201304	0.11136431	1.6057711	17.5945392	0.01826416	0.34559861	0.01822955	0.08361427	0.07657202
M229T169	13.6675253	1E-18	2.74315495	0.39167443	0.0393039	1E-18	1E-18	0.06676332	1E-18	0.0091226	0.07383802	1E-18	1E-18	2.72078672	10.7059001	0.0028893	0.83680367	1E-18	1E-18	0.05038228	0.16138963	0.31536374	0.17512658
M349T685	1.08	1.14	2.14	0.92163193	0.197	0.84225435	0.19238802	0.0804	0.17126367	0.12873331	0.04051489	0.203	0.089	0.0948965	0.0328	0.06682417	0.499	4.44	0.18129346	0.06743056	0.19	0.268	0.366
M139T83	1.33146714	0.09874405	2.27475664	0.17574912	0.07730448	0.07843281	0.30280056	0.02812434	0.01962906	0.191232	0.05447555	0.03381738	0.06242782	0.06809857	0.09049232	0.06726667	0.05997228	0.65116294	0.1450213	0.6409248	0.36588905	0.63451413	0.35328338
M313T734	1E-18	0.01855888	0.09078921	0.00538985	1E-18	0.05949656	0.08347609	1E-18	0.03224399	0.03832975	0.01166885	0.04416861	0.00619566	1E-18	0.03188893	0.00266296	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18
M269T899	0.317	1E-18	1E-18	0.00697865	0.01759905	0.23220712	0.34731574	0.00350912	1E-18	0.07211207	0.02671824	1E-18	1E-18	1E-18	1E-18	0.00296546	0.03184735	1E-18	0.00465263	0.01874568	0.00741	0.0118	1E-18
M169T84	2.01714724	0.35204082	0.2650929	0.13423351	0.30617092	0.33518144	0.28061915	0.17875092	0.06598079	0.03587016	0.0120688	0.0259341	0.03776869	0.25791697	1.45625966	0.03297361	0.42499968	1.10934315	0.04294501	0.20968562	0.46138878	0.17191574	0.58941708
M368T734	1E-18	0.00820475	1E-18	1E-18	0.02482887	0.07525764	1E-18	1E-18	0.02540681	0.00886711	0.00246	1E-18	1E-18	1E-18	0.03244098	1E-18	1E-18	1E-18	0.128	0.01468912	1E-18	1E-18	1E-18
M319T430	0.66783435	0.00899046	0.37969488	0.00548721	0.01783967	0.09091489	0.06964446	0.00427288	0.01188261	0.03144314	0.01642498	0.00889486	0.01389911	0.07833876	0.00863628	0.0063163	0.05459602	0.44656365	0.01003992	0.01864221	0.00884062	0.01233348	1E-18
M187T81	0.94240938	0.07847301	1E-18	0.02238482	0.05321493	0.34173128	0.12332784	0.02509405	0.02918644	0.02578196	0.00991609	0.33758181	0.05789587	0.03219918	0.04573051	0.01148426	0.55980664	0.79190061	0.03682549	0.05849433	0.03498855	0.05470228	0.03471779
M73T557	29.7924801	0.37910366	5.41529028	0.79030021	1.30803551	1.93069666	2.78512394	0.13232481	0.59862137	0.61366267	0.3015289	0.50282543	0.53698482	1.38032271	0.15602756	0.2196541	1.06961758	10.0960448	0.63724177	1.35788866	0.66300817	1.04860238	1.06950115
M269T901	1.19	0.10321348	0.10041668	0.01255943	0.01760077	0.42177234	0.44310893	0.0035092	0.00440882	0.10894506	0.03418147	1E-18	1E-18	1E-18	1E-18	0.00590735	0.03185106	1E-18	0.00465	0.0187	0.0108199	0.01179938	1E-18
M103T222	28.5195276	0.40978704	5.51693195	0.42003154	1.21595583	6.13718739	1.51324448	0.30076762	0.32969673	0.46362807	0.20846346	1.62685852	0.25937585	1.47868506	1.18003274	0.21015789	3.86106105	7.00649289	0.39084916	0.54699529	0.55860814	1.00154259	1.17447043
M670T1468	5.1855131	0.23404938	1.05056459	0.04167478	0.15242412	0.20309908	0.27091207	0.0390959	0.09635266	0.09793241	0.02487063	0.06195114	0.03330662	0.06921218	0.01336378	0.00382134	0.05289089	0.14571162	0.02623566	0.01260206	1E-18	1E-18	1E-18
M405T732	0.19156057	0.00427947	0.03044401	0.00283553	0.01159304	0.04114764	0.02630192	0.00717054	0.00040638	0.00318463	1E-18	0.00381788	0.00372326	0.00134519	0.0059703	0.00047055	0.01644198	0.377952	0.061409	0.02561204	0.00402969	0.00544079	0.00783357
M303T692	3.76864361	0.10566407	0.63114969	0.03317364	0.17965286	0.1741284	0.20321401	0.01797673	0.03206055	0.03684665	0.01640708	0.05967968	0.05468185	0.18223237	1E-18	0.01667046	0.159	1.01555442	0.01523025	0.1509947	0.04644924	0.09201187	0.10846623
M253T568	15.7341367	1.10722826	26.6375387	4.11860419	3.56052078	3.35001147	4.6199642	0.69097272	0.01486552	3.18244586	0.37799592	0.27520288	7.59277477	5.13872453	30.8883533	0.82828479	17.6142753	57.5765826	0.09087152	21.1228983	11.0885188	8.93367402	4.11644684
M117T67	14.1294462	0.28881939	2.58633338	0.40114939	0.40395545	0.22056234	1.09425608	0.25570645	0.29930851	0.14004714	0.01178137	0.03406404	0.03542742	0.10764964	0.01656369	0.06279514	0.64376882	1.34434059	0.22681443	0.0638003	0.2443928	0.28007428	0.53186189
M421T751	1E-18	1E-18	0.04833789	0.1502103	0.19680301	0.09506785	0.06088456	0.03246111	0.05889529	0.03199278	1E-18	0.04692024	0.09578453	0.06459139	0.19338942	0.03762492	0.74343037	4.78900642	0.02767792	0.99493126	0.07551064	0.2199723	0.287312
M281T617	0.99487717	0.013	1E-18	0.00851	1E-18	0.0983	0.268	1E-18	0.00608256	0.01075375	0.00878229	0.00452	0.00774	1E-18	1E-18	1E-18	1E-18	1E-18	0.00477	0.0356	0.167	0.10101656	0.04519855
M368T654	1E-18	1E-18	1E-18	0.0456	1E-18	0.08153444	1E-18	0.00449	0.01098377	0.0178881	1E-18	0.0278	0.00907	1E-18	0.01421842	0.00724	0.204	1.66	0.0553481	0.4645093	0.0473	0.0561	0.128
M119T458	1744.61865	3.36794248	312.287495	0.52025051	6.6990263	145.3073	170.616119	26.1069522	1.07700835	37.2530727	18.6530908	66.9000548	76.0874361	143.116401	41.5657201	17.5547108	106.710264	35.1751977	0.50155092	68.2248329	18.99564	3.16783758	0.99930602
M85T356	40.8701625	2.65858244	7.72074414	0.40993656	1.19340431	7.84866963	2.73014066	2.70252376	0.41346083	0.76017334	0.40630872	1.62793882	0.59596879	3.24095218	2.76814386	0.18259956	2.21867525	14.886375	0.63887971	3.45999498	1.53137699	2.6822681	1.15655385
M669T1574	50.8795639	0.81660986	14.1099292	0.36108186	1.20073657	1.25451743	2.72718087	0.60540409	0.44512924	1.04729683	0.20094782	0.33268654	0.68766093	1.40724788	1E-18	0.13908623	0.74873155	10.2962073	0.18741956	1E-18	1E-18	1E-18	1E-18
M303T711	4.03	0.09470826	0.581	0.04493708	0.16073692	0.17880766	0.167	0.0234	0.0321	0.0368	0.01641129	0.04763989	0.0546	0.195	1E-18	0.0167	0.157	1.15	0.0329651	0.167	0.042	0.107	0.08284687
M149T86	3.00874737	0.05404433	0.23663673	0.02254575	0.06722289	0.0768263	0.53056933	0.14000543	0.0233799	0.09164583	0.06975475	0.18620476	0.03301553	0.1495248	0.03559442	0.01341772	0.0548582	0.6214686	0.02066746	0.29139463	0.02517269	0.06649251	0.04760757
M165T407	19.9736961	0.70766472	3.68613372	0.38103092	1.02679672	0.36663496	2.51273027	0.0792068	0.10868044	0.21413892	0.21059437	0.30558726	0.43996503	1.61137693	0.15823443	0.19741559	1.00324674	1.46211371	0.34348537	1.19419585	0.23347625	0.35585278	0.33708764
M75T59	13.4955765	0.13783395	3.27304561	0.32421445	0.48135202	1.0207031	0.74305258	0.13294077	0.13246281	0.21297648	0.05369516	0.47295441	0.01364993	3.51540141	0.43140776	0.03204258	0.61261361	1.24048068	0.05216544	0.21947939	0.24437702	0.69522977	0.82009637
M397T730	0.14296371	1E-18	1E-18	1E-18	0.01540792	1E-18	1E-18	1E-18	0.01986621	1E-18	1E-18	0.00438175	0.00955992	1E-18	0.07388926	1E-18	1E-18	0.15181768	0.00696886	0.08479247	0.00594692	0.0142717	0.02805701
M310T1435	2.39283944	0.11900434	0.77443259	0.06875497	0.1855361	0.08009064	0.3179363	0.02272943	0.03735369	0.03318425	0.02215209	0.06154463	0.07503462	0.23805202	0.10658408	0.03131246	0.08732352	1.35713582	0.07010693	0.01849296	0.04785976	0.17395185	0.15498295
M247T83	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.03080489	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.05158366	0.059814	0.00348026	1E-18	1E-18	1E-18	1E-18	0.00323424	0.0190368	0.01177966
M155T552	49.7610876	2.19308208	12.8337838	1.57276106	1.53415644	5.10269002	5.00063165	0.35909198	1.00402471	0.40635044	0.45702328	0.44909365	1.15920395	3.6410586	1.30446676	0.92272487	0.57547916	7.69234009	1.26305041	4.69039184	0.86262609	2.80105802	1.78642079
M127T599	2.0284431	0.05101163	0.47638202	0.11464922	0.01535866	1E-18	0.03032622	0.09990493	0.0952485	0.10290314	0.00831596	0.46414677	0.40647293	0.14726244	0.01860333	0.00970381	0.51764884	0.243318	0.09100017	0.6631875	0.11905317	0.38386409	0.46589308
M88T193	7.8945098	0.01134047	9.66055032	0.20052737	0.03198627	0.17864809	0.02908839	0.27590125	1E-18	0.05093164	0.05505631	1E-18	1E-18	1.37951004	0.63584279	0.01401589	2.12126419	0.27270019	0.00462604	0.0201342	0.22314985	0.07532364	0.20927281
M669T1573	12.6608841	0.51319268	2.84765488	0.68556131	0.72129474	0.79771455	3.68464488	0.19439681	0.72629071	0.77606819	0.13448495	0.19702031	0.49073635	1.93444662	1E-18	0.19435529	1.01901668	5.9532497	0.37444005	1E-18	1E-18	1E-18	1E-18
M387T782	6.43840123	0.02373016	0.149	0.0112	0.32230033	0.135	0.4457841	0.02156036	0.0393	0.14472296	0.0111	0.06862135	0.1731878	0.108	0.0778	1E-18	0.67491101	0.5390515	0.037	0.18632071	0.12554001	0.34530206	0.35436393
M119T129	124.689099	18.098189	3.7208868	19.6106094	52.3544405	125.60585	52.1325384	1.7593422	25.7457661	0.05645254	5.40580212	0.4089032	1.41812023	56.9013193	9.13933028	1.85848346	0.35729702	271.452812	0.04069039	21.4045426	17.0916212	45.2412628	15.0436966
M327T631	9.71728563	1.66035185	1.57705633	0.13847038	0.15958726	0.64564453	6.64666678	0.02592572	0.05833893	0.1084163	0.05268387	0.08697349	0.0269754	1E-18	0.01313026	0.02491026	0.29137307	4.8832382	0.14629091	1.14419411	0.2729218	0.13463655	0.15415463
M154T89	1.49807851	0.07930744	0.2165665	0.10292415	0.06502504	0.15834962	0.11137654	0.03283961	0.03753222	0.09738113	0.0480078	0.03972763	0.18087038	0.54944044	0.20980858	0.02789504	0.63987563	1.22457609	0.30237297	0.15682187	0.12098195	0.10105456	0.10344747
M161T81	0.55124656	0.02201357	0.1304003	1E-18	1E-18	0.12702705	0.16080719	1E-18	0.03114723	0.0301543	0.00425464	0.01817254	0.02402414	0.21452753	0.27466612	0.050106	0.35256998	0.67899152	0.02739388	0.14781647	0.09376179	0.04470369	0.17370576
M159T1539	2.71726138	0.09585661	0.31018693	0.00790419	0.07427289	0.06308933	0.16875737	0.02037591	0.01289498	0.01960263	1E-18	0.02286422	0.04217981	0.14025418	0.05948048	0.01649696	0.23726186	0.49881922	0.0070043	0.04012682	0.02643015	0.23829245	0.20434201
M295T809	1E-18	0.00982	1E-18	0.0363	1E-18	0.468	1E-18	1E-18	1E-18	0.00734918	0.00224971	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.132	1E-18	1E-18	0.00283825	1E-18	1E-18
M233T84	0.81617066	0.26109293	3.25990019	0.46880766	0.08610026	0.51376818	0.56600914	0.22175706	0.33867158	0.32853454	0.00641807	0.06021096	0.08914251	1.96590543	0.09802811	0.0253305	1.74451022	14.9825341	0.01599156	1.73735531	0.32910173	1.51429511	0.98737351
M348T848	1E-18	1E-18	1E-18	0.0588	1E-18	0.114	0.43824834	1E-18	0.0429	0.15594699	0.0178	1E-18	1E-18	0.0599	1E-18	0.00326	1E-18	1E-18	0.0945	0.0995	1E-18	1E-18	1E-18
M195T91	2.06314352	0.06434936	3.60768093	1E-18	0.11419299	0.24695967	1E-18	0.02077009	0.02207898	0.03721624	0.126084	1E-18	0.01402966	0.35196457	0.10998529	0.00489243	0.0499535	0.30196875	0.04867745	0.01825691	0.01346943	0.04435404	1E-18
M217T442	67.3893127	0.99597963	18.5032019	1.12892875	9.15636933	1.57073772	8.28448352	2.58257227	0.17009824	1.16456308	0.09568717	0.19010302	2.0020491	1.69724516	3.7673302	0.86746848	4.55933378	6.62444726	0.02001769	5.28976885	3.26237107	1.54716521	6.31308318
M173T201	16.2496412	0.06326337	2.8170032	0.15505467	0.78209753	1E-18	0.02462299	0.29333993	0.00493072	0.04146027	0.01314689	1E-18	0.01609378	2.29358103	2.3650167	0.03484496	1.89936868	0.34010746	0.0119837	0.0781762	0.04207924	0.21047332	0.26293012
M217T607	2.58728394	1.16415197	73.960202	2.08505537	9.94351604	0.17745723	8.30005573	3.77217619	1.55833462	2.4040388	0.42800914	0.54215483	1.18202974	0.5814345	12.8376554	0.6731656	16.8477419	363.116655	0.16190767	59.5484348	11.8274395	3.71661496	5.14702577
M71T87	5.83893584	0.96141196	1.75915765	0.19182784	0.81063866	1.05381618	2.03493096	0.07265077	1.28120177	0.22709942	0.16028734	0.14258637	0.26260684	0.55469051	0.32918258	0.12155136	1.37723145	19.4314953	0.23250119	1.57241044	0.30369693	0.70957509	0.37502223
M311T726	6.59484191	0.46236386	0.37531173	0.99447537	0.06532975	3.91806902	0.8031555	0.22136292	0.02546484	0.02276845	0.10564099	0.02413544	0.43481385	0.36158537	0.20736849	0.04734909	1.80994303	13.4528564	0.03371099	0.0578528	0.46903823	1.12750095	0.26125816
M229T89	1.43539298	0.06693274	3.09284389	0.03820748	1.24419385	1.07009528	0.74866939	0.57669952	0.16995663	0.05121424	0.01281196	0.0710052	0.04245952	0.2351929	0.11067552	0.0806456	0.2403625	1.96122627	0.0457298	0.09049189	0.1100653	0.04654307	0.16357174
M168T85	1E-18	0.0100894	0.32806177	0.00988359	0.04260303	0.09043107	0.07903575	0.02358044	0.01774183	1E-18	0.01546701	0.01380921	0.01901833	0.11135692	0.05198021	0.00683724	0.08884219	0.68400326	1E-18	0.0475139	0.00931576	0.05401215	0.05774137
M319T1300	1.27980496	0.1207641	0.72566216	0.06677859	0.18300299	0.07918983	0.59525728	0.03497901	0.06942924	0.03428326	0.03139529	0.07953325	0.03020756	0.32821182	0.11012465	0.02746575	0.13076411	2.01847748	0.06993376	0.11810957	0.03531803	0.16530717	0.1345117
M198T1487	1.87262948	0.0486122	0.30483601	0.07767112	0.2992193	0.44039317	0.34028382	0.02667896	0.05738289	0.14106108	0.04451256	0.06877362	0.12395182	0.45667558	0.3405531	0.06161515	0.34482123	3.44128391	0.14682998	0.37464868	0.11956563	0.43398849	0.29901689
M198T1484	1.87262948	0.0486122	0.30483601	0.07767112	0.2992193	0.44039317	0.34028382	0.02667896	0.05738289	0.14106108	0.04451256	0.06877362	0.12395182	0.45667558	0.37502124	0.06161515	0.34482123	3.44128391	0.14682998	0.37464868	0.11956563	0.43398849	0.29901689
M153T89	42.1904505	0.63923141	0.88964787	0.85902935	2.78750372	0.2120407	4.22002942	0.02551282	0.58840743	0.053162	0.02964676	0.25199958	4.44070457	3.41840545	0.17782116	1.10902867	0.24788531	14.8736272	0.01539681	1.56050158	2.76959838	0.0570322	3.27781685
M197T84	22.6568751	0.09911315	0.3391927	0.05128261	0.02439722	1.23920773	0.21449795	0.00930693	0.02566341	0.04041772	0.02053537	0.04998092	0.03249824	0.10972077	0.05271571	0.20596185	0.05776156	6.63303309	0.22107138	1.08210531	0.04113037	0.06903833	0.11652745
M319T1411	0.54004519	0.02055019	0.12255325	0.00650599	0.01980222	0.04638471	0.03673809	0.0125514	0.01113354	1E-18	0.00557563	0.00499962	0.00739569	0.082362	0.04602555	0.00785807	0.05391854	1E-18	0.02481739	0.0311856	0.00301158	0.0262656	0.03012102
M406T732	5.32478517	0.02112019	0.75283446	0.20244154	0.21328648	0.27525367	0.2792362	0.04169927	0.02944087	0.07965429	0.00272782	0.06854396	0.06351993	0.10621719	0.07870195	0.0314453	0.28061482	2.42361165	0.17769834	0.67900728	0.05000025	0.18766536	0.22141843
M509T719	7.33875469	0.16315381	0.49892321	0.07375964	1E-18	0.36252497	1E-18	0.00293713	0.05822105	0.00716691	0.08072615	0.13351999	0.04432285	1.93180378	0.21794305	0.04848806	0.45291532	4.80132548	0.06888831	0.85132217	0.04904626	0.87534548	0.68013489
M397T728	1E-18	1E-18	0.74486386	0.02445696	1E-18	1E-18	1E-18	1E-18	0.01559488	1E-18	1E-18	0.03419366	0.0465	1E-18	1E-18	0.01581287	0.11640927	1.87335202	0.04698076	0.79904421	0.032	0.07527395	0.21271678
M241T652	41.7320558	1.61539013	23.9229263	2.49148695	5.55353559	3.66430411	2.98496594	0.85388856	0.35993063	1.40144457	0.61481835	0.35277849	0.84731892	0.84068682	10.2084701	0.46008013	1.88610416	68.1745312	0.60611326	4.58022878	5.11050424	1.05637735	6.4741123
M385T912	1E-18	0.0789	1E-18	0.27719159	0.04509969	0.02015107	0.33415007	0.01553783	0.00498588	0.03630503	0.00243	0.08983534	0.05314378	0.16788973	1E-18	0.01762645	0.29011077	3.72505511	0.09438238	0.56258324	0.034	0.15716968	0.27113534
M147T93	0.27424533	0.01017688	0.99407257	1E-18	1E-18	0.43850583	0.06229936	0.02116165	1E-18	0.35057879	0.0218136	1E-18	1E-18	0.53231325	0.69421116	0.00453651	1E-18	0.16805285	0.02389906	0.06433437	0.01028125	1E-18	1E-18
M265T639	0.80148723	0.00963801	0.43036785	0.02693419	0.02074818	0.10486531	0.06382767	0.02053552	0.05385247	0.51380517	0.20403093	1.21999234	0.78702714	3.36447027	0.93546838	14.510528	36.2301438	326.892698	4.27764428	13.1065937	1.69575392	21.6072078	37.42701
M557T587	0.62752246	1E-18	0.09065305	0.01860357	1E-18	1E-18	1E-18	1E-18	0.00723154	1E-18	1E-18	0.00653143	1E-18	1E-18	1E-18	0.008467	1E-18	1E-18	0.03304945	1E-18	1E-18	0.06227953	0.08713929
M266T639	1E-18	1E-18	1E-18	0.04870724	1E-18	0.05170368	1E-18	0.00430402	0.0131201	0.06473178	0.0067285	0.07504514	0.05731138	0.23562402	0.07586261	1.38509283	2.71646442	26.8699475	0.36495044	1.22576954	0.1682541	1.67586348	3.04240742
M353T694	1E-18	0.06289061	1E-18	0.01972116	1E-18	0.08916401	1E-18	1E-18	0.00517	0.00972659	0.00619608	0.01223105	0.00832	0.139	0.0378	0.0814	0.138	2.33	0.15784338	0.20533691	0.023	0.104	0.212
M265T640	0.80140107	0.00963093	0.41194207	0.01764754	0.02075947	0.10473322	0.06379862	0.02807734	0.05388788	0.49841511	0.24199482	1.37357369	0.80970263	3.60994189	6.89820089	14.8377572	32.0979997	304.221111	4.15989818	12.7081535	1.55137554	20.6665135	35.6361864
M207T93	1E-18	0.12703159	1E-18	1E-18	0.06238872	0.10133852	0.1697005	0.00850117	1E-18	0.04676872	0.0227016	0.01040387	0.01977143	0.09204247	0.34580503	1E-18	0.05425776	1E-18	1E-18	0.01917808	0.02163969	0.01605504	0.02429525
M87T426	42.9636951	0.35912004	8.9200347	0.39194423	1.60252314	1.08874689	1.85229302	0.63705316	0.738142	0.29883031	0.15745293	0.64644958	0.34116637	2.76034754	0.54327892	0.24757779	0.73371452	6.65438247	0.71031213	0.99230368	0.21395672	1.47664047	1.95213687
M307T938	0.25274392	0.1811397	0.17871953	0.10391737	0.08374992	1.66792373	1.8738414	0.03490869	0.01198391	0.54513683	0.11663956	1E-18	0.00653183	1E-18	0.01183364	0.00136728	0.0339718	0.95641797	0.05888268	0.19215916	0.16254594	0.04099176	0.05293127
M269T764	1E-18	0.05448952	0.46177526	0.02570301	0.04164619	0.13581956	0.22453229	0.02699757	0.03341346	0.00802038	0.0332726	0.0761	0.0245	0.0485	0.018	0.02001782	0.0171379	0.163	0.01470506	0.01322991	1E-18	1E-18	0.0134
M267T639	1.03442932	1E-18	0.48824733	0.14020035	0.06992371	0.06141813	0.23315823	0.05089659	0.00499461	0.11143551	0.00617319	0.01743562	0.02306397	0.03070497	0.10862175	0.38030405	1.08963376	9.98437846	0.1484983	0.43434264	0.09225495	0.70333748	1.18656366
M417T689	1E-18	1E-18	0.06722316	0.01644646	1E-18	0.02626422	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.00788359	1E-18	0.01285312	0.00260946	1E-18	1E-18	0.01268027	0.35558826	0.02865962	1E-18	0.01905231
M87T245	26.56194	0.3990989	2.69302687	0.30879111	0.45430254	2.036414	1.29470157	0.44294206	0.22443084	0.23954121	0.17926561	0.333159	0.15315422	1.69412841	0.63657649	0.0908979	1.1719082	3.7232531	0.46525746	0.93369496	0.22847018	0.76718537	0.5189958
M74T254	12.701536	0.24567751	2.0680511	0.20407516	0.54514269	0.72106943	0.75326218	0.14581951	0.21965985	0.25498713	0.08443785	0.17462651	0.1247977	0.87285986	0.34302658	0.05922655	0.74957559	4.189817	0.21282876	0.31743996	0.15485734	1.35227246	1.83213865
M181T528	7.93705572	6.22196952	70.6059159	1.69199913	1.497041	8.63123971	2.0960266	0.60365017	0.90127573	1.17959533	0.46795257	0.4760204	1.11503408	2.38986488	5.10113082	1.19020651	8.40274554	40.5471514	0.54599454	21.0239556	4.49284375	6.71348947	0.8986416
M385T821	3.95151186	1E-18	0.1507053	0.12	0.144	0.12850077	0.0708	0.0331	0.16687473	0.01179386	1E-18	0.03089618	0.0715	0.0935	0.0199	0.0628	0.443	2.39	0.05880831	0.10195192	1E-18	0.136	0.14987394
M235T92	1.9178648	0.11230517	0.32986178	0.04144328	0.10525019	0.10514293	0.03168374	0.02195912	1E-18	0.084771	0.00310426	0.00514163	1E-18	1E-18	0.01011259	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18
M217T520	27.5302962	1.15368917	1.34344644	0.89472157	3.30297442	2.71243048	7.09979159	1.18018589	0.14534062	1.3566747	0.07884634	0.70114714	2.29126724	1.90156981	2.08720978	5.76095491	3.0300443	61.1974907	0.13287436	9.44116393	0.59686907	4.97547823	5.07161375
M163T492	1.17662633	0.04114881	0.45153315	0.04325448	0.33961561	1.27000514	0.29382012	0.02479134	0.18837043	0.13641897	0.21448684	0.12083795	0.08179479	2.79305041	1.00313897	0.05968072	0.08503445	1.66227192	1.58356974	0.42975688	0.02548265	0.11478368	0.1472753
M219T89	2.27828501	0.05290391	2.8922222	0.02716149	0.11155794	0.06735664	0.16442654	0.4687421	0.03467903	0.04624525	0.06535366	0.03761353	0.07704036	0.15788504	0.15820944	0.03672937	0.44743544	1.36687419	0.05526266	0.29236218	0.41852662	0.28994199	0.05431658
M325T745	3.67344849	0.02416015	0.80457809	0.04036378	0.02121418	0.31586633	0.07360546	1E-18	1E-18	0.10044114	0.00353361	0.08868034	1E-18	0.1260965	0.01207991	0.00287605	1E-18	1.63265987	0.01034949	0.14329142	1E-18	1E-18	1E-18
M229T127	4.49825885	1E-18	0.31673996	0.29719354	0.04012387	1E-18	1E-18	0.0393217	1E-18	1E-18	0.08452997	0.01896861	0.00931256	2.25624166	3.48633099	1E-18	0.87312299	0.15857608	1E-18	0.14273988	0.24745361	0.65867513	0.12687233
M141T84	1.66803221	0.03574061	0.41375969	0.04924914	0.10672359	0.04888195	0.22727	0.03577831	0.07276022	0.04230283	0.01655813	0.04806627	0.02118532	0.14280457	0.14421091	0.01284647	0.87106995	1.13925872	0.22437312	0.16243143	0.13801472	0.06463934	0.04639652
M165T626	14.5890036	0.0695586	3.83228152	0.44063836	0.89794713	0.44397146	0.14163138	0.13781146	0.49241352	0.19049919	0.06880821	0.39476504	0.4034255	0.06258471	0.12932643	0.37208527	1.40654172	10.5103483	0.38705817	0.82924118	0.12823271	0.62743052	0.47636169
M139T94	0.74731378	1E-18	0.2941823	0.03145318	0.06357967	0.68761118	1E-18	0.02464325	1E-18	0.02780579	0.01771719	1E-18	0.08864546	0.15501753	1.57254112	0.02097874	0.74861739	1E-18	0.02934956	0.07268382	0.01969391	0.09410946	0.11232152
M250T543	0.35680037	0.0229419	1E-18	1E-18	1E-18	1E-18	0.02755101	0.02188721	0.00707204	1E-18	1E-18	1E-18	0.01647169	1E-18	0.30072063	0.01895597	0.01490082	2.41089711	1E-18	0.16393754	0.12258465	0.04234894	0.01431026
M111T293	74.9038079	0.51495137	3.55056953	0.50383361	1.25866085	0.72152112	1.05776344	0.44284796	0.6927238	0.35073772	0.20949982	1.10668017	0.15446728	3.62271196	4.08998713	0.32350056	3.27977615	5.95739648	1.72004978	1.00232091	0.18674957	2.09681903	1.97311501
M669T731	20.0881839	0.67049805	3.3328703	0.27473796	0.75015498	0.76142022	1.09268873	0.1717744	0.23065007	0.31012482	0.12483473	0.20102493	0.20854203	0.72965175	0.10638562	0.08001863	0.33463352	2.68217175	0.08768398	0.10045758	0.03575066	0.1650429	0.0935749
M243T91	1E-18	0.01067721	0.46454701	1E-18	1E-18	0.28849676	0.42571838	0.1338417	0.02278897	0.296041	0.06192126	1E-18	0.14248966	1E-18	0.03186945	0.01739773	0.08116629	1.30254734	0.0851416	1E-18	0.02213027	0.10496427	1E-18
M200T86	2.27662711	0.04616293	1E-18	1E-18	0.0567954	0.11383743	0.23897457	0.03114628	1E-18	0.0301535	0.04019175	0.04305717	0.06046196	0.58485728	0.06666059	0.02333654	0.15998738	2.83824176	1E-18	0.06001514	0.04638199	0.13375825	0.06939767
M196T90	3.76720383	0.10843372	1.34807816	0.07514298	0.22186041	0.2436819	0.53506019	0.06484701	0.09173016	0.05749753	0.0445862	0.08968562	0.10087225	0.31615432	0.15519925	0.03186792	0.26344754	0.77743083	0.0628537	0.09489479	0.05091192	0.17822432	0.12754853
M558T655	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.00618785	1E-18	0.00251298	0.00528732	1E-18	0.06141682	0.06745426	1E-18	0.07195847	1E-18	1E-18	0.07401141	1E-18	0.12515705	0.07850758
M108T83	8.89936746	1.14371896	0.82512359	1.23544105	0.25115937	0.26138509	0.82204637	0.07398296	0.21859368	0.11892712	0.14365095	0.50104885	0.36753611	0.43319481	0.23962184	0.08048878	0.16101823	16.4195331	0.48787295	1.3391679	0.67555148	0.26870671	0.22794093
M385T752	0.514	1E-18	0.08357341	0.10273381	0.183	0.23766725	0.1305459	0.0702	0.13220419	0.07975152	0.00555734	0.09357541	0.1730613	0.10914095	0.205	0.05099377	1.06104838	5.14580817	0.0111	1.30237874	0.11979787	0.226	0.62530554
M198T85	1.87948745	0.03536768	0.85612854	0.02182933	0.22164323	1.10588919	0.30722081	0.04810492	0.14457526	0.25458353	0.10929771	0.06506011	0.14870232	0.09801589	0.06126501	0.14273265	0.15106473	1.39142705	0.16428558	0.09826025	0.13433012	0.07209437	0.09234399
M87T485	51.0426436	2.39054271	16.5045064	1.27419841	1.91694071	8.38132438	8.27606465	0.41861054	1.55428156	0.28356827	0.70212674	0.97606621	1.22179138	5.63960195	0.44268567	0.61610621	1.93200929	29.9054847	1.43144765	2.09884437	0.68735233	3.29198629	1.65290971
M481T663	1E-18	0.23078354	1E-18	0.83598832	0.04310088	1.93535385	0.35506271	0.31445304	0.08540585	0.49337399	0.02914953	0.09486515	1.49573581	0.38525431	0.98643755	1.02820019	2.06527652	19.2998522	1.26231729	6.53134152	0.23248829	4.61684572	2.79043879
M500T620	1E-18	1E-18	1E-18	0.18031812	1E-18	1E-18	1E-18	0.00542576	0.03604956	0.13370193	0.09219803	0.35111293	0.50517347	0.20466377	0.20733359	0.40637331	1.12925886	11.1322822	0.95244677	2.35463636	0.10349437	1.39255073	2.42241563
M385T561	0.2766305	1E-18	0.06391126	1E-18	1E-18	0.50793912	1E-18	1E-18	0.10163651	0.01535336	0.03003702	0.01661268	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.06358072	0.02620831	1E-18	1E-18	0.02241586
M271T936	3.12097081	1.17548881	1.37	0.309	0.35555867	3.45339759	4.36588714	0.12779031	0.13718745	1.07992901	0.222	0.01952062	0.00731723	0.047	0.11	0.01736246	0.33627863	2.76641026	0.0726	0.33560834	0.3067465	0.14076873	0.161
M133T243	14.5187956	0.06544611	1.575246	0.14792775	0.09528483	0.08288069	0.14515349	0.1040504	0.02823725	0.01905594	0.00714735	0.53504155	0.06206287	0.35915902	0.47495175	0.05174469	0.38154144	1.08040156	0.09153102	0.18694886	0.02342865	0.23184995	0.47576819
M395T693	0.329	1E-18	1E-18	0.0403757	0.112	0.02600437	1E-18	0.00976	0.015	0.03198369	1E-18	0.10468723	0.09656825	1E-18	0.0314	0.10672114	0.7349557	5.59631317	0.0496	1.33532025	0.04727831	0.211	0.52202002
M168T78	0.93336931	0.05249014	0.38807324	0.02992176	0.11597081	1E-18	0.07656005	0.01817401	0.03110664	1E-18	0.01551203	0.01378262	0.03362399	0.15659614	1E-18	0.02004719	0.16211253	0.77215605	0.01929281	0.06970322	0.00930285	0.11033529	0.05779909
M119T1536	2.53947401	0.04120451	0.38672707	0.02987336	0.06932554	0.1497973	0.038474	0.0262	0.0076907	0.03409343	0.01736891	0.02057842	0.06535046	0.14948527	0.08642545	0.00793531	0.09709307	0.79806468	0.04731715	0.03589436	0.02085508	0.08483894	0.09293836
M125T255	2.61988334	0.05794757	0.57416967	0.05909556	0.10125757	0.24372351	0.1370205	0.03802583	0.02208991	0.04133243	0.00789479	0.07817773	0.04650022	0.09284094	0.07369962	0.01988906	0.09057998	1.21123531	0.05378411	0.23062652	0.02132541	0.09565229	0.12498277
M283T529	0.352	0.0258	0.30125163	0.0337	0.01508944	0.0412862	0.3	0.11555045	0.00854321	0.0886	1E-18	1E-18	0.0793	1E-18	0.56292244	0.03114357	0.0722694	4.92	1E-18	0.09664259	0.153	0.3544699	0.0765
M216T543	0.23835744	1E-18	1E-18	1E-18	0.01741733	1.0739105	1E-18	0.00364299	1E-18	1E-18	0.06034637	0.12640534	1E-18	0.51853994	1E-18	1E-18	1E-18	0.21713664	0.315468	1E-18	1E-18	1E-18	1E-18
M315T780	1E-18	0.0186609	1E-18	0.00269748	1E-18	0.25517604	0.27478928	0.00379735	0.00990821	0.04142219	1E-18	1E-18	0.00565563	1E-18	1E-18	1E-18	0.00892615	1E-18	1E-18	0.01851084	1E-18	1E-18	1E-18
M187T189	1.03022933	1E-18	0.24225633	0.02574622	0.01358881	1E-18	1E-18	1E-18	0.00554386	1E-18	0.00213334	1E-18	1E-18	0.60036342	0.29565377	1E-18	0.13506203	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18
M421T802	1E-18	1E-18	0.23256219	1E-18	1E-18	0.13932641	0.03073784	1E-18	0.18887668	0.00763295	1E-18	1E-18	1E-18	0.15211416	1E-18	1E-18	0.01693503	1E-18	0.0190941	0.03063738	1E-18	1E-18	1E-18
M543T471	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.02770712	1E-18	0.04546534	1E-18	0.02504178	0.11865073	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.19757272	1E-18	1E-18	1E-18	1E-18
M323T497	1.09726642	0.05014943	1E-18	1E-18	0.02024528	1.72164574	1E-18	1E-18	0.10164225	1E-18	0.29342666	0.34769446	1E-18	0.01915886	1E-18	1E-18	1E-18	0.1448491	0.01340052	0.01488391	1E-18	1E-18	1E-18
M308T976	1E-18	0.01859196	1E-18	1E-18	1E-18	0.36623359	0.02891079	1E-18	1E-18	1E-18	0.00237406	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.12811594	1E-18	1E-18	1E-18	1E-18	1E-18
M484T679	1E-18	0.06464562	0.02185846	0.14287284	1E-18	1E-18	1E-18	0.00878619	0.1995179	0.07610788	0.00618431	0.05682793	0.48196431	1.07874952	0.29233577	0.1507368	0.55204322	3.02098741	0.03318897	1.28550672	0.14076057	1.20043648	0.42463821
M407T736	5.1307121	0.0545752	0.71867144	0.19382515	0.20211758	0.24541965	0.30356094	0.03922439	0.07584394	0.09035972	0.00990872	0.07426293	0.0810937	0.1641143	0.12870225	0.03042675	0.27992058	2.3159693	0.03112378	0.66729885	0.05113074	0.16224525	0.21874482
M143T239	10.2805204	0.20036324	8.04920876	0.59974229	0.4037434	5.50649763	1.63179409	0.29106711	0.29136029	0.17826649	0.24523978	0.55255639	0.06553125	1.38940478	0.28042643	0.08193342	1.44290227	3.42884514	0.35273948	0.76012459	0.13135744	1.03639196	1.00732675
M95T148	232.617974	4.23356035	1.35558911	5.84585104	3.10489132	9.98800569	27.4553913	0.34885893	1.20864867	2.73361625	0.82135755	8.6118581	1.57130737	6.50321588	27.3607567	1.55284488	8.9220218	24.3670782	3.83259892	38.0702182	0.83205783	11.9109684	11.7372158
M223T90	1E-18	0.08200293	0.13929386	1E-18	0.16160223	0.09121349	0.21607842	0.00960124	1E-18	0.04461183	0.01421833	1E-18	0.00849054	1E-18	0.05592012	1E-18	0.13293594	1E-18	0.02447519	1E-18	0.01047712	0.01851942	0.0123045
M120T482	54.622828	7.02105463	12.295246	1.68979693	6.82755044	0.96342702	15.9679311	7.21537229	1.48475275	1.94021376	0.85836798	0.92847744	4.63244712	3.84508613	5.11409338	0.40648026	5.6625537	21.9974425	3.06105317	5.24582751	1.20210621	7.34685923	4.99018
M297T1469	0.493	0.68122561	0.531	0.0726	0.0326	1.77217124	1.58904717	0.0506	0.0072074	0.64307435	0.06446517	0.019	0.014	0.0209	0.0228	0.00618	1E-18	0.313	0.00501	0.07418063	0.03925704	0.117	0.0460533
M126T86	3.22916577	0.06267329	1.52642048	0.0890078	0.30834216	0.20454997	0.29351679	0.23585545	0.06314657	1.6463923	0.01441118	0.04297086	0.06677806	0.19774745	0.21543869	0.02159565	0.68334843	1.87948333	0.17482964	0.2651301	0.02573811	0.03701462	0.03841405
M272T936	1E-18	0.11727789	0.05472071	0.00588967	1E-18	0.42457051	0.5022776	1E-18	0.00277744	0.12421063	0.01356035	1E-18	0.00810996	1E-18	1E-18	1E-18	0.01942556	1E-18	1E-18	1E-18	0.03485454	1E-18	1E-18
M272T937	1E-18	0.11727789	0.05472071	0.00588967	1E-18	0.42457051	0.5022776	1E-18	0.00277744	0.12421063	0.01356035	1E-18	0.00810996	1E-18	1E-18	1E-18	0.01942556	1E-18	1E-18	1E-18	0.03485454	1E-18	1E-18
M555T669	1E-18	0.57659712	1E-18	0.10493597	1E-18	1E-18	0.24096641	0.18583772	0.70045222	0.72982028	0.1038412	0.66664957	1.77052945	1.79061605	0.49582381	0.82101875	3.03191619	28.2695784	0.0741495	7.66831548	0.39535558	7.00783966	3.50118436
M347T754	14.4935436	0.13407294	1.20718224	0.09778469	0.97257918	0.11072958	0.60409536	0.5591112	0.12101054	0.22694728	0.10687574	0.18902783	0.03301733	0.82297431	0.28083667	0.03193395	0.20222269	1.42612393	0.05763428	2.47316703	0.18107927	1.22634806	0.62603882
M218T90	1E-18	0.01271806	1.54538015	1E-18	1E-18	2.05689211	1.46695716	1.40736391	0.06563413	1.80305571	0.01502761	1E-18	0.31139975	0.61239385	0.03322881	0.00935145	0.16290625	1.62470877	1E-18	1E-18	0.0185355	0.10296954	0.01184243
M59T671	58.9659904	0.67057383	5.25737715	1.11395416	3.36200952	5.42047319	6.79813552	1.07666981	1.41129939	1.35992297	0.47533365	0.91578046	1.3905537	4.51413363	0.56706469	0.74895785	3.76912587	37.9477206	1.0162201	2.00927083	0.95181792	2.80496586	1.96630182
M133T83	0.58520865	0.07495262	5.31815499	0.0131283	0.07095489	0.1750848	0.13842395	0.2538717	0.03245627	0.13888098	0.11429717	0.03857382	0.04117639	0.35847095	0.08463979	0.12792172	0.07334535	0.49040708	0.57065795	0.07727216	0.02841404	0.13435899	0.05297947
M343T654	42.7778288	1.30136425	5.66056581	0.0217361	2.78531516	0.64146465	22.8418712	0.51625069	0.06778677	0.08477042	0.09501616	0.12335525	0.61302189	0.03895339	0.21452991	0.0149902	1.93603948	18.5280974	0.06077683	8.06994514	0.73177226	1.91368873	0.18057046
M345T76	0.92714296	0.10853028	0.10304625	1E-18	0.0327473	0.02947701	1E-18	0.00443	0.01454413	0.0089189	0.00265	0.00737	1E-18	0.0289	1E-18	0.00805257	1E-18	1E-18	0.0145	1E-18	0.00503425	1E-18	1E-18
M181T637	18.2208798	2.73455996	49.2349587	6.33173321	4.39117678	7.61984791	40.5112085	4.13891263	0.81315703	0.79245644	1.98349517	0.75804439	4.32111888	1.22437175	7.21964488	3.84786221	21.096797	126.133126	0.60467491	5.14392739	13.6520771	25.9148724	0.80987762
M127T84	4.04517356	0.15149194	0.29984405	0.21957346	1E-18	0.16710105	0.23650357	0.50268611	0.03150538	3.82013125	0.01190771	0.0582533	0.07164915	0.25240345	0.07191904	0.01533154	0.28997582	5.11983052	0.00614779	0.47265899	0.01973271	0.09497803	0.14961198
M196T89	4.76910655	0.05675672	1.2181542	0.03682683	0.05627157	0.07489108	0.25008247	0.02944765	0.08815713	0.190128	0.05554386	0.01613195	0.02889424	0.24124117	0.07068177	0.01228352	0.11832614	2.38050238	0.09486049	0.2813434	0.0438643	0.0611487	0.06679149
M159T205	0.71838291	0.04958584	0.53751325	0.02714515	1E-18	0.05698554	0.0414047	0.00726208	0.00576493	0.01339288	1E-18	1E-18	0.00762952	0.18401595	0.10477434	0.00881499	0.10057909	0.32830422	0.00699073	1E-18	0.00654959	0.07385947	0.03815681
M481T662	1E-18	0.17589275	1E-18	1.35563388	0.229699	1.03715305	2.08222794	1.79413997	0.43927323	1.06331523	0.12845307	0.40959697	2.77169819	0.96313119	0.62221382	2.07029173	2.62653633	30.7450186	0.07090426	10.8621896	0.3592045	7.61914053	4.29626311
M85T232	88.1592082	0.87073663	5.92705491	1.27959118	1.09796876	7.20948968	1.55509393	0.24112566	0.6416995	1.2156453	0.3102707	0.51331664	0.37677957	10.4097075	16.2974354	0.47257543	6.73485692	18.7798656	0.48024888	2.72409904	0.72513159	2.22974492	3.45598833
M339T528	0.2766008	0.03158835	1E-18	0.01054609	1E-18	0.12811506	1E-18	1E-18	1E-18	1E-18	1E-18	0.01415042	1E-18	1E-18	0.02578249	0.00506231	1E-18	1E-18	0.01016503	0.06728096	0.00610201	1E-18	0.01425326
M243T87	0.98008568	0.17417915	0.21832515	0.22462063	0.27979475	1.22859554	0.33616602	0.1186599	0.0575174	2.37959963	0.0587267	0.32171251	0.20451637	1.70066477	0.14065521	0.13031456	1.52346674	1.04227954	0.57511202	0.35235186	0.01564714	0.12557098	0.20386306
M146T85	1.17529201	0.04703124	0.5116187	0.07217452	0.14790759	0.32439473	0.12552214	0.0529648	0.0354623	0.05331924	0.08506448	0.01077047	0.01536123	0.119527	0.0575405	0.00408993	0.06978824	0.49910706	0.02227908	0.10252162	0.05402765	0.03704117	0.06005815
M319T87	2.76536217	0.06562612	0.63563744	0.02865187	0.06994842	0.10099877	0.27371649	0.01999682	0.02045371	0.02837756	0.01582902	0.02643279	0.05685711	0.12402105	0.03890727	0.01154225	0.28914828	2.62312218	0.02197941	0.09746314	0.02041129	0.27322159	0.13285905
M219T670	11.0749199	0.36862195	3.86420151	1.06043618	5.09079175	3.95877396	6.83979441	3.32551847	0.17201278	2.13744598	0.10230781	0.02405972	1.36526938	0.02335227	3.37867069	0.62148327	8.68037087	39.3587355	0.19963575	12.3492438	0.62609193	5.82220252	6.04707455
M113T357	15.6226107	0.47181664	3.90558169	0.21686966	0.52714479	6.29122226	0.64508581	0.18936267	0.19677859	0.22700668	0.08818694	0.18642457	0.16481623	1.187934	2.13591905	0.06458284	0.95918889	3.44024608	0.18619775	0.2725834	0.10361912	1.12337891	0.70792799
M305T724	2.64236364	0.18679617	1E-18	0.17911477	0.0144704	1.29594587	1.32729627	0.00336443	0.07176939	0.27591735	0.01050301	0.09504038	1E-18	1E-18	0.01991499	0.00561768	0.0175888	0.41350511	1E-18	0.34125737	0.00493378	1E-18	0.04390741
M153T84	1.30179034	0.09993386	0.30362582	0.11944364	0.13250288	0.07273338	0.26238126	0.19820704	0.04472499	0.0295507	0.02372838	0.02928147	0.04779754	0.2159748	0.93120298	0.00749414	0.86689592	4.03493939	0.05674759	0.24024682	0.03037383	0.05075522	0.09980045
M333T718	23.5886375	0.85102281	2.93321804	0.23719353	0.96666295	3.26229937	7.82150165	0.37970795	0.0877102	0.24102799	0.2095003	0.25034348	0.27782816	0.69276762	0.35517045	0.05645693	1.16641264	7.42373459	0.76137561	2.3032764	0.21992142	0.64516354	0.41260285
M295T925	0.2786649	1E-18	1E-18	1E-18	1E-18	1E-18	0.214	0.0035	1E-18	0.198	0.00251	1E-18	1E-18	0.0241	1E-18	1E-18	0.0154	1E-18	1E-18	0.04610685	0.0153	1E-18	0.00952152
M126T85	3.22661887	0.22355249	0.21035721	0.08908137	1.46923266	0.39904627	0.27841831	0.01861803	0.69192548	3.71774896	0.03675529	0.0429446	0.09016245	0.09136567	0.03655355	0.03553768	0.12253273	10.044964	0.09685213	0.19682575	0.06995746	0.11647354	0.26399873
M593T585	9.62883434	1E-18	2.14496774	0.10759302	0.57333027	1E-18	0.17503737	0.20405983	0.20135335	1E-18	0.01397011	0.10076447	0.20537494	0.44038432	0.2134151	0.09560744	0.33456924	2.84005455	0.36785872	0.14765879	1E-18	0.24244068	0.11937298

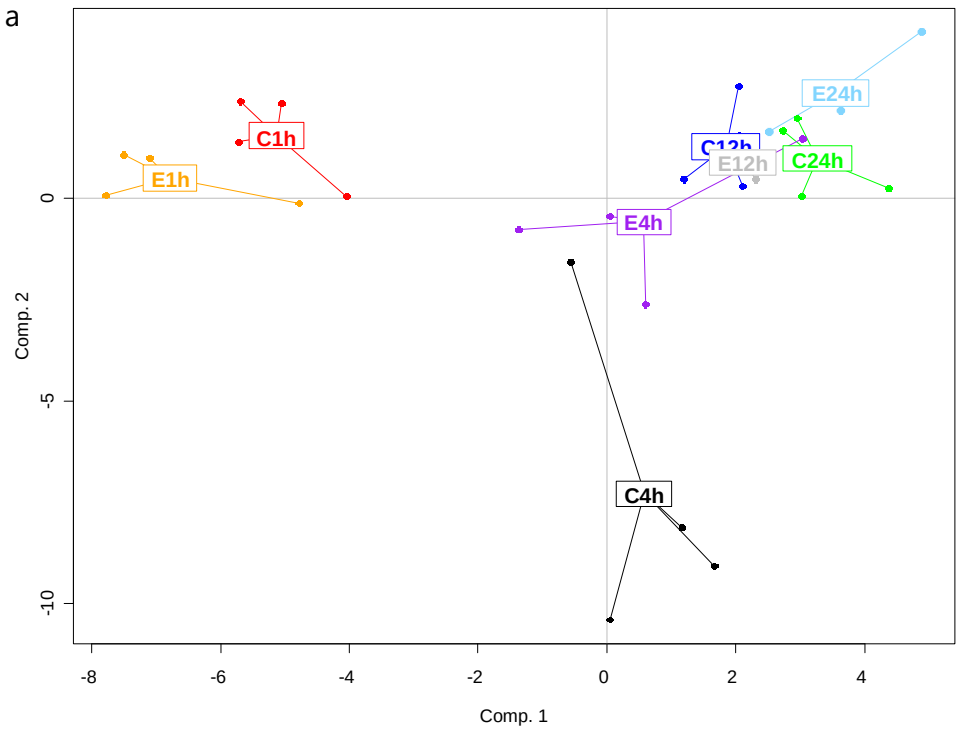
M271T1000	1E-18	1E-18	0.04811704	0.04806299	1E-18	2.13540469	0.17049093	1E-18	0.03816198	0.04994452	0.01442713	1E-18	0.00543667	0.02196107	1E-18	0.00301718	1E-18	1E-18	0.10233533	1E-18	0.0234323	1E-18	0.01339667
M135T1447	6.0248749	1.30123953	5.95643311	0.6352649	6.96135483	4.71026374	3.42238898	0.10348819	0.50472846	0.23401404	0.33445961	0.12929682	0.24940935	0.95266857	1.3714792	0.15459917	0.47526257	4.9140597	0.16925372	2.127875	0.12921774	1.31099677	1.36242576
M319T1367	1.57516772	0.0355191	0.24692536	0.0344587	0.05137189	0.0786036	0.1361637	0.01562458	0.03288778	0.0280672	0.01464889	0.01961911	0.02901985	0.04958091	0.02321879	0.01661935	0.0395596	0.43980691	0.01339246	0.05821884	0.01181196	0.09098819	0.0868087
M97T142	24.4723148	1.64537773	34.7898921	3.03204053	4.39679787	1.00190957	8.02575525	0.53016232	0.69550498	1.89134818	0.15648188	1.4435054	0.4579261	3.80164038	7.40248511	0.50512793	3.00802751	24.4656673	0.78937829	7.00763065	0.44226909	5.40644616	6.42222534
M151T245	3.58205673	0.12714372	0.78695197	0.09985372	0.25287162	0.48042182	0.27660348	0.04886435	0.0786638	0.09461112	0.01716399	0.1041948	0.02648485	0.26350083	0.18063971	0.03761765	0.1631415	1.1896911	0.09811043	0.33112815	0.04464187	0.23431784	0.17921904
M88T142	5.6928991	0.01734897	4.45301539	0.32552612	0.23993851	0.28955054	0.18380722	0.15046784	0.05822876	0.08304919	0.12064368	0.02238846	1E-18	1.56124143	0.71736914	0.00406975	3.6335903	1.27748259	0.00574798	0.09944262	0.41438723	0.17642853	0.13375705
M220T91	2.19291123	0.04024989	0.52187527	0.02449538	0.04502543	0.12195067	0.6092947	0.03392422	0.40447	0.02806114	0.42155824	0.04347016	0.02239933	0.50789796	0.18100773	0.01348266	0.59817258	1.68242019	0.35293412	0.08796775	0.02299515	0.0522867	0.05773724
M327T744	1E-18	1E-18	1.12991411	0.01758077	0.03137339	0.04320095	0.76099908	1E-18	0.0105368	0.47017653	1E-18	0.01683242	1E-18	1E-18	0.00809372	1E-18	1E-18	0.5769729	1E-18	0.55633247	0.00604686	1E-18	0.05119496
M293T87	0.779	0.0305	0.339	1E-18	0.0959	0.297	0.40304275	0.01158789	0.0331	0.02265841	0.026	0.0468	1E-18	1E-18	0.0605	0.00958268	0.184	0.544	0.025235	0.01835449	0.04480928	0.116	0.03408702
M120T1561	81.0738145	2.93468746	22.1423775	0.61573382	3.27264525	3.35866321	6.8623742	1.38499645	1.57348819	1.1796209	0.78638125	1.20642139	1.40214534	3.54330754	4.13036127	0.37977954	3.46264008	30.9115563	2.20469137	3.09874756	0.84196498	1.97769771	2.11499496
M219T524	17.8823272	1.147588	5.79733901	1.27187579	9.44454943	0.72228208	3.90944562	0.54999715	0.17708485	1.59759904	0.45780621	0.18701955	1.83015748	0.49201239	3.30374539	2.2320415	5.00255965	28.6471939	0.03808767	56.0616439	0.44353909	16.074394	7.99628526
M239T594	35.7995763	0.97552568	12.4624989	1.00946476	1.98645627	2.27692753	5.03358039	1.06244214	0.158512	2.59977029	0.28599051	0.16142375	2.42236073	3.91933169	5.98901715	0.53981215	5.77210916	33.5342571	0.2609873	5.28951593	1.63793604	5.81741076	3.40768602
M483T729	2.87777325	1E-18	15.1532831	0.07445193	0.04572499	1E-18	1E-18	1E-18	1E-18	1E-18	0.00307516	1E-18	1E-18	0.02767774	0.00945366	1E-18	1E-18	1E-18	0.00508767	1E-18	1E-18	0.04251929	0.02020857
M319T1444	2.01190517	0.07853246	0.37035179	0.04620439	0.10654582	0.16647199	0.12748337	0.03121753	0.05499745	0.05112268	0.02093574	0.0289855	0.05583092	0.10962645	0.04945679	0.01453468	0.07268208	0.49045479	0.03782983	0.1186832	0.0274703	0.10280777	0.10166102
M131T326	5.34730174	0.06279168	0.75255525	0.07105905	0.12620422	0.06761756	0.19131081	0.09709859	0.01357162	0.07262692	0.02628533	0.02558531	0.07513444	0.30090882	0.56348182	0.02586699	0.15820128	0.12047098	0.02656471	0.10410119	0.03247871	0.17300899	0.26298292
M103T190	50.8135549	0.65900697	4.03250232	0.61253595	0.70167715	2.30261241	1.47226176	0.48594016	0.3411474	1.00040934	0.19991552	0.2253141	0.36745098	2.85877395	2.44942192	0.18610784	3.32605165	8.13121275	0.27971489	1.17966801	0.62254554	1.79294389	1.30449719
M483T687	1E-18	0.04392448	1E-18	0.09740536	1E-18	1E-18	0.10170433	0.08273392	0.0919555	0.51159029	0.19824807	0.05588477	2.14414756	0.99424752	0.11265689	0.60881371	2.66183766	16.7284188	0.82754762	5.6656837	0.60026323	5.10232209	1.69273644
M307T678	85.0175292	0.57063238	13.3574639	0.24917572	5.327654	9.77656035	82.350521	1.41592714	2.13342489	0.8136474	0.21576756	0.23129437	1.54717883	1.34441868	0.2169966	0.60385825	9.48549262	93.7852711	1.66181835	0.70955699	1.5569522	5.66056186	1.11063361
M75T538	27.0982972	2.66379604	3.77629278	2.35528926	14.3179602	2.53564651	8.40366571	0.4796329	1.51277939	1.6713673	0.17004277	0.68726765	1.34477511	3.31037648	1.43459835	0.79855459	4.62434719	37.9362772	1.14270538	1.16318537	0.97886847	2.94295306	2.16133387
M75T556	34.4061023	1.90293197	6.04375708	1.26409952	2.32017895	1.78841852	6.42815124	0.28916161	1.15480448	1.14894689	0.45344944	0.65288132	1.12347795	2.96573941	0.64017544	0.41647129	1.76212093	23.2624082	0.8066897	1.93188844	0.81928643	1.38593435	1.89361584
M124T599	25.8680997	1.3108971	14.1690925	0.72518511	3.0182393	3.73853098	5.41728878	0.670303	1.59578454	0.72583422	0.38012305	0.82431371	1.17561961	3.75134982	1.3167169	0.83333847	2.08965445	24.8495904	1.05284827	0.44270954	0.44809721	1.74589833	1.25949918
M101T304	30.6837549	1.05952859	6.89292204	0.6805912	1.83263438	0.65275156	2.17132709	0.3682035	0.34940473	0.45070231	0.32399794	1.30519053	0.24943463	4.51355517	2.44350681	0.22066725	1.30821412	8.34666245	0.52254129	0.87263162	0.31986176	1.92917343	1.04563065
M349T749	17.158703	0.18664868	1.40142854	0.2300515	0.01752023	0.2577998	1.33681084	0.0870672	1E-18	1.5351963	0.00235442	0.00626251	1E-18	1E-18	1E-18	1E-18	0.42282128	5.83136767	0.02813876	1.32061629	1E-18	0.16324114	0.11325176
M215T89	1.54836376	0.06349046	0.4093694	0.02478444	0.10156316	0.04459299	0.19422866	0.04447209	0.03603356	0.0435755	0.04659892	0.03308222	0.02774136	0.07454144	0.03632169	0.1157657	0.43477646	2.03052187	0.02194859	0.12448789	0.03166912	0.12063831	0.11214459
M319T81	1.64928708	0.04304772	0.34957806	0.02698804	0.06040774	0.28542913	0.49749961	0.07209293	0.02851483	0.0692755	0.0120928	0.01857125	0.03354431	0.0982743	0.03413398	0.06071274	0.06392954	0.45205044	0.02641885	0.07419265	0.06351017	0.2613093	0.08936641
M156T307	3.43635988	0.11364284	0.49855906	0.05811025	0.13487407	0.01909358	0.24205943	0.02298037	0.0579429	0.05633124	0.01404888	0.03120162	0.00961435	0.16814447	0.23370282	0.01584706	0.1680136	0.41460735	0.03063169	0.1006213	0.02290102	0.20884445	0.12356983
M181T574	125.032257	2.78188898	71.501015	10.8540543	7.40589802	6.79679657	115.337642	11.1081189	0.74526703	1.81159427	0.24824851	0.31204381	1.83430292	2.12881852	6.32085005	2.78570198	10.5470536	231.933276	0.42991268	21.1938117	70.3349841	22.5462228	1.73411326
M482T663	1E-18	0.09240792	1E-18	0.06278732	1E-18	1E-18	1E-18	0.03844458	0.10658074	0.03223219	0.03982403	0.14135914	0.12317692	0.32887663	0.54270549	0.09426337	0.11506375	1.16228648	0.2613386	0.53876256	0.00610306	0.3435329	0.19807955
M229T86	1.22692586	0.05553645	2.99962993	0.03808594	0.137523	0.09611243	0.17023495	0.07064899	0.01794505	0.04279171	0.04779452	0.03904505	0.19536007	0.94598587	0.32395372	0.03510191	0.17490496	1.536194	0.09410203	0.13833481	0.10396005	0.21799006	0.04368835
M218T190	14.4056814	1E-18	0.75686362	0.02381005	0.01184665	1E-18	1E-18	0.15806034	1E-18	0.00814233	0.00762086	1E-18	1E-18	0.28642205	2.96829985	1E-18	1.15467037	0.13365731	0.00477365	1E-18	0.01224772	0.07288542	0.02228417
M185T90	1E-18	0.03597936	1.167545	1E-18	0.02478714	0.46629104	0.09885546	0.10670067	0.02592085	0.83250091	0.01450881	1E-18	0.03165034	0.14593589	0.05497378	1E-18	0.12380331	1.22129007	1E-18	0.03141592	0.01182993	0.1492553	0.04169009
M95T1586	85.5155796	0.20888858	16.5288326	1.44750195	12.9997729	19.4255685	9.20864567	1.01586088	0.39838981	18.2599617	0.44425203	0.7609691	1.48382931	12.4565926	0.76344189	0.21143941	17.6559658	55.2497546	0.23560415	11.3217862	0.87797527	3.20171836	0.88902267
M483T679	1E-18	0.30814773	0.17582559	0.05193042	0.06200694	0.04883864	0.80314677	0.76828659	0.61482066	0.82703069	0.07040491	0.01765609	2.48789419	2.33695394	0.23765351	0.73450626	2.72888791	17.3449129	0.21753834	6.01719724	0.60492742	5.49101602	1.83539761
M81T82	7.13323187	0.02108226	1.87556159	0.14790881	0.12976553	1.20344144	0.81008159	0.27630613	0.13550399	0.11143298	0.07893145	0.04365884	0.37610002	0.85223957	0.42495345	0.14056049	1.50968331	3.18610509	0.49467804	0.74903492	0.28685724	0.0786774	0.61946646
M87T1521	64.0443294	1.13547415	9.02474767	0.66801506	1.90225547	2.53542865	4.88317025	0.96821562	0.97765119	1.00139173	0.38701338	0.77526985	0.8636308	3.22264163	1.01165105	0.506843	2.09059718	16.88707	1.17052792	1.78644573	0.52088174	1.85904662	1.76665202
M160T85	0.88111599	0.01922247	1.23122355	0.00811214	0.01618771	1E-18	0.04537678	0.11407793	0.07206082	0.01134262	0.0156426	0.02333032	0.04335717	0.06106498	0.03747756	0.00937227	0.09796652	0.71918461	0.05121505	0.0733187	0.01176855	0.03782706	0.02451984
M355T822	0.11602363	1E-18	1E-18	0.01111157	1E-18	0.07622571	0.28306862	0.00201	1E-18	0.24247036	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.1141361	1E-18	0.00576552	0.28693655	0.04073161	1E-18	0.0105
M165T773	17.4558736	0.12800764	2.41398525	0.03662352	1.13343446	1.34907725	0.97930648	0.0717699	0.10249672	0.23051598	0.02622383	0.24411111	0.24031536	1.40495615	0.07080213	0.11326633	1.1006798	7.18925601	0.35440338	0.72730211	0.02679193	0.66062131	0.66112402
M352T669	5.75769262	0.19274536	0.54293389	0.06512611	0.1119114	0.81696774	0.52083936	0.02851823	0.08062917	0.52932974	0.0536757	0.07767514	0.06996515	0.05582462	1E-18	0.04632089	0.38169401	3.40241922	0.06675999	0.5260447	0.10122634	0.09446549	0.07669545
M509T715	3.25367285	0.10474398	1.29686586	0.11703481	1E-18	0.59666025	1E-18	0.00293704	0.47476944	0.00716873	0.29642741	0.05205376	0.04432676	0.22148295	0.1113986	0.04849262	0.4529329	4.80163061	1.14121547	0.85127717	0.04905058	0.87533178	0.6801735
M97T478	83.5748796	1.55718319	2.85373623	1.94166242	1.83495969	4.55693754	7.79672067	1.54141883	1.0685645	1.21740404	0.60156597	1.08795594	0.70181195	2.22540592	1.55666456	0.34272566	1.51459789	69.4161351	1.80412326	5.96457159	0.25632846	3.7015658	4.504325
M197T90	0.54453816	0.05042703	0.16125494	0.03237498	0.08621926	0.11276906	0.13339835	0.06600827	0.05286362	0.65740651	0.08031588	0.00517259	0.1383182	0.08913086	0.29646841	0.01168217	0.49285662	0.91846988	0.14857772	0.18449061	0.04361553	0.18194399	0.13424459
M122T283	4.75615668	0.44922779	2.59966386	0.20786729	2.28758054	0.48601328	1.28843008	0.17477392	0.21838479	0.48393878	0.10974082	0.2629886	0.35912443	1.00216293	0.54249211	0.33597236	1.00452594	5.53497815	0.62300699	0.99753697	0.5100498	2.89415687	0.78973318
M232T89	1E-18	0.03674353	1.58594059	1E-18	0.02319656	0.04899867	0.03056011	0.00930539	0.01162084	0.02055984	0.02369679	1E-18	0.04497614	0.3540412	0.14350884	1E-18	0.18439874	0.18940344	1E-18	0.05932127	0.12363257	0.2248787	1E-18
M387T723	11.7725824	0.27220829	0.3666175	0.0207197	0.190999	0.08690956	1.08590594	1E-18	0.00544631	0.12357962	0.00334039	0.07561432	0.09991452	1E-18	1E-18	0.0212	0.55675366	2.86111849	0.01470825	0.19503319	0.16542915	0.06352963	0.17953906
M83T509	64.4350029	5.54010377	13.22057	3.05490508	1.88903554	2.55334952	15.7073626	0.31346439	1.57724518	1.19967064	0.26212624	1.62121749	2.66066584	1.97573366	1.81771049	0.79282424	4.45653745	29.248061	1.64742873	3.74548665	0.31841678	5.89709459	3.04292215
M171T87	7.12074366	0.0294531	0.31645541	0.0699307	0.67320802	0.04792919	0.08125334	0.02100224	0.10601377	0.04566975	0.00753884	0.05128945	0.19360405	0.89662396	0.07339993	0.02573926	0.44377158	3.45897128	0.04851632	0.1504709	0.0635891	0.04494371	0.03242608
M211T89	1.27124465	0.06868888	1.80731625	0.00945101	0.10820782	0.05329012	0.13502972	0.15951528	0.02356316	0.86518721	0.01097226	0.05029319	0.27560736	0.34540365	0.19496113	0.01366827	0.07079647	0.67796015	0.07011688	0.22576481	0.11257215	0.06698058	0.07513082
M124T452	19.591326	1.61047849	18.3539196	1.09507869	2.49423137	1.60350342	2.89871003	0.32324179	0.50547152	1.02469199	0.23460268	0.44275804	0.85605001	1.62072483	1.58092711	0.55828758	2.09400121	10.7049603	0.94577435	0.35432754	0.45235979	2.69893626	1.68971607
M244T91	3.69222688	0.33836505	0.25594507	0.01558498	0.06526215	0.1212759	0.44446092	0.89267579	0.04554257	0.04307279	0.02492405	0.07200631	0.02498937	0.78592809	0.05861703	0.0179722	0.15431569	0.79055132	0.38987064	1.96236322	0.01791378	0.1381688	0.31178588
M175T89	0.92385228	0.08246498	0.26017553	0.8233161	0.07709384	0.64632335	0.13499522	0.26762294	0.10532189	0.03569186	0.01606719	0.0495043	0.02589252	1.8224219	0.04707334	0.06345536	0.52535769	0.93612081	0.1495894	0.35438429	0.0148657	0.07430727	0.11286362
M215T191	14.5843401	0.00754499	0.8016091	0.03725115	1E-18	1E-18	1E-18	0.08583054	1E-18	1E-18	0.00863105	1E-18	0.00707839	0.79581241	0.67637985	1E-18	0.67347139	1E-18	1E-18	1E-18	0.00965111	0.04276799	0.17041537
M319T890	0.90218918	0.06416244	0.38826659	0.02940616	0.06384083	0.17987259	0.2170592	0.02976127	0.05691682	0.04890798	0.01048296	0.03890969	0.03573454	0.157355	0.09054851	0.02994874	0.17535269	1.36188771	0.06374476	0.10147877	0.02440554	0.09865087	0.06964785
M383T749	5.94554466	0.0105	0.33872144	0.03922246	0.10898294	0.15683412	0.16544515	0.12944573	0.1227772	0.1029196	0.0196885	0.01975229	0.05699272	0.11525146	0.11639872	0.01719791	0.19136035	0.54543966	0.08480549	0.13284664	0.01940292	0.24002451	0.30039383
M393T700	1E-18	1E-18	1E-18	1E-18	1E-18	0.14410207	1E-18	1E-18	0.0433	0.0732871	1E-18	0.03681531	1E-18	1E-18	0.182	1E-18	0.0674	0.469	0.01475467	0.38	1E-18	1E-18	0.0171
M185T83	1E-18	0.03151366	1E-18	0.04030315	0.0914566	0.81269808	0.48234074	0.11307842	0.04608148	0.09797101	0.00980998	0.04235971	0.05275328	0.31573983	0.20511836	0.03577432	0.13999009	0.98483258	0.0719699	0.15217949	0.0934915	0.07334371	0.23419297
M373T774	0.335	0.159	1E-18	0.13360132	0.0172	0.63055242	0.382	1E-18	0.11419582	0.245	0.00325	0.00946	1E-18	0.3279673	1E-18	1E-18	0.03546546	1E-18	0.09527301	0.50937518	0.00361	0.01410824	0.05328449
M500T616	1E-18	1E-18	1E-18	1E-18	1E-18	0.08865029	0.3776916	0.20494783	0.4366482	0.37070897	0.15977966	0.55138299	0.53644288	0.28984744	0.04189135	0.30040387	0.63149989	8.29268548	1.07109466	2.04096007	0.07102451	1.08099413	1.96549391
M365T756	5.09894374	0.01004928	0.46263192	0.15513055	0.17193311	0.33186991	0.07945817	0.04829263	0.09420955	0.01608441	1E-18	0.01779485	0.03221024	1E-18	0.08347672	0.00949765	0.08603693	1.09447024	0.03699844	0.10900295	1E-18	0.04265607	0.04960745
M361T732	0.292	1E-18	1E-18	0.0226	0.0142	0.09172291	0.03059096	1E-18	0.00807	0.02159137	1E-18	0.01038573	0.04490155	1E-18	1E-18	0.00316	0.081	2.47	0.02216148	0.709	0.0208	0.0375	0.159
M69T417	30.1346126	1.03258355	10.937262	0.5195334	1.23786949	10.6949146	2.06315404	0.08072064	0.54883312	0.41764879	0.13802185	0.59448835	0.46194686	2.02892488	1.25029084	0.2187769	1.0620815	5.76758474	0.60808546	0.9692143	0.14764122	1.64714937	0.88705226
M187T89	7.36576992	0.0307905	0.18660786	0.15671582	0.0754351	0.09514633	0.28150995	0.01251482	0.04211176	0.35083846	0.01128418	0.11916806	0.03431751	0.14429355	0.05963123	0.02542273	0.13337332	0.71476277	0.07927903	0.0888591	0.01305859	0.06180009	0.05257353
M335T845	6.55123944	1.21042307	1.96816036	1.2559476	0.4665388	0.49631559	1.66664154	1E-18	0.04559188	1.11598837	1E-18	1E-18	0.08869733	1E-18	1E-18	0.0357369	0.91256062	2.97206684	1E-18	1.72540651	0.15834977	1E-18	0.15215788
M555T665	1E-18	0.07702648	1E-18	0.08536409	1E-18	0.76540741	0.84690849	0.76360839	1.39938936	0.79068831	0.08625302	0.2295586	1.65357701	1.89648128	0.083788	0.63118482	1.00600437	17.4947875	0.05723523	6.53631417	0.21138897	4.77103555	1.94169221
M133T326	4.09223982	1E-18	0.68503909	0.0679436	0.03370003	0.03928581	0.03837361	0.08569665	0.01230139	0.08239567	0.01247465	0.04061447	0.04095491	0.26011621	0.45347696	0.02524846	0.33124282	0.64788462	0.02668764	0.18254563	0.02730545	0.15244492	0.29352091
M164T484	1.19878569	0.1043345	0.86271948	0.01388056	0.16443956	0.22294568	0.18692921	0.26904284	0.01127619	0.02727381	0.0144297	0.01880414	0.00553458	0.0182104	0.38591978	0.02038811	0.05723434	0.12161122	0.06195534	0.10371289	0.01804993	0.21614969	0.07373926
M89T87	1.37025679	0.01013292	0.90059285	0.03821293	0.08506242	1.52842752	2.15143776	0.23435017	0.12295557	0.40780414	0.09369718	0.0611213	0.09138682	1.30856137	0.35253209	0.0486436	0.48806351	5.66149382	0.22771478	1.0257887	0.1479153	0.1158599	0.1088513
M113T77	5.76744332	0.0661536	0.26518233	0.02466264	0.34073732	0.04505028	0.97947044	0.11053693	0.04858448	0.22496439	0.02593512	0.10576097	0.05461183	0.52318808	0.13493951	0.16867435	0.42103964	2.33045581	0.04603875	0.36647323	0.09433483	0.35467443	0.73572592
M341T580	1.08883842	0.09598422	1E-18	0.05085015	1E-18	0.32151613	0.04987707	1E-18	1E-18	0.06770849	1E-18	0.0055801	0.00873818	1E-18	1E-18	1E-18	1E-18	1E-18	0.00677341	0.01872761	0.00453425	1E-18	0.01418928
M501T610	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.12638112	0.0496785	0.07846533	0.03164344	0.00917755	0.07347727	0.07152443	1E-18	0.02638475	0.02670149	1E-18	0.76804474	0.14486288	0.23752516	0.00445625	0.08839759	0.17969285
M119T59	8.09238276	2.58472647	1.22539113	13.1075927	10.5956842	74.6574847	39.401194	0.51366793	0.3203919	2.56121926	4.0107356	1.01759231	3.01544391	30.9083745	24.3122877	1.14519334	2.6947143	9.40080393	2.889467	17.2800325	0.24573322	27.316082	82.6103579
M139T598	9.35950107	0.61809877	9.14560046	0.58983963	3.1814672	2.40114145	2.38376283	0.44377286	1.58949555	0.79035697	0.26497918	0.34036994	0.28721285	4.50595604	0.91113618	0.83199374	1.74086958	11.3326653	0.37766058	0.38937284	0.1284778	2.0544255	2.26983126
M319T301	2.58346532	0.04640447	0.24749006	0.02848663	0.05690628	0.13399012	0.19397906	0.01973166	0.02964625	0.04112402	0.01429539	0.03542247	0.03544397	0.19029491	0.05533962	0.02228809	0.2074741	1.28985988	0.05102346	0.08472127	0.02693081	0.04662709	0.0721387
M327T739	1E-18	1E-18	1E-18	0.0050603	0.01489749	0.04548364	0.03970522	1E-18	1E-18	0.07785505	1E-18	1E-18	0.00608021	1E-18	0.00809032	1E-18	1E-18	0.17933127	1E-18	0.04197663	1E-18	1E-18	0.00988674
M92T190	3.47057747	0.28178749	1.38087596	0.19875862	0.7530464	0.46622652	0.54874492	0.21027324	0.11225361	0.36176759	0.11897781	0.7968735	0.16082914	1.2866129	8.33742282	0.11157859	1.35486085	4.54090824	0.0716589	1.30441182	0.18307434	0.55815195	1.0555589
M128T89	1E-18	0.01005704	3.06585904	1E-18	1E-18	0.08784644	0.03333177	0.07328252	0.05671726	0.01110731	0.01038167	0.03476573	0.03526258	0.10612008	0.14599024	0.02888282	0.05079561	1E-18	0.18987196	1E-18	0.05250002	0.11070791	1E-18
M210T90	1.30490744	0.04276345	0.78867961	1E-18	1E-18	0.10657115	0.14167653	0.11739384	0.01622853	0.06080953	0.09117274	0.00476112	0.0087466	0.15441633	0.04597743	1E-18	1E-18	0.29651339	1E-18	0.08635001	0.00721117	0.09315742	1E-18
M201T97	0.79687852	0.05973969	0.65855568	1E-18	1E-18	0.09055491	0.11537168	0.00837272	0.02033717	0.13069238	0.0123259	1E-18	1E-18	1E-18	0.12739378	0.11759435	0.1279364	0.15831218	0.0271632	1E-18	0.01353055	0.04896774	1E-18
M510T709	0.21255469	1E-18	1.00638316	1E-18	0.01119314	0.07009898	1E-18	1E-18	0.10774136	0.00812253	0.05725561	0.13620995	1E-18	0.60533633	0.2200934	1E-18	0.03464163	0.48460367	0.12705199	0.08855382	0.00276604	0.18497033	0.14158621
M37T654	0.228	1E-18	0.06	0.01076982	1E-18	0.06580539	1E-18	0.00767	0.02771115	0.4527425	1E-18	1E-18	0.05015724	1E-18	0.0242	0.00413358	0.19074689	0.92581701	0.05554112	0.21237754	0.00885357	0.051	0.08027126
M196T661	0.26746863	0.02251696	0.13438756	0.01531325	0.03337139	1E-18	0.11443521	0.00409616	1E-18	1E-18	0.00324063	0.00394194	0.01299621	0.02154289	0.06069862	0.01146872	0.06564395	0.43297906	0.01874732	0.02095025	0.00725467	0.09603551	0.01629716
M556T660	1E-18	0.09826637	1E-18	1E-18	0.01406722	1E-18	0.06050082	0.13310206	0.01642216	0.16226002	0.03830355	0.10999107	0.43327984	0.1412131	0.18866371	0.18316675	0.39561116	4.2555352	0.04383074	1.79642567	0.05295669	1.52952294	0.49634362
M183T89	1.82519471	0.07152032	1.42443356	0.03911032	0.36187715	0.7919809	0.83845006	0.0878551	0.04120611	0.33047419	0.12932637	0.01880868	0.06288687	0.36577877	0.39618248	0.01028291	0.28198232	2.49170634	0.07527348	0.17350951	0.0719703	0.23290784	0.06076537
M252T569	0.86619225	0.0109396	0.05833616	0.02221942	1E-18	1E-18	0.08872029	1E-18	1E-18	0.01787315	0.10720012	1E-18	1E-18	1E-18	1.63511404	1E-18	1E-18	1E-18	1E-18	0.17999699	0.01929355	0.01617396	0.05927861
M319T1373	3.84032552	0.16365534	0.47174828	0.08980661	0.24136432	0.34497879	0.53561107	0.06598141	0.09596283	0.10767556	0.02370098	0.06344866	0.08629075	0.25110997	0.18766168	0.0399254	0.18943148	2.13218021	0.07811246	0.09195396	0.05401759	0.27588526	0.14434598
M395T726	1E-18	1E-18	0.0448	0.0128	1E-18	1E-18	1E-18	1E-18	0.07745575	1E-18	0.00254	0.00526245	1E-18	0.0189	0.13817384	0.0122	0.04263091	0.76463564	0.05533604	0.13420263	1E-18	0.0128	0.04499086
M215T189	21.1040198	0.0075027	0.7571557	0.0372505	1E-18	1E-18	1E-18	0.10336997	1E-18	1E-18	0.01367507	1E-18	0.00708334	0.73053107	0.89689013	1E-18	0.37551673	1E-18	1E-18	1E-18	0.01748539	0.04275006	0.22812124
M403T652	1.57691041	0.02162226	1E-18	0.19895241	0.15894061	0.8596201	0.16877076	0.02702909	0.09594585	0.41996629	0.00779837	0.0381514	0.18764971	0.03839059	0.12371503	0.07507323	1.24868629	7.24449607	0.21385223	2.12527717	0.28267395	0.36419817	0.80827096
M113T305	23.3108986	0.47229657	3.48084344	0.26663476	0.6274304	0.28767528	0.76459064	0.15756014	0.30663535	0.17407058	0.1190378	0.28431636	0.09041873	1.80721668	2.11556684	0.08838478	0.94690523	3.27370857	0.19805908	0.44209674	0.12142904	0.97340458	0.36616797
M129T85	1.11216133	0.50631951	0.45669001	0.07921486	0.19483908	0.50301387	0.58608965	0.01848693	0.02400133	0.79829278	0.00557968	0.05112162	0.02598719	0.07383482	0.08084011	0.01721085	1.60570647	1.29519967	0.063382	0.06737396	0.05676808	0.1664137	0.04990157
M326T509	2.49629836	0.19325005	0.08733145	0.07706847	1E-18	0.59557081	0.68523736	1E-18	0.00690395	0.22500941	1E-18	1E-18	1E-18	1E-18	0.00379792	1E-18	1E-18	1E-18	0.01120819	1E-18	0.00525903	1E-18	0.05999976
M326T598	2.76294164	0.19323688	0.15399714	0.07112656	1E-18	0.69681647	0.71945799	1E-18	1E-18	0.2250395	1E-18	1E-18	1E-18	1E-18	0.00379687	1E-18	1E-18	1E-18	0.01120443	1E-18	0.00526136	1E-18	0.06001479
M193T89	1E-18	0.05535486	0.25825248	1E-18	0.0361644	0.35503897	0.03203722	0.03132032	0.02866659	0.03061781	0.3017815	1E-18	0.13619202	0.09242235	0.05444044	0.02399209	0.06305568	0.74512731	0.02195755	0.03011775	0.02559671	0.07473701	0.07706323
M167T83	2.30883429	0.38620078	0.31060333	0.15873629	0.19989804	0.4695384	0.3437016	0.06034718	0.01746275	0.8333418	0.09599863	0.0932521	0.04786196	0.24310879	1.51438278	0.04348877	0.52550808	1.02985601	0.055587	0.1270892	0.08608628	0.18413729	0.24922346
M450T680	0.73077547	0.01100387	0.55805266	0.00603645	0.08184162	1E-18	0.16559127	0.04772012	0.10053687	1E-18	0.00279536	1E-18	0.01789813	0.25260822	0.07507939	0.00765652	1E-18	0.16417669	0.06906209	0.03556784	1E-18	0.05431937	0.01492285
M249T81	0.34637378	0.01320576	0.15575983	1E-18	0.04336886	0.04229454	1E-18	0.04462774	0.01932757	1E-18	0.01691665	1E-18	0.01044123	0.0749272	0.27236305	0.00560244	0.07489863	0.17645225	1E-18	1E-18	0.00319678	0.09436395	0.03608501
M176T89	0.59772949	0.03245905	1.6490595	0.01091997	0.11676693	0.65115505	1E-18	0.02326804	0.0071388	0.03125672	0.00909773	1E-18	0.03138336	0.37269547	0.470973	0.01817829	0.88038191	1E-18	0.04363097	0.04080291	0.00923907	0.08551214	0.24981465
M241T534	79.3248249	2.23000045	27.5789209	5.69498905	2.65539132	2.43043611	13.2498128	1.52748268	1.00462515	2.83710336	0.3498429	0.90113592	0.74314257	1.54052801	7.21287397	4.18969548	8.29030065	1298.18489	0.39200034	2.08381015	0.87864174	11.5849066	5.04957758
M79T83	3.22449847	0.71563727	7.93949639	0.07815002	0.17836146	0.52882442	0.44302809	0.24424061	0.10244555	0.58853607	0.02955694	0.17441787	0.16531362	0.16148775	0.42783968	0.07017248	1.17467271	16.8198006	0.9719109	0.57364417	0.14558324	2.3415441	1.05307304
M121T346	6.52108194	0.07065178	1.0681627	0.07431844	0.23377561	0.15375978	0.236546	0.07083821	0.08463551	0.13022451	0.04114573	0.09532766	0.06612592	0.03134505	0.1637743	0.04554163	0.27056174	2.09577232	0.05640235	0.19677371	0.0613568	0.2508697	0.18883304
M103T361	11.5009039	1.55703292	6.50627299	0.6560865	3.66219089	1.0668709	1.66593427	0.4989826	0.40154447	0.61421867	0.29951717	1.04049446	0.3792816	6.50584407	1.36459871	0.2582302	4.6599569	7.44853275	0.25332831	0.83854655	0.37437062	2.36446057	0.9910682
M62T296	341.037241	8.53968207	15.0422346	1.44989017	3.78728581	3.23260353	17.2172341	1.78669473	1.37336279	3.45113873	0.71638298	0.827049	1.49215027	7.69862967	5.61168301	0.37734995	7.17215408	20.9139218	1.74403446	5.19228048	2.64313338	27.3043129	6.69304167
M151T87	0.51094979	1E-18	0.37565403	0.00586282	0.01613766	0.36366121	0.09164367	0.03819475	0.05288453	0.09747291	0.04224276	1E-18	0.04141606	0.05421781	0.1813464	0.01890235	0.10483708	0.21982631	0.03487981	0.04402853	0.00821445	0.05128646	0.06051156
M148T173	1.17460814	0.13202826	0.20705379	0.0601336	0.13937485	0.1193202	0.37230108	0.01347874	0.09209187	0.08098566	0.00592551	0.02599865	0.03587738	0.16001955	0.14562874	0.03436143	0.07464611	1.19449533	0.07534182	0.17380364	0.02473387	0.1715483	0.12520613
M400T92	1E-18	1E-18	0.42443208	1E-18	1E-18	1E-18	0.221	0.0716	1E-18	1E-18	0.012	1E-18	1E-18	1E-18	0.0553	1E-18	1E-18	0.14655859	0.00594449	1E-18	0.04017511	0.02623425	1E-18
M557T663	1E-18	1E-18	1E-18	1E-18	0.15658187	1E-18	1E-18	1E-18	1E-18	1E-18	0.00554725	0.0198966	0.06433176	0.18108867	0.05756376	0.01449048	0.02236361	1E-18	0.03965576	0.13217529	0.00797861	0.17527838	0.03117966
M353T643	0.58561136	0.124	1.7	0.11514991	1E-18	0.25732924	0.0976	1E-18	1E-18	0.12554156	0.00275	0.00659	1E-18	1E-18	1E-18	0.00341	0.0159	1E-18	0.0122	0.16	0.0203	0.029	1E-18
M140T87	1E-18	0.07225237	0.27471743	1E-18	0.01926117	0.17261787	2.02544705	0.01997295	0.0185302	0.03513124	0.03741966	0.01787881	0.03684886	1.75876902	0.02898087	0.0049471	0.07141808	0.4385294	0.06265981	0.04863945	0.0806454	0.0703686	0.02496682
M119T405	205.114958	8.27562672	44.0463494	22.8381956	3.293349	2.63713931	118.232249	15.5611859	0.25849065	31.1216777	3.81452057	22.3046149	8.02937336	11.5306134	30.5397371	2.85560159	2.3124918	434.035385	11.0570872	29.1067122	12.7492873	5.14084069	0.67644672
M375T649	1E-18	1E-18	0.01626629	1E-18	1E-18	0.05369793	1E-18	1E-18	0.03788962	0.17612963	1E-18	1E-18	0.02012757	1E-18	1E-18	0.03720717	0.07028295	3.2242889	0.10850719	0.15108431	0.01903101	1E-18	0.04072291
M129T510	8.56751133	0.36374283	1.99871343	0.20494	0.9407254	0.83626036	0.94023724	0.08554919	0.19166275	0.32781098	0.13266358	0.16714332	0.30460848	0.54571443	0.28358577	0.15028409	0.87003681	7.32116209	0.19310938	0.69119743	0.13574358	0.71790627	0.54946679
M345T767	1.52216467	0.73542896	1.3154388	0.96995104	1.21060166	0.70401827	1.33377025	0.00476821	0.00593118	1.18289279	0.00263311	0.01357773	1E-18	1E-18	1E-18	0.192218	0.87834454	6.12703923	1E-18	0.62903288	0.00359183	0.01382211	0.35418898
M312T749	1E-18	1E-18	0.05573152	0.02549544	1E-18	0.4106104	0.05451092	1E-18	0.01946644	0.00909437	1E-18	1E-18	1E-18	1E-18	0.0242863	1E-18	1E-18	1E-18	0.00515716	1E-18	0.00277752	1E-18	1E-18
M239T93	0.2986441	0.0552725	0.05512462	1E-18	1E-18	1E-18	0.38876859	0.00545666	1E-18	0.01963254	1E-18	1E-18	1E-18	1E-18	0.03583351	1E-18	1E-18	1E-18	1E-18	1E-18	0.00217718	0.04169524	0.04096047
M95T84	12.8066657	1.16061284	4.96650501	0.75079234	1.27431845	0.33464265	3.5022553	0.46372273	0.045919	0.06322209	0.1469216	0.09679731	0.13938631	3.34684781	0.13058691	0.14470173	0.27761454	1.14853323	0.30200269	0.2502767	0.04491178	0.30348475	0.62118336
M127T87	2.00717595	0.01331064	0.79630346	0.01631297	0.06842553	0.31569782	0.41901086	0.10263116	0.01023689	0.03176482	0.01588366	0.01925588	0.07221709	0.3364406	0.0870863	0.01610325	0.06637868	1.65426742	0.04385792	0.09069063	0.02008228	0.10649731	0.04596615
M153T92	1.11077335	0.03384183	0.56249829	0.04344094	0.37953349	0.81273545	1.82891677	2.67016139	0.05293721	0.97357221	0.05735614	0.42476136	1.86692392	1.96745804	16.8080323	0.01611447	0.1338588	1.37845208	0.04962572	0.13098319	0.19062817	0.07679574	0.23513423
M177T144	34.8887823	0.18894227	11.4516117	0.7862928	2.18594888	0.8314486	0.91285765	0.28410941	0.2317047	0.59383704	0.20696335	0.32742164	0.03652419	1.1293544	16.2526583	0.15863834	4.96796123	4.36383265	0.14521575	0.63394441	0.66915953	2.53065064	0.52111612
M268T640	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.0992	0.0545	0.0846	0.743	1E-18	0.03806377	1E-18	0.02002909	0.11096656
M345T706	15.733836	0.24077236	3.00304493	0.21338479	1.29756345	1.52150993	1.06339052	0.38645521	0.1714756	0.46496502	0.03896154	0.22541164	0.3824332	0.25855574	0.21494954	0.18583651	1.90999341	15.5826353	0.17785221	2.8005184	0.15241439	0.95468359	0.6646833
M103T135	30.4179588	0.59292776	6.04990595	1.41880192	1.52677443	2.9648876	1.42706928	0.04252513	0.53764912	0.73278845	0.11880502	0.23028105	0.29454203	1.33611576	1.54374143	0.232845	5.10167138	8.0327386	0.29523109	0.83996004	0.40169549	1.35749028	0.77914244
M308T658	17.742178	0.13190432	2.27802356	0.06181947	0.91572992	1.02314068	14.4448151	0.25367968	0.03426744	4.05881185	0.06860624	0.0169546	0.27336736	0.25487154	0.1398869	0.08958633	1.47060261	14.0795669	0.08946142	3.19596704	0.30009546	1.10249574	0.28567655
M111T84	5.72843018	0.47444415	1.08532356	0.58979468	0.13599003	0.39210484	1.53131049	0.49012999	0.08068416	0.10541846	0.03341042	0.11118084	0.21323129	0.44564098	2.38498317	0.04319389	2.25865892	1.33451486	0.27960214	0.17575961	0.02061362	0.22913172	0.55253644
M105T500	79.5167049	1.31903668	9.75899551	0.85856883	2.79881174	2.62244196	12.8936544	1.04816873	1.0705898	0.96942532	1.36171673	0.57439106	2.18598678	7.96631538	3.83383285	0.93516607	4.34365117	33.7083017	1.4614502	8.41513405	0.47042981	3.02369184	4.78533711
M381T661	5.49421881	1E-18	0.864	0.12110033	0.27089646	0.03976977	0.21194118	0.04276667	0.0692353	0.0814	0.00396639	0.09540588	0.10055462	0.0686	0.06219418	0.0341688	0.14545216	1.2230472	0.04291513	0.57686523	0.02864485	0.108	0.23322065
M319T572	5.82558001	0.37341299	1.42250919	0.03508241	0.13413507	0.44614717	0.921179	0.12460151	0.14011981	0.25777098	0.06692984	0.12054389	0.23580916	0.51709163	0.06451533	0.1032376	0.44466704	0.55680147	0.13626085	0.49886787	0.13851414	0.44964691	0.29833583
M337T820	0.11083569	1E-18	0.06207661	0.10536554	1E-18	1E-18	0.03063559	0.04539926	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.04887441	0.28776296
M253T229	1.15700447	0.0392758	7.78836129	0.21722338	0.03272932	0.27559058	0.05190044	0.03419104	1E-18	1E-18	0.04026095	0.13251419	1E-18	0.54253063	1.92801307	1E-18	0.04076325	1E-18	1E-18	0.01520364	1E-18	0.25127388	0.08790121
M196T85	2.89582393	0.02123709	0.43405489	0.01105544	0.08606767	0.78465897	0.7393397	0.06683929	0.08575118	0.0552537	0.07881697	0.08318042	0.10474091	0.14465093	0.09754558	0.03591335	0.2162747	7.33555327	0.08746528	0.14014967	0.1504731	0.8965947	0.11052428
M363T770	1E-18	1E-18	0.06309	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.0646709	1E-18	1E-18	1E-18	1E-18	1.52	0.01116975	0.0149	1.12395807	1E-18	0.20682954	0.02199646	1E-18	1E-18
M194T83	0.84915176	0.06366539	1.19348513	0.02037027	0.12357063	0.10943027	0.45743566	0.16015401	0.03089848	0.17281402	0.01951707	0.03011331	0.08528474	0.06418973	0.30285113	0.05001452	0.09552678	0.61315729	0.03841381	0.11592183	0.02433179	0.54262966	0.30376248
M110T233	3.87940529	0.00978536	1.62380685	0.047054	0.04855596	0.18169805	0.05479918	0.064178	0.00875736	0.03318379	0.0101735	0.11200896	0.00697142	0.18798183	0.23207665	0.00824353	0.02157731	0.28373752	0.02720714	0.20206545	0.0183377	0.07179489	0.31033264
M137T125	40.8402212	0.26221266	16.7467964	0.59029763	1.38431659	0.62248185	1.55744247	0.27475813	0.16155683	0.76765845	0.12372624	0.32858358	0.0798042	2.40015312	2.17865009	0.21236548	1.25862292	11.1903304	0.52342235	1.86561535	0.18520517	1.2918726	1.6137574
M121T1583	12.2427572	0.43661349	2.74215803	0.03673847	0.44249456	0.90017481	1.81657838	0.16699457	0.24370493	0.29009724	0.13630687	0.01985683	0.30327581	1.30969393	0.33105192	0.08684517	0.97057452	4.46492608	0.20367531	0.7131017	0.18718219	0.52916158	0.32702776
M251T531	2.12758805	0.03780646	0.27284399	0.03074095	0.17057846	0.66286274	0.30267094	0.06805784	0.02184778	0.05650715	0.01323097	0.06385366	0.18424802	1E-18	0.63624769	0.01570697	0.14627207	3.0019689	0.05456385	0.28649821	0.09813538	0.22388577	0.01321933
M80T299	2.84099205	0.0931175	0.58649016	0.04930379	0.13698218	0.20912791	0.27753756	0.01880838	0.05039322	0.0839331	0.0172694	0.06410961	0.09049932	0.16030553	0.09057483	0.02753506	0.14255151	1.30568536	0.03964108	0.2543213	0.04131086	0.15754178	0.09567668
M96T81	6.91744583	0.2242855	1.67628226	0.33180905	0.58886433	0.60839668	0.6517809	0.09023444	0.29543144	0.10681508	0.12703738	0.14781124	0.48938151	2.53165572	0.86614976	0.1109092	3.60568898	10.187737	0.61317083	0.59935715	0.08226423	0.85673578	0.27014392
M153T202	18.1692004	0.0127786	4.46977819	0.70853428	1.06209864	0.08686501	0.06787382	0.39419249	0.00332043	0.07478747	1E-18	0.16933142	0.09998491	1.23242382	17.0567599	0.06270442	1.65043212	3.52230633	0.03772357	1.30399323	0.07213842	0.16989491	1.20242672
M616T678	1E-18	1E-18	1E-18	1E-18	1E-18	0.12843288	0.30680913	1E-18	1E-18	0.08308832	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.02943333
M122T345	4.35990998	0.311493	1.71234494	0.21569455	1.51760318	1.63641745	1.03812122	0.17228712	0.29506023	0.6878245	0.06848749	0.16515292	0.29331821	0.80179639	0.30865088	0.28471788	0.60911222	8.38640388	0.82076899	1.18332504	0.32230792	1.08856938	0.76580821
M253T140	10.546836	0.00967116	0.23236864	0.25318795	0.02158589	0.15824791	1E-18	0.08120457	0.00882354	0.13665341	0.0234815	0.00473606	1E-18	1.53226392	1.85806553	1E-18	2.67542181	1E-18	1E-18	0.01566836	0.22556986	0.62093003	0.03488191
M125T86	2.19737296	0.02957644	1.53645677	0.02320077	0.09951375	0.12015726	0.93738126	0.56562227	0.0734147	0.03605344	0.01752639	0.05310653	0.02908783	0.7020168	0.054935	0.06293747	0.90333899	3.58917636	0.34611626	0.07328123	0.05887573	0.37834506	0.04254614
M114T97	2.09514396	1E-18	0.22910701	0.02366019	0.68352649	0.49288843	0.72814057	0.1464205	0.15849468	1.54910721	0.03466618	0.01931272	0.06668138	0.9382565	1.02025852	0.04523761	0.45730616	0.75988396	0.02241432	0.54654409	0.13301737	0.33947271	0.29590256
M229T218	10.926188	0.00855296	8.76534535	0.14732366	1E-18	1E-18	1E-18	0.03915121	1E-18	0.00764781	1E-18	0.2810816	1E-18	0.38540387	1.32087834	1E-18	0.25762138	1E-18	0.00472909	0.0146302	0.0029957	0.03516938	0.22272506
M69T449	29.7593016	1.27062212	8.15839976	0.91637176	1.17362601	2.44307097	2.39790156	0.72732864	0.64627799	0.48778033	0.27127273	0.45231414	0.42708662	2.11608679	1.34337057	0.38559506	1.13758907	11.7031525	0.32001072	0.69583244	0.19603037	1.59496368	0.82103036
M349T838	0.495	1E-18	0.19106039	0.1036947	0.08496148	1E-18	0.03803675	1E-18	0.06505436	1E-18	1E-18	0.00418895	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.01788683	1E-18	0.0137	0.10751077
M85T289	32.9632929	1.70232466	6.40614561	1.55929827	2.88672293	1.42569188	1.58100604	0.17409059	0.39174758	0.34853596	0.40798473	0.7790263	0.2416827	20.8086017	2.23155265	0.2416549	2.80439023	22.760717	0.39668447	0.79836749	0.34064255	2.56130921	0.6978423
M379T675	18.6763889	0.123	2.70819119	0.65889737	1.04310802	0.24367578	1.34138188	0.14260512	0.078372	0.32253136	0.0218	0.0726	0.28359847	0.0949	0.0266	0.18040524	1.28779816	10.3039789	0.15723765	1.97042936	0.15893673	0.337	1.11
M75T417	50.3124881	2.35547009	14.9712233	0.59021883	1.27816845	8.52680237	2.4258252	0.50254715	0.67543837	0.25834059	0.3941611	0.62768609	0.47899254	2.99124734	1.35466481	0.26289535	1.65313574	16.5681449	0.50955293	0.96818649	0.22430654	4.13781879	0.92686667
M309T768	17.1162269	1.61971824	3.12700362	0.80931428	0.32581744	2.04760588	2.14800319	0.0900373	0.12896214	1.04527472	0.0766711	0.20542336	0.17921321	0.02065328	0.20603919	0.17198175	0.52789126	3.97387882	0.02803677	0.84655986	0.08966025	0.03434205	0.45668883
M377T793	4.87999073	1E-18	0.561	0.07190417	0.917	0.18653052	2.03345943	0.00321	0.0117	0.285	1E-18	1E-18	0.0824	1E-18	1E-18	0.0535	0.341	2.35137473	0.05431712	0.54959143	0.108	1E-18	1E-18
M267T481	10.4	0.147	1.81	0.08608304	0.194	0.64511958	0.271	0.14324726	0.01764738	0.10790431	0.04763613	0.03689219	0.07788778	0.02052521	0.68631781	0.01637036	0.25023222	3.55432475	0.06627974	0.80547478	0.03241928	1.54338946	0.30784174
M110T84	5.46373212	0.27457151	0.65081426	0.21202125	0.26484857	0.82757954	0.38169736	0.17070361	0.06548089	0.15832356	0.02864985	0.06337884	0.16931611	0.59495542	0.27898882	0.05160826	0.24288039	3.13667551	0.09502676	0.74315294	0.09484337	0.54526259	0.15541494
M333T784	4.72928216	0.02429084	1.31186002	0.07732709	0.07073055	1.6450665	1.86473724	0.01893367	0.07282781	0.52379381	0.02977012	0.54362708	0.41924699	0.02082473	0.03651149	1E-18	1.65322736	1.05797103	0.03754407	1.15772969	0.08842408	1E-18	0.19945554
M319T88	1.7542279	0.05679472	0.52352891	0.0184722	0.06062967	0.10214782	0.17487396	0.03150633	0.04297616	0.05251353	0.0191125	0.04059081	0.03607028	0.13444212	0.04694598	0.01487377	0.11966101	1.34198861	0.02141191	0.08086716	0.02437348	0.06630526	0.11491966
M85T168	35.742963	1.69001894	10.6340892	0.74791113	3.97346488	4.09135332	1.24079183	0.23085286	1.83789432	0.76349487	0.77223613	0.36548841	0.38769943	3.20162822	1.43063353	0.58932206	4.30848217	15.4700563	0.48116842	3.55498067	1.24934966	0.98801398	7.18207022
M377T671	31.8283351	0.262	4.35320273	1.07505535	1.77	0.20164591	2.03	0.24421009	0.06552631	0.58482684	0.0161	0.0522	0.50685972	0.0642	0.06157601	0.30861475	2.08699259	15.8790568	0.01442593	3.50971182	0.24794801	0.59881969	1.75390512
M185T89	2.13584928	0.06577142	0.18215094	0.01937954	0.15865358	0.12246442	0.14159724	0.06759929	0.00907329	0.10681606	0.05433029	0.13207756	0.0639541	0.48370639	0.24449154	0.01130965	0.06531191	0.65933015	0.11932789	0.1121068	0.05632173	0.30048371	0.14172939
M105T395	100.988772	1.45111122	6.15564936	1.60250799	5.60444514	1.83086059	6.59967318	0.27608254	1.4597863	1.15075517	1.39238034	2.6963495	1.34615639	10.1562632	3.29498224	0.96895435	3.31455168	23.245509	1.58013868	1.69907108	0.27317029	3.33014331	6.55932993
M59T1387	3.8355076	0.09009358	0.61592686	0.08465606	0.27145887	0.15081935	0.16986407	0.09454175	0.13901418	0.13745325	0.04715165	0.06774605	0.05932896	0.34989941	0.05492491	0.02804098	0.33152921	3.76125364	0.08854027	0.14765359	0.06183969	0.27688424	0.17339765
M271T1567	1E-18	3.78	0.10992803	0.10025762	0.0905	14.9463137	4.71367114	0.122	0.03749001	0.68905475	0.06324696	1E-18	0.0066437	0.16371638	0.0997	1E-18	0.0324	1.59	0.00725961	0.176	0.01684344	0.182	0.25340867
M172T190	4.64110677	0.02017092	0.37450747	0.05889393	0.22333882	0.08356006	0.03100308	0.04822928	0.01246124	0.00837009	0.00262615	1E-18	0.03090743	0.28510965	0.37792603	0.01787662	0.1182956	0.21533272	1E-18	0.17596995	0.01244362	0.02212812	0.2331078
M95T77	1E-18	0.01969149	1E-18	0.01803925	0.05406275	0.12353691	0.1158735	0.01724857	0.00555736	0.0213791	0.00270111	0.00621274	0.02785746	0.04569281	0.06707147	0.00853888	0.18089778	0.3203067	1E-18	0.01613875	0.00442813	0.03479291	0.03943524
M151T190	6.45504703	0.11766443	0.61510034	0.12172996	0.41901707	0.39564793	0.37986982	0.05479036	0.07036965	0.20298746	0.04306847	0.03529036	0.0933104	0.38509692	0.37496903	0.04591588	0.4278223	2.46506715	0.097066	0.33269529	0.07331766	0.28269193	0.33004991
M311T660	2.68655463	0.04071864	0.31271703	0.11144306	1E-18	0.14823057	0.32234309	0.01864339	0.02282729	0.12646233	0.0493429	0.20076552	0.10672273	0.27668138	0.01052221	0.12242839	0.25712265	6.30229301	0.06873232	0.35757077	0.08510328	0.43314425	0.13110231
M161T494	10.7981469	0.40672747	4.440489	0.2244197	0.09420914	0.87127537	2.79361499	0.37215952	0.58513724	0.39099987	0.06616476	0.11714164	0.75542396	0.48227304	0.90920529	0.15404083	0.52107594	16.6412998	0.68375426	1.15520826	0.30261218	1.36898213	0.2898215
M60T675	19.5606006	1.38342001	7.44723104	0.69456808	2.00401662	2.81126956	4.1673261	0.5623617	0.58866312	0.92041605	0.33079113	0.30141836	0.73273499	2.76596379	0.44542129	0.65424986	2.53095098	19.2485357	0.34740428	1.48967224	0.37770883	1.81678242	1.23975681
M499T633	0.23906231	0.00108129	1E-18	0.11812563	1E-18	1E-18	0.03825555	0.00866878	0.01256918	0.15299902	0.12452886	0.48412647	0.89370185	0.38753136	0.10295662	1.20534146	6.23574401	45.9167481	1.53905027	5.07622743	0.61163196	4.41091266	0.07232962
M163T619	11.3418668	0.04849109	15.7519144	0.14845018	0.59752071	0.1042111	0.51388557	0.04160156	0.12785399	0.86149763	0.02625698	0.07792749	0.68308236	0.36295192	2.76224394	0.55151326	0.28406548	2.02283823	0.15263333	0.29122726	0.16161464	0.10140765	0.22781234
M283T458	0.53464317	1E-18	0.20543976	0.00556103	0.03866885	0.20959226	0.02902742	0.11820383	0.00440833	0.04512895	1E-18	0.077	0.01030627	1E-18	0.4676369	1E-18	0.01709895	1.0079888	1E-18	0.16801988	1E-18	0.90949654	0.13574495
M341T675	25.0547844	0.07123946	2.70587636	1.07891216	0.94202572	1.23152931	1.83926036	0.15929335	0.06084684	0.44750121	0.06663604	0.07152454	0.33138577	0.22817282	0.17399419	0.15456122	1.7997748	12.1543025	0.11570124	2.35165913	0.18150162	0.50856349	1.55120578
M349T606	34.2488042	1.24163421	5.28159215	1.10449229	1.03774718	6.60950395	5.37427919	0.20732247	0.10344575	3.15338526	0.03348894	0.48629019	0.35148932	0.58970723	0.06250148	0.25017798	1.76123902	10.4151181	0.11889003	1.9680609	0.45574573	0.7522532	0.45912153
M387T663	17.7	0.0953	2.49	0.303	0.482	0.54362722	1.58	0.0824	0.1305507	1.41	0.05683203	0.16601066	0.204	0.258	0.157	0.0801	0.885	8.37835006	0.08823559	1.63081188	0.368	0.385	0.0576645
M337T665	5.9778099	0.06961267	0.56349339	0.3080316	0.04171806	0.03873353	1.18807024	0.08673484	0.14966369	0.12296085	0.01195385	0.0376705	0.02063721	1E-18	0.02661997	1E-18	0.31148045	2.27250102	0.13446027	0.56468136	0.06310028	0.11445968	0.39592664
M165T89	1.23269113	0.02262916	0.57195575	0.0054377	0.07639567	0.18756049	0.40598845	0.02285603	0.0298368	0.19299718	0.01281773	1E-18	0.05445787	0.0562669	0.02344461	0.01817677	0.04731859	0.54805327	0.04254606	0.09416324	0.00780873	0.04928629	0.06454505
M99T86	12.3134953	0.07799442	1E-18	1E-18	0.03479251	0.45176644	0.1551697	0.0285033	0.08911803	0.37657812	0.01386877	0.11492623	0.03632897	0.46283992	0.03698186	0.104913	0.16797334	3.67360747	0.05138997	0.07754951	0.01029432	0.08538708	0.11654504
M215T138	23.6262605	1E-18	0.6407193	0.03843521	1E-18	1E-18	1E-18	0.13054	1E-18	0.02880801	0.22236044	0.00568876	1E-18	1.2727776	1.44473802	0.00350974	0.43839933	1.26956248	0.0098952	0.02455055	0.05349596	0.44694868	0.31299294
M255T1469	5.42489016	0.46880073	1.2864721	0.20767707	0.45275197	1.54181956	1.08142582	0.11897134	0.20121278	0.70837125	0.08017035	0.13687071	0.13040213	0.25447683	0.10406473	0.0578031	0.37151789	2.63908477	0.14245013	1.02984765	0.0839428	0.18943108	0.26694408
M95T442	31.3430416	3.041669	27.3120268	1.00133458	2.58679346	3.92969056	11.4754245	1.7597062	4.15835682	3.34324876	1.27450227	1.979112	12.5242666	85.8304058	2.13879922	0.75020251	6.31572237	16.9182376	0.78865164	15.9184695	2.26676067	4.78246707	30.0254733
M161T613	29.0910061	0.52716185	1.41852957	0.88315817	0.86744383	1.21626798	1.07552202	0.2360762	0.48766883	0.42643706	0.15222832	0.40232418	0.08329073	1.98281569	0.64191175	0.35712054	0.28918008	2.20406902	0.67150905	0.62012107	0.1264839	0.75166803	0.73930386
M195T88	1.88361949	0.08567314	0.32610008	1E-18	0.27378753	0.33433351	0.1763466	0.11148589	0.01939019	0.13472076	0.03665239	0.0062293	0.04558797	0.46097231	0.02454177	0.01153046	0.07185694	0.86219504	0.16126786	0.11240059	0.02785042	0.0904397	0.01429329
M405T888	3.47782103	1E-18	0.12730544	0.05294795	0.13725615	1E-18	0.20900576	0.07882195	1E-18	0.06943532	1E-18	0.02433812	0.05802747	0.06425624	1E-18	0.0221211	0.90073458	6.78678608	0.02206535	0.60767063	0.04252364	0.3594239	0.05762484
M117T213	20.6630107	0.23393352	0.61311682	0.21482787	4.82159408	0.76083556	0.45324889	0.18102442	0.04186291	0.15309968	0.01465473	0.2488753	0.10467299	1.27861873	0.84436716	0.08445523	0.50124065	3.55544973	0.11380453	0.04099364	0.07789516	0.2789709	2.34405047

M166T608	0.27343858	0.00981173	0.16158717	0.0072449	1E-18	0.06019902	1E-18	0.00399657	0.00593124	0.02329809	0.00295762	0.00458129	0.00537686	0.02607011	0.00978853	0.00423229	0.01661918	1E-18	0.00594678	0.02352791	0.00450503	1E-18	0.01192962
M80T1498	1.93896038	0.06169408	0.42290287	0.0624663	0.11563693	0.12618636	0.34466825	0.02328949	0.04237602	0.06607121	0.03112196	0.03896182	0.06215258	0.14580513	0.05124951	0.0295796	0.13655206	1.43172043	0.06059136	0.14528284	0.02192675	0.12731834	0.11209132
M105T335	46.7248194	1.04448859	52.1950382	0.85357907	2.69958219	4.22631597	1.50010969	0.28104835	1.3988766	0.67543092	1.48357423	0.97803145	2.50368124	1.88297194	1.07312331	0.49642169	1.84048071	22.0532522	0.75260938	2.27893365	1.99907478	2.45345144	0.70485681
M233T83	1.78939504	0.03425284	1.06456241	0.17608431	0.0960861	3.29777592	0.12171698	0.21546693	0.01831167	0.04410537	0.06121271	0.01476889	0.56225447	1.68491092	0.13192384	0.05827108	0.0511285	0.9064132	0.12189757	0.12890907	0.12190297	1.06698066	0.05804502
M241T607	17.1952063	2.12116825	18.8370424	4.8888232	11.7684799	4.41220784	19.7401447	0.67094989	1.103797	1.95432569	0.89168844	1.0342637	1.19043025	1.09669955	8.36091409	0.46266736	2.28306003	273.580715	0.31519738	10.1569483	3.19496728	0.47034143	7.93639685
M361T794	4.70942599	0.0101	0.0555	1E-18	1E-18	0.22463039	1E-18	0.00359	1E-18	1E-18	1E-18	1E-18	0.03108517	1E-18	1E-18	1E-18	1E-18	0.18635323	1E-18	0.14719954	0.0033628	1E-18	0.04766093
M339T628	0.25019859	1E-18	0.07682023	0.020915	0.0176574	0.05507326	0.04998756	1E-18	0.00533969	1E-18	1E-18	0.00499494	1E-18	1E-18	1E-18	0.00381642	1E-18	0.39556805	0.04723581	0.16258368	0.01012641	1E-18	0.14677859
M372T774	1E-18	1E-18	1E-18	0.01058304	1E-18	1.17244578	1E-18	1E-18	0.03147878	1E-18	1E-18	1E-18	1E-18	0.17588776	1E-18	0.00626768	0.01824118	1E-18	0.03123919	0.19522081	1E-18	0.01221654	0.18255455
M310T701	6.67637624	0.31001399	0.23953176	0.32209651	0.4888878	0.73896612	3.69310676	0.16622276	0.08357569	1.10422208	0.0705444	0.21514067	0.14908355	0.20655497	0.13454585	0.03819157	1.02288228	7.0796214	0.12524038	1.27136458	0.08739163	0.43480686	0.19837533
M183T360	1E-18	0.42486685	1E-18	1E-18	1E-18	1.05765201	1E-18	1E-18	0.1336493	1E-18	1E-18	0.20106581	1E-18	1E-18	0.01075366	1E-18	0.01549642	1E-18	0.14886042	1E-18	0.0026911	0.03348044	0.55683673
M405T786	1.82297304	0.0020603	0.20636042	0.13003277	0.05441398	0.16683433	0.05081326	0.00183536	0.38703757	1E-18	1E-18	0.04062946	0.03204242	1.00193424	0.05172203	0.00092138	0.07002833	0.41988849	0.12853935	0.2410023	0.00771789	0.00787807	0.25321284
M319T157	1.62727226	0.04728157	1.0817878	0.0926626	0.18461155	0.27442259	0.57754367	0.07931427	0.01741514	0.05507296	0.0344855	0.04618218	0.07896616	0.25387861	0.18175648	0.06732332	0.28349157	1.55178882	0.07344812	0.27041823	0.02148964	0.08506825	0.16121952
M209T91	15.3626815	2.15828552	0.21629926	0.02783717	0.23344289	0.18773218	5.90517338	0.3286297	0.02453462	0.08712177	0.01128816	0.04843982	0.02613223	0.21882137	0.04899285	0.02444964	0.11371239	11.2818881	0.01573763	0.22258908	0.18754551	0.06589553	0.0520176
M103T87	18.2519071	0.23231661	2.68476782	0.35315377	0.39298746	0.75863905	1.27045961	0.51754308	0.08764337	0.09711989	0.29580762	0.3063881	0.19991329	0.26237734	0.07763409	0.04277121	2.05595578	16.114106	0.13116862	0.21637692	0.37311226	0.3353899	0.16299124
M556T665	1E-18	0.04261926	1E-18	0.00957909	0.01406357	0.39834626	0.13433273	0.18863534	0.43364389	0.16218423	0.13282634	0.32103359	0.41789887	2.72013296	0.69618559	0.16527482	0.24267639	3.2238588	0.06969471	1.7011023	0.03157972	1.05296665	0.33919233
M307T792	19.8082488	0.5361918	0.28866421	0.37066863	0.78354231	4.40169834	0.73674068	0.11185079	0.02564925	1.99522433	0.09336147	0.1100825	0.37124834	1E-18	0.07208066	1E-18	1.04944507	31.6635532	0.021229	2.1073786	0.25247617	0.55408458	0.51990137
M249T535	1.62661434	0.02305502	1E-18	0.01795495	1E-18	0.59468645	0.11452452	1E-18	0.10374739	1E-18	0.05019158	0.04961244	1E-18	1E-18	0.06315769	1E-18	1E-18	0.23681724	0.05180566	1E-18	1E-18	1E-18	0.02254363
M165T748	14.6463919	0.4565311	2.96969565	0.22016226	0.64088063	0.96347836	1.58699299	0.15520841	0.17376334	0.33558606	0.1619362	0.20334736	0.33184947	1.17808695	0.38337616	0.16687113	0.7528535	8.26524535	0.24059032	0.83593549	0.11654629	0.74136165	0.49650797
M325T631	3.59465978	0.55524582	0.80323161	0.55520864	0.0460017	1.44372829	1.51421248	0.00891958	0.02822957	0.63967571	0.06318021	0.03765881	0.07827802	1E-18	0.18351698	0.01609929	0.26109219	2.78144953	0.05257251	0.65475115	0.14123623	1E-18	0.30556025
M269T500	7.45	0.156	4.24	0.115	0.624	0.226	1.44	0.468	1E-18	0.29	0.006	0.0241	0.415	1E-18	1.18046981	0.09720739	0.43231323	11.0528034	1E-18	1.08265541	0.234	1.97818823	0.16302303
M135T1110	19.5439352	0.98984898	10.6765455	0.77670466	2.13823985	4.52317849	3.87767208	0.45840513	0.80405417	1.07545727	0.44281964	0.5730129	0.59515225	2.47068882	0.54781582	0.29772107	1.93984444	11.9910926	0.7679644	1.94814665	0.56946195	1.57273097	1.25512602
M403T654	0.78838856	1E-18	1E-18	0.06565996	0.05482031	0.24647445	1E-18	0.01347823	0.03448247	0.23486348	1E-18	0.0399685	0.085753	1E-18	0.04750031	0.05092976	1.09739145	5.81719158	0.0948873	1.14799424	0.13033671	0.16311741	0.46566756
M211T92	0.75330895	0.10373002	0.33388193	0.0205004	0.14808834	0.16357066	0.12916641	0.08556063	0.01889836	0.63150308	0.0217954	0.05977682	0.04367492	0.29817748	0.02205129	0.06902714	0.19991368	0.68595221	0.03099994	0.18209744	0.05665791	0.04993111	0.26424814
M173T148	14.0124001	0.07341425	1.95768957	0.04023072	2.1927751	0.08829334	0.62014919	0.57290606	0.05051233	0.67084503	0.05055438	0.10244234	1E-18	1.03228077	3.8054453	0.13065639	1.88736455	2.6658778	0.02160752	0.29403837	0.26390948	2.41580282	0.61819328
M241T576	77.3701363	1.40943701	59.3207285	7.67619427	8.3879436	4.4187269	9.38206108	3.06943631	0.4983128	10.522214	1.64925679	0.37661571	0.91702628	1.678993	25.8891835	14.8853821	11.0667264	242.947883	0.29620206	5.48423833	1.36620474	3.70250724	4.45437665
M165T85	3.2654079	0.16530726	0.33852165	0.18780816	0.10328096	0.10138749	0.22224404	0.03290713	0.21050418	0.02631764	0.11194444	0.08001058	0.0799049	0.28890412	0.1756164	0.036777	0.13881443	0.68520928	0.07569964	0.84123074	0.04096999	0.14394444	0.17355125
M117T85	4.66764731	0.06626028	1.71933818	0.0735984	0.36229249	0.15412354	0.25573797	0.0207845	0.09622932	0.07967739	0.08293127	0.30824571	0.05640227	0.77506647	0.03797685	0.01475683	0.10546378	2.49264922	0.01675652	0.11492789	0.03500612	0.16612379	0.21353447
M145T85	1.43010725	0.10269261	1.25651229	0.02059915	0.10161673	0.09242055	0.19048688	0.11520462	0.01533887	0.3742099	0.01370141	0.0174975	0.05180742	0.07287105	0.08378764	0.04298211	0.51771269	1.45839314	0.10605993	0.03020326	0.0161088	0.16420246	0.03789267
M143T146	13.8566935	0.03466243	2.14987519	0.38987214	0.16333808	0.20959493	0.23807421	0.01260896	0.08513458	0.62790742	0.0224956	0.00477688	1E-18	0.05411428	0.073178	0.00400575	0.36919523	0.78987252	0.01495444	0.41408948	0.10655857	0.10916269	0.19477763
M80T1245	1.99298429	0.09681143	0.45814563	0.05057135	0.14030776	0.19965708	0.27852936	0.02680689	0.04411202	0.07337581	0.02328183	0.0504163	0.0570561	0.20889049	0.07839054	0.02566941	0.16749094	1.21527963	0.0575814	0.14555363	0.03141333	0.15512778	0.08168196
M199T91	1.54055369	0.08843315	1.26231649	0.09204184	0.15101525	0.06119068	0.8491366	0.2921026	0.03503287	0.02496512	0.05014704	0.02910314	0.08828221	0.42390447	0.65619331	0.19850674	0.63264525	1.94503707	0.21657976	0.14833078	0.01588434	0.31332858	0.07364785
M193T83	1E-18	0.0156399	1.19218907	0.00680988	0.09874323	0.09241229	1E-18	0.0584094	0.10267146	0.04453292	0.00954269	0.02214848	0.02473991	0.37214486	0.12080076	0.01629232	0.17134003	0.41341433	0.03466492	0.08134523	0.02189552	0.11103556	0.03991572
M227T82	18.858247	0.3739725	1.05099797	0.69730695	1.15277284	0.77322998	2.55810049	0.08660257	0.14738097	0.02057486	0.0583275	0.50395858	0.03305312	0.1345387	0.03294717	0.02896471	2.93072393	0.96469352	0.01697848	1.68770488	0.02049162	5.40322973	0.23281175
M101T361	60.1780491	1.75303751	3.24078496	0.0265768	2.09756537	2.91934207	1.5327884	0.64241866	0.3349972	0.73276391	0.17950743	1.42076457	0.35565746	1.18557707	2.74660327	0.92729172	0.78111486	8.67276181	0.38040178	0.96605402	0.45889281	2.55957968	0.55930473
M305T659	11.8159585	0.41840102	1.10678813	0.41980514	0.17240677	1.27144067	1.76732056	0.03826312	0.03309569	0.15353505	0.07416239	0.17492623	0.13885721	1E-18	1E-18	0.01737382	0.44490373	3.99561931	0.07773353	1.44194505	0.11504029	0.13547296	0.77438552
M197T123	1E-18	1E-18	0.12892981	1E-18	0.02211288	1E-18	1E-18	0.00584378	1E-18	0.00930087	1E-18	0.00675755	1E-18	0.34528547	0.03042349	0.00669441	0.02818043	1E-18	0.00481323	1E-18	0.00275539	0.11574355	0.01026363
M380T685	0.63061012	1E-18	1E-18	0.03328812	0.09652204	0.07834609	0.0297	0.00450917	0.00652945	1E-18	0.00331849	0.01201046	0.0275	1E-18	0.02164015	0.01881905	0.1605941	1.67999795	0.02639285	0.21648982	0.00694144	1E-18	0.13104429
M137T190	19.4460479	0.27906024	2.60540123	0.2798203	1.3088171	0.3261695	0.48477439	0.30248967	0.05651723	0.32505878	0.04098819	0.33189603	0.23704285	1.72141063	1.43244916	0.08696143	0.75687115	6.16869438	0.19396028	0.86570541	0.08219991	0.35035917	1.66883241
M122T86	1.72836182	0.05756539	0.41747021	0.04127091	0.15636177	0.24342096	0.20519191	0.01049973	0.05165295	0.06568696	0.00993251	0.03454356	0.0276308	0.17477063	0.05992942	0.02323818	0.08938444	0.66367	0.0297828	0.08618201	0.0279277	0.33277771	0.05909695
M165T1560	9.86403996	0.46796587	3.14354345	0.32348395	0.71987884	1.01654105	0.66591264	0.18964728	0.24227441	0.46957937	0.04880244	0.24879485	0.15504839	0.98436784	0.41278188	0.16204444	0.73175045	4.44504916	0.28961649	0.34499161	0.12966594	0.78385973	0.57507218
M357T724	4.99129614	0.0235	0.188	0.27435257	0.332	0.37493523	1.02122015	0.11290389	0.136	0.30307504	0.0385	0.0419	0.134	0.0353	0.0608	0.0269	0.31213509	3.39	0.1896758	2.47705592	0.06220712	0.343	1.11048212
M309T83	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.15932772	0.03527649	1E-18	0.11674788	1E-18	0.05533933	0.06612313	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.02120532	0.02695286	0.03041409	0.01144092
M319T828	5.26448655	0.04282831	1.67624896	0.10434887	0.4717783	0.31695952	0.5689603	0.08261284	0.11862646	0.17010806	0.05641721	0.10154472	0.11785992	0.52378568	0.16909254	0.05446206	0.32670261	0.7325564	0.10536292	0.30235354	0.05815306	0.39450951	0.20581328
M69T483	25.3359186	0.64529471	9.84237807	0.84735261	3.65495074	2.40341669	1.83388309	0.34760133	0.98209609	1.1142964	0.60598042	0.75256148	1.23591436	3.3945611	0.4349791	0.55743944	5.20537407	21.0820966	0.95828397	1.87381432	0.39697604	2.86543982	1.57204139
M357T605	4.2081479	0.04530695	0.0868773	0.03875509	1E-18	0.23313265	1E-18	1E-18	0.00354588	0.01132714	1E-18	0.01129423	1E-18	1E-18	1E-18	0.00424343	1E-18	1E-18	1E-18	0.08358789	0.01394055	1E-18	0.06811049
M399T89	0.88245467	1E-18	0.978	1E-18	1E-18	0.135	0.696	0.609	0.06072529	0.101	0.0257	0.20469723	0.08991244	0.228	0.07218235	1E-18	0.0555	1.41358863	1E-18	1E-18	0.15962521	1.38792005	1E-18
M549T694	0.2783899	1E-18	0.22661477	0.00728296	0.10699959	1E-18	0.22032841	0.25613296	0.11159162	0.07620535	0.0055652	1E-18	0.06018486	0.31174601	0.11802035	0.00255601	0.13357003	0.42402618	0.00714472	0.08276033	1E-18	0.18214825	0.11691929
M201T92	1.41715856	0.04114528	1.38834104	0.01751658	1E-18	0.16311263	0.25394969	0.040116	0.04834781	0.03844426	0.01016216	1E-18	0.06797322	0.17617102	0.15329723	0.02175363	0.22110161	0.41163619	0.0278103	1E-18	0.1062459	0.06556393	1E-18
M215T1568	2.42638373	0.11288931	1.00878434	0.02430585	0.19560606	0.09109489	0.27028713	0.06219001	0.06810241	0.07026537	0.00965888	0.02659183	0.05444439	0.47025009	0.25213427	0.0241049	0.07655697	1.14162394	0.00524407	0.0423289	0.01653746	0.33177001	0.07520419
M81T85	11.2114623	0.43806142	1.03027551	0.08411001	0.36089148	0.79434145	0.62459484	0.10751121	0.06897385	0.29227781	0.03947076	0.01715413	0.04675637	0.56114549	0.4383784	0.17363266	0.51534609	4.06462724	0.10743953	0.32176401	0.05647885	0.42477876	0.48677749
M322T762	0.28631833	0.17308944	1E-18	0.05163722	1E-18	1E-18	1E-18	0.00366734	1E-18	1E-18	1E-18	0.00513014	0.00592907	0.02028204	1E-18	1E-18	1E-18	1E-18	0.01804866	0.01894925	0.00243526	1E-18	0.16670203
M339T779	3.66572512	1E-18	1.02446775	0.03951462	0.01623472	0.02043963	1E-18	0.00314021	1E-18	0.00831357	1E-18	0.03955385	0.01467269	0.32340094	1E-18	0.00298373	1E-18	0.06038829	0.09627183	0.0685958	0.01578114	0.01346418	1E-18
M285T416	1.09298363	0.231	0.147	0.0972	0.01422785	0.51507552	1E-18	0.17490365	1E-18	1E-18	1E-18	0.12326837	1E-18	1E-18	0.292418	0.0181	1E-18	1E-18	0.10158835	0.03474335	0.00323	0.24204423	1E-18
M62T359	183.629963	5.62136577	12.7049441	0.13934237	3.74377295	2.70368318	10.3620715	0.50088436	1.20048458	2.27120145	0.1321819	0.89758234	3.06602905	3.80966127	5.81176749	0.99936292	4.4845103	25.8254409	1.02562905	8.80908754	0.29348281	6.46097303	2.65200705
M227T84	1E-18	0.0477597	0.1351973	0.05086927	0.2578551	1.0892126	0.39411642	0.07321359	0.03320961	0.40290259	0.02000428	0.03407977	0.09564815	0.10900591	0.09099611	0.01429104	0.17476799	0.46645407	0.01675646	0.23477431	0.17017326	0.04504685	0.06108182
M155T598	34.3203222	1.84116279	11.0183609	1.24768066	2.88661967	3.09160416	7.22811591	0.3277499	0.32810027	1.56849126	0.30370067	1.38391851	1.27469809	4.43579571	3.38649928	0.79373861	3.07758443	12.4378235	0.9642268	6.08751512	0.84207767	1.16580602	7.98820842
M325T701	6.17498187	0.12028683	1.51758917	0.1570932	0.01447269	0.74647132	2.54817141	0.05288057	0.00471734	0.30366644	0.04606372	0.03695928	0.01871293	0.88857807	0.05459023	0.08402216	0.23409497	8.77286179	0.29824716	0.60811443	0.26466822	0.12599994	0.79564648
M141T1584	6.12967578	0.74838589	1.97491603	0.16837999	0.81940137	0.4803529	0.90235034	0.01908884	0.13288771	0.29581385	0.11398063	0.7092243	1.02814109	0.67388029	0.50801462	0.70362459	0.2044732	6.63809753	0.93649121	0.16722636	0.04548878	1.99416224	0.39983752
M241T86	1.13971471	0.43319612	8.37238925	0.49231104	0.69152591	0.11726005	0.24613538	0.3274699	0.19774345	0.13204659	0.01304709	0.05740289	0.03265947	10.0418159	0.17956154	0.1194543	0.0634181	0.54871436	0.02166683	2.77901515	0.07164032	0.08417853	0.54273063
M146T81	1.44625763	0.07575314	3.2336784	0.0423642	0.10029283	0.38186569	0.36348272	0.31772569	0.14514921	0.06046143	0.17131092	0.04484416	0.05507857	0.26812339	0.11405068	0.02442752	0.3117138	2.47258435	0.12872261	0.16412881	0.08307224	0.29786709	0.17840751
M217T89	2.33596272	0.05268374	1.17920476	0.06080705	0.4136864	1.47976395	0.92283268	0.05466571	0.07204061	0.04909409	0.02614083	0.08152281	0.10726966	0.32540441	0.07602916	0.04341122	0.11415188	1.77024323	0.08169545	0.19028075	0.02803571	1.72392348	0.05682454
M351T656	58.8167671	0.29534405	7.41085073	0.15792102	1.08183467	1.66624225	5.962596	0.27588425	0.15701858	4.79821106	0.10517017	0.19620498	0.66895951	0.31242624	0.10808425	0.0370785	2.07637383	25.7053292	0.06615879	5.07522561	0.98775831	1.21255742	1.05725686
M333T795	2.82369429	0.37183395	3.99612797	1.66379206	0.40163923	0.71409272	3.24020277	0.01931609	0.09751382	0.35376174	0.03264704	0.04234227	0.20263065	0.02082676	0.03642398	0.09768506	0.80634689	0.69403708	0.0207536	0.91087434	0.42947835	1E-18	8.11645127
M309T692	71.134154	0.37926503	11.1834432	0.28424093	4.66221299	0.65768264	30.1695849	1.27209408	0.0949857	8.58492465	0.1591326	0.20040865	1.51099414	0.4913976	0.14690414	0.09782678	6.67386015	43.7595665	0.42225136	9.50338844	0.78183098	3.88847808	0.39653645
M219T82	5.92033866	0.24595186	1.34488714	0.03523707	8.30602584	0.8669566	0.28911993	0.10060639	0.20019778	0.03808737	0.02463702	0.04013382	0.04735614	0.66120236	0.20039676	0.00845969	0.35441187	1.87375337	0.06079152	0.15172087	0.05106466	65.5369451	0.19103814
M615T676	1E-18	0.04905875	1E-18	0.00619691	1E-18	0.06802396	0.90291331	1E-18	1E-18	0.24550171	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.01738474	1E-18	1E-18	0.02950588	1E-18	1E-18	0.12221253
M571T693	1.50438578	1E-18	0.67342171	0.05343837	0.33512244	1E-18	0.69740733	0.72749448	0.00995321	0.25400061	0.00975486	0.02766929	0.17342927	0.60402023	0.11608708	0.00340305	0.48293747	1.61328563	0.01207776	0.19986065	1E-18	0.62828886	0.35296312
M195T132	1E-18	1E-18	0.1290321	0.00578258	0.16819855	1E-18	0.0311483	0.05070702	0.00708215	1E-18	1E-18	1E-18	1E-18	1.2917548	0.58210458	1E-18	0.28442797	1E-18	1E-18	1E-18	1E-18	0.41360966	1E-18
M383T800	1E-18	1E-18	1E-18	0.09923595	1E-18	1E-18	1E-18	0.0433	0.0384035	1E-18	1E-18	1E-18	1E-18	1E-18	0.00916547	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.233
M421T809	3.11604492	1E-18	0.32442971	0.20763631	0.06112545	0.10849616	0.02449399	1E-18	0.07027843	1E-18	1E-18	0.01271289	1E-18	0.14806106	1E-18	0.03453138	1E-18	0.99656881	0.12006185	0.26466898	1E-18	1E-18	0.1931634
M381T703	1E-18	1E-18	0.0563	0.02033032	1E-18	0.0984	1E-18	1E-18	0.0519	1E-18	1E-18	0.04954646	0.00889645	1E-18	0.04801893	0.00759	0.03884717	0.22516848	1E-18	0.14574274	1E-18	1E-18	0.01668441
M305T885	1E-18	1E-18	0.0644441	1E-18	1E-18	0.06551868	0.03810547	0.0099868	1E-18	1E-18	1E-18	0.00439345	1E-18	1E-18	1E-18	0.00333231	1E-18	1E-18	1E-18	1E-18	1E-18	0.01453061	1E-18
M311T565	0.35382529	1E-18	1E-18	0.01582561	1E-18	1E-18	1E-18	0.00509642	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.10857888	1E-18	1E-18	0.19020386	1E-18	0.03919285	0.02979132	1E-18	1E-18
M500T731	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.03555277	0.12726966	1E-18	1E-18	1E-18	0.06500459	0.06164367	1E-18	1E-18	0.61385079	3.17404856	0.0562876	0.26209527	0.09120632	1E-18	1E-18
M327T579	0.81123187	0.01217045	0.07271094	1E-18	1E-18	1.20642708	0.08133062	1E-18	1E-18	1E-18	1E-18	0.14330093	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.01119055
M342T687	1E-18	1E-18	1E-18	0.01826032	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.06864292	1E-18	0.02025044	1E-18	1E-18	0.03273379	1E-18	1E-18	0.06806025	1E-18	1E-18	0.01135353
M294T757	1.93	1E-18	0.141	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.00311	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.01940872	1E-18	1E-18	0.00986596
M312T674	0.23618814	1E-18	1E-18	1E-18	1E-18	0.04088521	0.04033756	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.13271567	1E-18	0.01915346	0.16612509	1E-18	1E-18	1E-18	1E-18	0.02436049
M326T540	0.2490733	1E-18	1E-18	0.00734397	1E-18	0.0203094	1E-18	0.00903283	1E-18	1E-18	1E-18	0.0073422	0.0061368	1E-18	1E-18	1E-18	1E-18	1E-18	0.00686847	1E-18	1E-18	1E-18	0.00948032
M285T415	0.48274985	1E-18	1E-18	1E-18	0.0142291	0.17114873	1E-18	0.09486553	1E-18	1E-18	1E-18	0.05890217	1E-18	1E-18	0.19647259	1E-18	1E-18	1E-18	0.08498236	0.01556018	1E-18	0.13638029	1E-18
M371T837	9.7663198	0.36701834	1.05902707	0.43220572	0.30594099	0.54205351	0.9322858	1E-18	0.01139881	0.14558896	1E-18	0.07616741	0.0426841	0.02294975	1E-18	0.04617402	0.3494402	2.71222437	0.00468958	0.23393183	0.17509727	1E-18	0.50363182
M199T464	8.5789372	0.09621766	1.20284722	0.07047838	0.25802642	0.62353534	0.23832307	0.01680651	0.03019578	0.02189601	0.0034501	0.01657319	0.0148837	0.02182174	1E-18	0.00688515	0.01649593	0.16690972	0.0134987	0.10346178	0.01200189	0.01525841	0.05508586
M199T467	8.5789372	0.09621766	1.20284722	0.07047838	0.25802642	0.62353534	0.23832307	0.01680651	0.03019578	0.02189601	0.0034501	0.01657319	0.0148837	0.02182174	1E-18	0.00688515	0.01649593	0.16690972	0.0134987	0.10346178	0.01200189	0.01525841	0.05508586
M571T689	1.504967	1E-18	0.75541085	0.05344015	0.33508899	1E-18	0.73901491	0.73676719	0.03216137	0.27072666	0.00975781	0.02768485	0.18141125	0.95639706	0.03596631	0.003403	0.48272023	1.61307596	0.08060682	0.24103905	1E-18	0.67392072	0.37022357
M323T608	7.62335512	0.03763932	1.60623415	0.2653222	0.15376247	0.29356463	3.12447228	0.01446284	0.10186459	0.21245135	0.04093137	0.22140455	0.08849036	1E-18	0.05204144	0.05452014	0.91731214	1.34793317	0.36060482	1.0921295	0.07996108	0.16505154	2.22728626
M345T646	13.463356	0.6399047	1.98475749	0.44691041	0.7815785	0.84870798	3.73595441	0.14724189	0.09185096	1.37772276	0.11498742	0.03591579	0.30303728	0.0760711	0.06634446	0.08358265	0.87083355	6.53900559	0.08590735	2.13114682	0.1864393	0.58840787	0.32529678
M117T126	46.5806839	0.54143408	8.74276978	0.2445394	2.08716208	1.52291142	3.0447	0.43388775	0.25190312	1.58013633	0.13564949	0.68082644	0.16876227	4.12791213	0.77693513	0.3923492	2.69520526	22.6280938	0.72846305	3.583916	0.34306409	1.87752623	2.84206964
M319T93	1.37046148	0.04840653	0.25458686	0.03740007	0.06940564	0.18275267	0.15935244	0.01322414	0.02846181	0.03496018	0.02606987	0.02671761	0.03011298	0.2057449	0.02861961	0.01398806	0.08591448	0.90320068	0.07036298	0.08672143	0.01144682	0.13899179	0.05262288
M87T434	27.8471073	2.96283951	70.3912242	0.98749589	3.92034232	4.9971877	4.54225529	3.06366968	1.30257702	0.93115563	0.76625544	1.66935497	0.89963951	2.53885568	1.59386671	0.44642784	2.06368287	25.5768419	2.5305516	1.48546036	0.24682665	2.61882094	1.72780739
M499T586	114.621239	0.88333281	21.241372	0.29129991	8.59126009	0.53199241	0.16772913	1E-18	0.30612576	1E-18	0.08225785	0.27221668	1E-18	0.62157585	2.37367301	1E-18	1E-18	1E-18	0.0316528	1E-18	0.00353421		7.15971036
M122T129	15.7085795	0.67456269	3.364681	1.14047196	2.64591082	1.05376276	3.2043033	0.29977581	0.48101069	0.55155365	0.13871532	0.50221508	0.36506363	1.21761186	1.12070939	0.292303	1.04715957	17.7561081	0.46464979	2.01908683	0.20344558	1.86662286	0.5124658
M405T654	1E-18	0.01691989	1E-18	0.04429154	1E-18	0.23360861	0.03326841	1E-18	0.01639077	0.09702875	1E-18	0.00786422	0.0534262	1E-18	0.01582654	0.01959542	0.27912655	1.38152601	0.07715492	0.65286579	0.08234175	0.02889969	0.22980964
M219T358	0.38837615	0.13199899	0.20683656	0.00885196	1E-18	0.16629353	1E-18	0.02333586	1E-18	0.00846216	1E-18	1E-18	1E-18	1E-18	0.15743146	0.00279182	0.03778713	1E-18	1E-18	1E-18	1E-18	0.47467362	0.00277003
M325T710	2.07753244	0.09419914	0.21863791	0.12981353	0.01446988	0.22440754	0.62064861	0.00371955	1E-18	0.34340645	0.03479858	0.12077554	0.00796025	0.62973128	0.03171211	0.05337445	0.12401885	6.15673048	0.08140986	0.46704636	0.06779546	0.4180811	0.63264398
M164T87	1E-18	1E-18	2.94871507	1E-18	1E-18	0.115618	0.10810061	0.95764772	0.11198959	0.0197925	0.0144675	0.04615806	0.03329138	0.19985341	0.06396755	0.06956626	0.0339526	0.5217311	0.31437737	1E-18	0.034545	0.15107256	1E-18
M111T366	23.3688203	1.16844036	6.8654439	0.54162565	1.80521546	1.34717244	2.05939696	0.53751461	1.05788738	1.11361495	0.46206353	0.58976244	0.57693773	1.07158517	1.30526103	1.01988342	2.71710608	10.9586647	0.8373119	1.06862987	0.49046754	1.25644582	2.03107812
M80T938	2.67094788	0.09298337	0.77315484	0.08183805	0.07638872	0.27643095	0.15393119	0.04058044	0.06773398	0.09777674	0.02104307	0.0508636	0.09631369	0.19359692	0.14001231	0.03683963	0.12534399	1.44072182	0.06659064	0.23169099	0.03954241	0.10084944	0.15501179
M243T86	5.83429445	0.04024874	1.0511137	0.2045153	0.06677247	0.64866767	0.42365266	1.17838805	0.45838494	0.54088507	0.15608284	0.20668319	0.14260254	0.1186607	2.91555577	0.12224913	0.36252984	3.32577237	0.13359521	0.04810275	0.16326221	1.23468793	0.61237684
M60T750	22.6863884	0.60749113	6.27233575	0.36727626	1.21221216	1.69845964	2.01882911	0.25908502	0.4777795	0.81948518	0.29263612	0.40147219	0.67697439	1.76658492	0.42557868	0.41739826	5.51937666	9.8842513	0.4110867	1.13946899	0.23113212	2.73278121	0.91882875
M187T139	0.94256424	0.01131468	0.32599347	0.01156386	0.1806223	0.10381497	0.03629282	0.09307084	0.13220233	0.01592962	0.0033649	0.00747944	1E-18	0.70948642	0.26735428	0.00319898	0.50738407	0.13423942	0.00571146	0.03733984	0.01122112	0.3937006	1E-18
M255T1080	1E-18	0.01716246	1E-18	0.02668596	1E-18	0.8892143	0.15700704	0.00888824	1E-18	0.29011507	0.01085219	0.0170703	0.00627061	1E-18	0.02390592	0.00299315	0.0853806	0.288071	0.0232729	0.55274859	0.08567069	0.01200551	0.10613268
M613T565	10.4449998	1E-18	2.51223054	0.02000225	0.51929034	1E-18	0.0269222	0.06657412	0.16215204	0.0090773	0.00596434	0.09810992	0.15611499	0.23442364	0.16741872	0.10630242	0.18529383	1.67679414	0.17036575	1E-18	1E-18	0.01674863	0.03607765
M275T85	1.99	0.17936106	0.38220014	0.01512868	0.104	0.06422064	0.25601372	0.0807	0.18347032	0.17607308	0.01169654	0.0938	0.07587437	0.113	0.0452	0.0112	0.40959173	4.6	0.0428	0.164	0.01408252	0.0806	0.039
M237T438	4.87365859	0.09615895	1.2525467	0.03663993	0.0164598	0.06529523	0.2078728	0.04922569	0.02378574	1E-18	0.00319783	0.00486112	0.00928352	0.05417195	0.07440059	1E-18	0.03527087	0.15303737	1E-18	1E-18	1E-18	0.12254497	1E-18
M113T434	40.7221349	1.34502835	12.3614226	0.88652526	2.42669862	5.05970555	2.28030143	0.38250163	0.11831966	0.67656097	0.48216086	0.52581309	0.58371696	2.86571129	1.26988066	0.31480875	1.46537742	11.0642063	0.23448374	0.93155952	0.17171683	1.58487673	1.20859035
M145T352	1E-18	1E-18	0.1936355	1E-18	1E-18	1E-18	1E-18	1E-18	0.00513572	1E-18	0.00303312	0.32029808	1E-18	1E-18	0.00934944	1E-18	0.05604402	1E-18	0.4225343	1E-18	1E-18	0.11000849	1E-18
M252T595	2.4604749	0.04533888	0.17407868	0.03892546	1E-18	0.16637483	1E-18	1E-18	1E-18	0.08667507	0.01384504	0.00574558	1E-18	1E-18	2.01391168	1E-18	0.01569208	0.17638065	0.00622409	0.25401902	0.03017305	0.0161806	0.04067889
M311T811	4.93274433	0.04974636	0.9869459	0.21356236	1E-18	0.20205958	0.89105861	0.00676422	0.00611868	0.04107093	0.01913754	0.05109629	0.21951952	1E-18	1E-18	1E-18	0.51271109	1.70349789	1E-18	0.48187855	0.04874199	1E-18	0.44378032
M404T654	1E-18	1E-18	1E-18	0.0075757	0.01874215	1E-18	1E-18	1E-18	1E-18	0.02048293	1E-18	1E-18	0.02694141	1E-18	1E-18	1E-18	0.14335011	1.15293199	0.01736219	0.05932261	0.02374483	1E-18	0.05759155
M245T84	1.83363424	0.20577231	0.51321182	0.03562016	0.04924187	0.10870702	0.23218032	0.10266764	0.00727615	0.2587259	0.0670959	0.0227865	0.02023508	0.11375407	0.46875095	0.01748493	0.26724604	1.12027306	0.0079656	0.1042729	0.02847699	0.18423962	0.06985213
M125T496	13.4606502	0.46439505	1.69170983	0.20595612	0.50152476	1.01492879	1.45025045	0.07931293	0.18978214	0.31244386	0.06879945	0.070126	0.24417595	0.59802388	0.30623648	0.16237096	0.46521237	4.29163368	0.36469422	0.469416	0.10699297	0.64410117	0.30998788
M95T81	22.1478033	0.6148407	19.542436	0.25777509	0.78943054	14.2482372	1.06470326	14.58591	0.70934791	1.43882029	0.17928098	0.47012955	0.0556566	155.681366	3.59266005	0.08460719	16.3075676	24.7768263	2.95929525	1.49605795	0.02524712	0.53309899	31.276648
M387T582	1E-18	1E-18	1E-18	1E-18	1E-18	0.32726438	1E-18	1E-18	0.07562051	1E-18	0.0063018	0.04212193	1E-18	1E-18	1E-18	1E-18	1E-18	0.205	0.05036817	0.032	1E-18	1E-18	0.17679851
M95T78	0.2489981	1E-18	1E-18	1E-18	1E-18	0.17257201	1E-18	0.02833259	1E-18	0.07411686	0.00445235	1E-18	1E-18	0.0249395	0.05720054	0.00398436	0.05467841	0.62925373	0.00510068	0.01768529	1E-18	0.02257535	0.03218776
M160T89	0.2866926	1E-18	0.11974183	1E-18	0.02073241	0.02218598	1E-18	0.03000213	1E-18	0.00876033	0.00786029	1E-18	0.02188648	0.26632361	0.0439202	1E-18	1E-18	1E-18	0.04233021	1E-18	0.01268192	0.11456262	1E-18
M218T89	0.88007919	0.077183	0.40146802	0.17335509	0.08849206	0.20064943	0.23481763	1.16172919	0.19137947	0.07628942	0.17695919	0.05423718	0.02271617	0.17753509	0.05892702	0.01534246	1.06129495	2.23977757	0.74984879	0.1613479	0.01687188	0.29196041	0.40914324
M164T131	1.22906503	1E-18	0.39468314	1E-18	0.02049221	0.08009937	1E-18	0.15215885	1E-18	1E-18	0.01038331	1E-18	1E-18	0.22324184	1E-18	0.00512655	0.18028865	1E-18	1E-18	1E-18	1E-18	0.26352356	1E-18
M108T129	9.07705396	0.2191791	2.74935018	0.62658377	1.3865187	0.8443726	1.13345695	0.30481516	0.10214443	0.96870374	0.21049658	0.26530507	0.0950185	2.63727864	2.16393653	0.12862358	1.60726307	11.2370961	0.30212599	1.5044827	0.17225555	1.17711605	2.12378363
M181T674	53.2395405	3.85703688	34.6611331	5.68618222	11.9163287	10.8897942	3.52042616	5.79211757	0.46586696	9.79602303	0.46915715	0.38280288	1.20242607	1.12983844	10.1545658	0.06549217	3.42485719	702.588233	0.5750433	12.3459501	1.48758034	4.70985066	0.92597757
M349T825	4.1753842	1E-18	0.29479108	0.15474223	0.20922909	1E-18	0.03803395	1E-18	0.11117563	0.02170878	1E-18	0.00418925	1E-18	1E-18	1E-18	1E-18	0.01620854	1.78037046	1E-18	0.01547623	1E-18	0.01374382	0.21500674
M548T687	0.34334104	1E-18	0.40655703	1E-18	0.14157438	1E-18	0.1761422	0.27459898	0.0796754	0.11657502	0.00406207	0.00461769	0.05570364	0.32759557	0.05977781	0.00313791	0.15055878	0.38232794	0.02209652	0.1387223	1E-18	0.22622954	0.1655326
M133T190	51.1979617	0.09296701	1.96129596	0.2181633	1.06615157	0.33348697	0.34424868	0.18340194	0.02402612	0.33476856	0.07291347	0.01973989	0.27311763	0.93813467	1.10755918	0.09502866	0.6686516	4.53236591	0.1092476	0.95867919	0.07787695	0.31049066	1.25866963
M118T91	1E-18	1E-18	0.06261606	0.01313548	1E-18	0.05290929	0.0808703	0.0995399	1E-18	0.02951362	0.00570905	1E-18	1E-18	0.16816138	0.05360794	1E-18	0.01682668	1E-18	0.02112228	1E-18	0.0326972	0.13472435	1E-18
M323T779	1.12313949	0.01369929	0.1131624	0.04694575	0.01707668	0.0556263	0.09126545	0.04239706	0.0455264	0.11001711	0.00814713	0.03934105	0.04230755	1E-18	0.07215505	0.00869967	0.18637407	1.1408539	0.02814032	0.62088311	0.00516509	0.10647091	0.43953113
M547T689	1.62288621	0.03237565	1.94381898	0.08365938	0.57165125	0.05725384	1.32274451	1.02406823	0.02356073	0.49796888	0.03914455	0.01146936	0.31165179	0.413163	0.14516974	0.02832507	0.74749828	3.62013445	0.0266396	0.55608288	0.00961374	0.96646821	0.70301832
M319T90	2.16606162	0.02879384	0.84673037	0.02484201	0.09237756	0.24712489	0.08793294	0.01932035	0.03883037	0.02659723	0.02575576	0.02460005	0.03163351	0.16737596	0.05104178	0.02662066	0.09150872	1.25841876	0.03639151	0.07385246	0.01135731	0.06533256	0.0455489
M207T83	0.25884197	0.05153004	0.87327789	1E-18	0.04959636	0.16023939	1E-18	0.01051114	0.0157867	0.02277966	0.02136943	0.00998573	0.04439384	0.06947491	0.03927733	0.01200949	0.02597091	0.30908731	0.01406026	0.03888971	0.02300487	0.04377821	0.02012391
M484T734	2.98143662	1E-18	1.63925502	1E-18	1E-18	1E-18	0.02870356	0.00386475	1E-18	0.00677802	0.01619675	1E-18	1E-18	1E-18	1E-18	0.00692953	1E-18	1E-18	1E-18	1E-18	1E-18	0.01403829	0.18334826
M124T489	73.1133378	1.47874458	5.40645074	0.82399881	2.99274638	3.34858361	4.84020127	0.73490044	0.74766936	0.83666272	0.33750616	0.53568168	2.55625437	4.01604837	2.93819544	0.36494847	4.12603865	41.5812766	1.07412569	0.27224448	0.37865677	3.48181147	0.66199525
M310T700	13.116991	0.52033711	2.00802081	0.57867128	0.87461664	3.17982716	5.93567039	0.21997943	0.24606469	1.66588393	0.11071981	0.32204772	0.22015602	0.26832061	0.0344796	0.04288477	1.13503771	7.79152448	0.32899332	1.90459416	0.14113405	0.74614445	0.45275951
M323T599	20.5419377	0.75980428	2.22765743	0.20096178	0.08983152	0.57826621	1.29650849	0.0111028	0.0839355	0.46665168	0.05474059	0.19886761	0.0250929	1E-18	0.0520655	0.05451786	0.68296712	1.91837311	0.26238706	2.19589929	0.23088495	0.14810189	2.88478551
M133T85	8.98136552	0.08989575	0.28346481	0.02230581	0.07275461	1.99506227	0.27514401	0.0960581	0.03054309	0.04163195	0.02664981	0.04968	0.04023219	0.35021785	0.12126392	0.04979128	0.75082793	2.39998751	0.02293534	0.10664834	0.02367585	0.12008788	0.04076363
M251T83	1E-18	1E-18	1E-18	1E-18	0.05044503	0.07376411	1E-18	0.0235469	0.03387087	0.01514423	0.00298861	1E-18	1E-18	0.08111938	0.03703983	0.00323859	0.02020663	0.13344795	1E-18	0.0184519	1E-18	0.07167894	0.01571456
M367T731	1.1125507	1E-18	0.05909184	0.13309579	0.02868698	0.0837998	0.03310485	0.02303381	0.07168045	0.06963959	0.00729	0.02341587	0.03098724	0.199	0.16984009	0.03922664	1E-18	0.41013273	0.03452085	0.20225192	0.0485	0.02620587	0.18350489
M319T432	3.27160602	0.10190596	0.82203415	0.04738994	0.18230438	0.21279126	0.3439249	0.034039	0.03589006	0.08842067	0.03751246	0.02183728	0.0565005	0.29588021	0.11975056	0.04319765	0.16413998	2.03267756	0.05311292	0.13421236	0.03190704	0.28076978	0.08612489
M261T89	1E-18	0.00972117	2.63442859	1E-18	1E-18	0.06485592	0.1945716	0.0964813	0.02275047	0.0365634	0.00925307	0.02516379	0.04535648	1E-18	0.05917253	1E-18	0.02118079	1.02456755	1E-18	1E-18	0.01644359	0.49331107	1E-18
M131T687	9.93382319	0.4216399	3.08306123	0.2941738	0.61267885	1.02734482	1.9146656	0.19339759	0.27791902	0.38192164	0.15738669	0.23338787	0.40184225	0.74929331	0.27924804	0.15609551	0.61777784	6.40089453	0.31097549	0.75046312	0.19261679	0.52519414	0.40960743
M231T174	4.46691953	1E-18	0.29058463	0.05908615	0.01375948	1E-18	1E-18	0.00392632	1E-18	0.00767068	0.0035022	1E-18	0.0061425	0.95333194	1.23679951	1E-18	0.87556498	1E-18	1E-18	1E-18	0.02847641	1E-18	0.01103295
M241T85	0.32602259	0.0589631	0.07156806	0.02723369	0.06971943	0.12990226	0.17409963	0.04101393	0.05112214	0.14945347	0.02304244	0.0268766	0.04437018	0.22256187	0.06391312	0.00413943	0.16782893	0.95222511	1E-18	0.13766867	0.03282015	0.07895191	0.04074307
M267T753	1E-18	1E-18	0.18051771	0.00662945	1E-18	1E-18	0.06160289	0.04300274	0.01502472	0.09762935	1E-18	1E-18	0.02380731	1E-18	0.27046624	0.01824762	1.02674728	0.67754593	1E-18	0.19305491	0.04576585	0.01624997	1E-18
M303T538	1E-18	0.01144991	1E-18	0.00532628	0.03270858	0.25846763	1E-18	1E-18	1E-18	1E-18	0.01137393	0.03389394	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.03383342	1E-18	1E-18	1E-18	0.17442771
M97T427	48.4678691	1.18609885	15.6379521	2.18507892	5.63915809	2.29140468	1.79430266	0.84896251	0.53950393	2.52381848	0.78569501	0.32428567	10.5761796	1.74901453	1.12542704	2.72110522	1.29516645	12.1981106	0.57574158	0.75913441	0.2410134	3.39169808	6.09765781
M153T143	24.2773659	0.08881475	0.29924649	0.18404547	1.04513537	0.10312004	0.88559663	0.35712346	0.01361205	0.61282343	0.12485755	0.40834262	0.02958724	4.83114427	0.7797363	0.09953321	6.09448201	5.54400979	0.26109284	0.83135228	0.24649702	3.62450099	0.28201243
M319T1114	4.29812336	0.16934207	1.38371932	0.07027889	0.23533544	0.37317744	0.33658754	0.04389187	0.09726199	0.14732755	0.05626426	0.08882219	0.09490286	0.09540088	0.08903097	0.04876868	0.26934064	2.35394674	0.09506138	0.23246669	0.05383851	0.21117804	0.19150326
M83T484	83.6531116	3.90038668	32.8004407	3.87725523	7.75683305	1.74191145	12.1071131	1.18074627	1.33336073	3.88838365	0.61804159	1.60553185	4.01466995	9.31363383	0.84206177	0.34062721	3.83969326	27.7021813	2.51494159	4.52224289	0.95312719	6.70081758	4.24159033
M188T89	1E-18	1E-18	0.49744715	1E-18	0.11647017	1E-18	0.15858464	0.01462467	1E-18	0.07670594	0.00877455	1E-18	0.03892773	0.28784109	0.05972247	1E-18	0.02150143	1E-18	0.02540435	0.055347	0.01837799	0.04611513	1E-18
M119T1562	232.758879	17.2206473	23.874277	1.91175273	2.91709828	13.6669028	6.71759644	19.0172823	26.5010099	1.74431112	2.31282444	13.0473636	3.26342163	18.9449845	11.7566097	7.33004205	22.4777006	584.221007	1.56681735	7.86195916	0.74731232	0.14766184	44.4754504
M579T471	1E-18	1E-18	1E-18	1E-18	1E-18	0.0965222	0.03305296	1E-18	0.22801897	1E-18	0.01709397	0.08389834	0.00524905	0.14374041	1E-18	1E-18	0.01576942	1E-18	0.07036607	1E-18	1E-18	1E-18	0.23684456
M220T88	3.86396496	0.04546191	0.85481983	0.04614366	0.13192154	0.92837519	3.15010587	0.09986646	0.26784856	0.45357077	0.12252593	0.07467057	0.40163419	0.6246354	1.24201359	0.03236938	0.53283656	6.11051766	0.03702385	0.20270321	0.44382146	2.15751961	0.02314128
M83T426	75.6920474	0.69359648	17.5780831	2.15946241	1.69776367	1.31772233	2.85798657	0.76750787	0.08239812	0.90586294	1.00596199	1.08322243	0.54121043	6.02560391	1.34903408	0.40447481	1.11813135	17.9033421	0.19725387	1.68548886	0.30126568	1.54867132	3.48668375
M152T87	2.17783223	0.05025842	1.0557905	0.00987299	0.0635824	0.27446168	0.12385176	0.06976833	0.01210471	0.2775093	0.00906137	0.04983892	0.04375202	0.2017898	0.02868696	0.01859231	0.16976145	1.35005293	0.0563983	0.11562352	0.08324643	0.0751036	0.06500576
M279T640	0.557	1E-18	0.20220854	1E-18	1E-18	1E-18	1E-18	1E-18	0.00462565	0.32765981	0.00287739	1E-18	1E-18	1E-18	0.00875877	1E-18	0.02499121	1E-18	1E-18	1E-18	1E-18	0.0141946	0.18602579

M183T85	4.61784049	0.221801	0.28347237	1E-18	0.1708602	0.25546846	0.33044414	0.00977175	0.02123283	0.03167292	0.01929002	0.0203477	0.0309989	0.09358381	0.04114246	0.05219471	0.06877916	1.87942481	0.13110822	0.12138073	0.01521757	0.16471176	0.02489077
M247T155	0.25827209	1E-18	1.44719999	0.00494032	0.13375976	1E-18	1E-18	0.11202442	0.12258026	1E-18	0.05058359	1E-18	1E-18	0.57023137	0.26076637	0.00560113	0.40454779	0.11178745	1E-18	1E-18	0.01176469	0.51865739	1E-18
M251T595	29.3731325	0.49918074	5.52519235	0.42878253	0.78834571	1.08627884	1.96356167	0.22174866	0.22124323	1.00122271	0.06982893	0.13977986	0.32154482	0.73823482	0.07817751	0.06070052	0.8219583	9.86849255	0.33462253	1.69310666	0.24806173	0.46610123	0.13997543
M321T816	0.7366389	1E-18	0.42246133	0.1532734	1E-18	0.04008952	0.14719219	1E-18	0.04627784	0.01443765	0.01925962	0.23889786	0.01036225	1E-18	1E-18	1E-18	1E-18	0.14645187	1E-18	0.32756074	0.00312514	0.01172048	0.24053881
M279T86	1E-18	1E-18	1.75357583	1E-18	1E-18	1E-18	0.04054371	0.0207628	0.05269177	0.03714586	0.01335225	0.04209108	0.01577276	0.18692063	0.07781998	1E-18	0.02850136	0.4804576	0.01707291	0.02790474	0.02827179	0.13617217	0.01431891
M117T182	24.0987462	0.12806481	1.21545086	0.23728466	0.77731271	0.49020194	0.59675473	0.20068044	0.08151513	0.48792467	0.05563296	0.07173057	0.23811865	1.20774582	1.02725971	0.10350389	0.80480393	4.27122095	0.18530068	0.8370523	0.10312278	0.31023813	2.06434807
M351T723	2.77	0.25691856	0.76282322	0.13118391	0.083	0.49605393	0.69517228	0.00742	0.0345648	0.6772747	0.00223	0.08065857	0.0912	0.12671135	0.00972491	0.0477	0.44379165	1.46932379	0.0215189	0.76160183	0.10461203	0.0849	0.29137496
M400T91	1E-18	1E-18	0.4732642	1E-18	1E-18	1E-18	0.1906124	0.07157855	1E-18	1E-18	0.02863485	1E-18	1E-18	1E-18	0.05530415	1E-18	1E-18	0.14655859	0.00594	1E-18	0.08950102	0.02623425	1E-18
M101T251	49.4635052	1.38522282	2.85230488	0.97839726	1.78899975	10.3792685	2.56046472	0.48509788	0.7067481	0.72122033	0.18777465	1.74359757	0.46725909	3.4095526	1.94995887	0.15499346	2.04217953	9.43267264	0.59449874	1.60247678	0.41956967	2.08009068	0.37524743
M361T600	5.176887	0.11664089	0.199	0.0732	0.02067733	0.51986527	0.283	1E-18	0.0149	0.332	0.00680063	1E-18	1E-18	1E-18	1E-18	0.0276	1E-18	0.285	0.02149279	0.15059763	0.0393	1E-18	0.18476242
M209T90	11.1684407	0.06144834	0.40427824	0.23697412	0.09356258	2.17209263	3.83171634	0.21984955	0.21006896	0.12932234	0.01859406	0.15243578	0.3662893	1.32628373	0.1035956	0.04807159	0.93991743	0.36913189	0.27517597	0.10425291	0.01277845	0.14209715	0.29284552
M59T1476	8.42220464	0.44040426	1.26769447	0.43908571	0.39849324	0.55066802	1.23413877	0.06541894	0.27377134	0.38721007	0.16546085	0.26531189	0.28408133	0.94594501	0.29498541	0.1281268	0.88231382	4.75103259	0.3264921	0.82022471	0.17655288	0.55511501	0.43014264
M326T700	1E-18	1E-18	1E-18	1E-18	0.01346372	0.01917546	0.38697148	0.00340502	1E-18	0.09700235	0.00280633	0.01138194	0.0065315	0.05146551	0.00891947	0.00767003	1E-18	0.46028296	0.01834942	1E-18	0.00368294	1E-18	0.05967384
M342T667	4.53749299	0.03110734	0.42355247	0.24101039	0.19766812	0.04255797	0.29366709	0.02616195	0.04135709	0.06703242	0.0044475	0.0580875	0.04483729	1E-18	1E-18	0.01217601	0.21318591	1.4881707	0.03059021	0.40369359	0.02957734	0.02125744	0.28652251
M247T153	0.25821768	1E-18	1.77043557	0.00494	0.29162038	1E-18	1E-18	0.20859742	0.1307115	1E-18	0.0883594	1E-18	1E-18	1.05314452	0.45594187	0.00566068	0.67306985	0.11174093	1E-18	1E-18	0.01176083	0.86222247	1E-18
M283T652	4.3017898	0.181	0.62783157	0.10156184	0.07815589	0.94021921	1.64654325	0.01305656	0.02473642	0.54564916	0.0346	0.07367696	0.05196382	0.0261	1E-18	0.00415	0.10913754	2.39212515	0.0949	0.75449469	0.0801	0.13205715	0.25498837
M120T86	4.12328836	1.13417553	0.79752069	0.06011624	0.06104344	0.08966133	0.29755215	0.16429778	0.07773645	0.94585806	0.02727123	0.0116448	0.11053022	0.27788703	0.05960826	0.13763579	1.48502922	3.02357166	0.02496501	0.22104998	0.04277492	0.05918582	0.55055494
M134T85	1E-18	0.04494306	1E-18	1E-18	0.06957287	0.07156512	0.1383831	0.01472154	0.03556468	0.04839163	0.01306888	1E-18	0.03652145	0.07642811	0.12998265	0.01607827	0.04651336	0.39123799	0.02045498	0.06664216	0.00955964	0.10314127	0.05348404
M227T514	10.2382403	0.25888128	5.98895826	0.25926061	1.55450879	0.99275335	2.59248309	0.70021988	0.03418613	1.20970201	0.02268566	0.07342941	0.8140167	1E-18	2.33826469	0.40829909	0.99727097	33.2925166	0.08761632	2.98195448	0.38804743	5.08845569	0.43952576
M163T79	0.78250687	0.03122349	1E-18	1E-18	0.02683499	0.10598799	0.11851738	1E-18	0.02855355	0.15570286	1E-18	0.02177284	0.03025946	0.18122531	0.25845786	0.07800905	0.19125361	0.85133751	0.01444555	0.16019948	0.00726197	0.04952639	0.05614756
M136T83	1.19703014	0.16663042	0.7756252	0.01978238	0.03759497	0.11435111	0.15696709	0.0248928	0.04623694	0.06099545	0.01871531	1E-18	0.01553908	1E-18	0.0996301	0.02041148	0.09114624	1.87418812	0.05750975	0.37517344	0.01235727	0.16629739	0.05978067
M140T136	11.1393718	0.21687698	0.1535888	0.31077137	0.10360763	1.28384534	0.53042881	0.14813832	0.1747238	0.37836328	0.05483617	0.12622378	0.1039616	0.71172644	0.47445171	0.06641882	0.53719814	3.7692804	0.13817027	0.46179972	0.07627619	0.45349439	0.5558292
M163T78	1E-18	0.01082478	1E-18	1E-18	0.01457013	1E-18	0.04260585	0.00610137	0.00598	0.03031776	1E-18	0.0059102	1E-18	0.03496911	0.05273636	1E-18	1E-18	0.13666462	0.00617701	1E-18	0.00729258	1E-18	0.01395727
M165T258	5.1152315	0.26441745	1.22393441	0.17125587	0.29543696	0.48629248	0.90533749	0.08001293	0.16594652	0.21411406	0.09632105	0.09770137	0.18874679	0.44501769	0.24430886	0.09917807	0.45225438	3.03585571	0.19133953	0.39133187	0.08198516	0.3055479	0.2719067
M337T770	6.82289882	0.68966424	0.12241759	0.45805911	0.08860665	0.3942935	0.71164793	0.01536358	0.02485083	0.05846377	0.0431463	0.05384806	0.00838873	1E-18	0.00801127	1E-18	0.25401769	1.70538284	0.07726477	0.25219606	0.04924946	1E-18	0.50636543
M223T656	6.40830221	0.15655291	3.46932103	0.22404523	3.53485692	1.93223788	4.47689888	0.326911	0.13863629	4.5939528	0.20819097	0.33899631	0.84540854	0.15475608	4.05538714	0.47395577	5.01204295	45.5776602	0.28529237	8.96737381	1.26013573	2.15069449	0.40170477
M343T692	0.91174658	1E-18	1E-18	0.01089655	1E-18	0.08947273	0.03053855	1E-18	0.00628092	0.01800051	1E-18	0.05214079	1E-18	1E-18	0.06701877	1E-18	0.03724407	0.29959928	0.0070765	0.17500126	1E-18	0.15533962	1E-18
M369T785	2.48053887	0.1225923	1.33115797	0.08097593	0.10304062	0.34235922	1.692382	1E-18	0.32299965	0.20730365	0.01142916	0.02173656	0.00982711	0.41371401	1E-18	1E-18	0.06247255	0.13367352	0.15234269	0.33693958	0.19649029	0.01672539	0.46224107
M99T1584	10.7706097	0.45922365	2.1963263	0.34243249	0.48314268	0.70355805	0.79511315	0.15052136	0.22619743	0.36379479	0.12648851	0.18361662	0.15398127	0.86895259	0.21463089	0.12270761	0.58596057	4.51240343	0.15932465	0.66944491	0.12232945	0.62446172	0.38290557
M319T1028	1.18417069	0.06527808	0.50948252	0.03245898	0.09378023	0.14689296	0.13004939	0.01287004	0.02890047	0.02971943	0.03264787	0.02791945	0.03666434	0.24251629	0.06182711	0.01060565	0.12288341	0.84348182	0.03607463	0.07888728	0.01409343	0.04547367	0.08294062
M149T190	12.9684904	0.0425407	1.92470404	0.26080134	0.68688644	0.36456537	0.46256186	0.17498507	0.00619966	0.38184276	0.08751904	0.00473871	0.06705411	0.94701291	3.74817954	0.19853465	0.77280951	2.72083149	0.08293128	0.59105068	0.08982899	0.30936254	1.03761898
M116T352	41.2515759	1.32788616	9.19754358	1.32580706	1.78476641	1.51643778	1.1688007	0.69451178	0.80459279	0.63753572	0.74950294	0.42930821	0.3855149	3.38905305	1.95201281	1.41359948	2.68191041	14.0251932	0.40127253	0.90601389	0.46984013	1.7837837	0.79701302
M174T89	1E-18	0.06015307	2.19132474	1E-18	1E-18	0.07047004	0.05425391	0.0920758	0.02094014	0.05150674	0.00955703	1E-18	0.03172499	1.18775872	0.08335988	1E-18	0.02923659	0.20769229	0.03249983	1E-18	0.03195641	0.2658075	1E-18
M103T445	60.5974318	2.06276958	4.87616131	2.90878451	1.74257448	3.5540328	4.68266213	0.99723842	0.58297813	1.55719486	0.37293837	0.05292068	1.18675832	4.52824189	0.76505971	0.77972004	5.18028209	7.75428508	0.41300548	0.98258384	0.65109615	3.18128081	1.12581598
M312T799	1E-18	1E-18	1E-18	0.12516447	1E-18	1E-18	0.45019386	1E-18	1E-18	0.0682537	1E-18	0.04200986	1E-18	1E-18	1E-18	1E-18	0.1319508	1E-18	1E-18	1E-18	0.0626965	1E-18	0.1652537
M405T843	4.62598083	0.1141221	1.82314935	0.12710274	0.09472517	1E-18	0.25887824	1E-18	0.00519488	0.02296291	0.02638491	0.05813114	0.16917778	1E-18	1E-18	0.07822584	0.24331966	0.36865573	1E-18	0.13336807	0.02576069	1E-18	0.19564305
M242T128	7.21227138	1E-18	1.59511268	0.00578901	1E-18	0.02099755	1E-18	0.11485867	1E-18	0.01117131	0.06835942	1E-18	1E-18	0.28396783	0.13661712	1E-18	0.15259812	1E-18	1E-18	0.02147491	0.05121038	0.2713192	1E-18
M385T641	11.4425532	0.20481902	1.0926447	0.04171174	0.16920177	1.09157119	0.79474561	0.06711538	0.08552129	0.59644395	0.02667085	0.06206435	0.08012413	0.02820272	0.06089499	0.04755366	0.30509523	2.03189912	0.16124233	0.96182437	0.07819022	0.05207155	0.34978498
M403T732	1E-18	0.01057629	1E-18	0.07454739	1E-18	0.07123699	1E-18	0.00480075	0.23523921	0.02093525	0.04303115	0.08138074	0.06573661	0.13151597	0.3194999	1E-18	0.12050466	1E-18	0.12969812	0.17822381	0.11025724	1E-18	0.06362206
M213T156	5.98858928	0.03676058	2.18213277	0.06685465	0.04676215	0.58288618	0.19809684	0.09079111	0.0989735	0.08195995	0.04879342	0.03035412	1E-18	0.12778779	0.24092817	0.11383074	0.59210555	1.55410151	1E-18	0.22998274	0.02828729	0.07669525	0.26378837
M337T791	1E-18	0.01273126	1E-18	0.00553777	0.01518266	0.06191989	0.03477607	1E-18	0.00912918	0.05594592	1E-18	0.00946806	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.07979894	1E-18	1E-18	0.03605343
M87T750	24.7049926	1.17671456	13.1219098	0.62195233	3.58352134	2.82268191	3.72160103	0.27766613	0.92022772	1.80485531	0.47362697	0.48225694	0.89300535	4.12812388	0.75721836	0.48866568	6.87445557	17.5755138	1.66320233	10.619796	0.24526547	1.03171895	1.03536444
M317T88	1E-18	1E-18	0.7542339	1E-18	1E-18	0.03263833	1E-18	0.18048785	0.02153756	0.02467988	0.02514964	0.0252582	0.04065343	1E-18	0.09355667	1E-18	0.02553221	1E-18	0.03769078	1E-18	0.04085193	0.10944765	1E-18
M68T1574	20.0228226	1.05827911	5.41642246	0.34887968	1.17251604	1.26447127	2.081306	0.3457091	0.28053442	0.60632595	0.19307265	0.3664938	0.35383725	1.62992104	0.51441749	0.195365	1.34261959	11.7658358	0.28448517	0.99312493	0.20227735	0.84048836	0.68604686
M337T760	7.71265367	0.92097192	0.05794791	0.72258096	0.15797256	3.25993461	4.35319129	0.01536007	0.02068459	1.24728602	0.05101126	0.08976311	0.04035964	1E-18	0.02768745	0.02594889	0.68692845	2.79888724	0.1473206	1.63562135	0.179471	0.07292307	0.7525344
M159T93	1E-18	0.05381749	1E-18	1E-18	1E-18	0.10018928	1E-18	1E-18	1E-18	0.0318397	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.01741513	1E-18	0.00505575	1E-18	1E-18	1E-18	0.02094823
M127T91	3.68220846	0.12033604	0.67024488	1E-18	0.04454193	0.97492894	0.08200086	0.0213782	0.01690931	0.85987363	0.03671208	0.01047619	0.03368847	0.07746711	0.04873876	0.27943184	0.5156809	0.60536199	0.02138088	0.11338886	0.05377387	0.05977665	0.55705336
M291T799	1E-18	1E-18	0.292	0.01090004	0.0143648	0.04176952	0.09905688	1E-18	0.03965063	0.01900244	0.0161104	0.00430903	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.01652046	1E-18	1E-18	0.23256158
M215T604	10.2331317	0.42735264	7.19403889	0.2637229	0.76681104	0.18280716	3.18246525	1.69728425	0.0891507	1.38504838	0.11706108	0.09757415	2.78271875	0.73716339	1.95213231	0.25693578	0.43094619	15.8374002	0.04421506	5.36193293	0.54481093	1.15465671	1.68894241
M225T153	0.23464361	1E-18	0.1490183	1E-18	1E-18	1E-18	1E-18	0.00834992	0.00493646	0.00764738	1E-18	1E-18	1E-18	0.20932119	0.41368111	0.00279094	0.14497711	0.13883789	1E-18	1E-18	1E-18	0.0505258	1E-18
M156T89	0.42594565	0.06449851	0.34918889	1E-18	0.16296514	0.15643136	0.17164692	0.05176544	0.02489458	0.06161926	0.02348134	0.02387524	0.05623131	0.04703434	0.11399082	0.05491527	0.13611402	0.99764586	0.03485485	1E-18	0.05623915	0.16708324	0.01232526
M177T96	1.38460776	0.20221772	0.24906237	0.09313347	0.31559562	0.68607547	0.12091438	0.03394493	0.07929272	0.09532113	0.10369619	1E-18	0.70688027	0.74617096	1.31567629	0.01474543	0.093168	1E-18	0.02701789	0.47510896	0.03371498	0.6691776	0.0665343
M237T462	7.01948658	0.22474049	1.44666715	0.08581351	0.17928369	0.22306728	0.45432868	0.04260432	0.02514168	0.02357162	0.01357257	0.0068282	0.06380787	0.02488577	0.09672481	0.00898558	0.01883565	0.60636641	1E-18	0.04719932	0.00359381	0.21167272	1E-18
M357T767	0.28419897	1E-18	1E-18	0.21052679	1E-18	0.11374026	1E-18	0.00362	1E-18	0.04491772	0.0155	1E-18	0.00656	1E-18	1E-18	0.00189349	1E-18	1E-18	1E-18	0.33720926	0.01320564	1E-18	0.30006154
M147T84	12.5775105	0.03514964	0.99474326	0.02281684	0.16052659	0.09539058	1.89322668	0.06020915	0.08084828	0.13694674	0.03177221	0.07374819	0.05800476	3.40112905	0.03269457	0.01014118	0.48557441	0.51597484	0.02319519	0.15476611	0.02581501	0.07412549	0.07534568
M199T584	2.03553219	1E-18	0.97985077	0.10241252	0.30213186	0.38893415	0.07106022	0.39962004	0.01210195	0.7889635	0.03628509	0.05126287	0.06715616	0.13177379	10.0706703	0.45030587	0.40412681	0.66704354	0.01256995	4.94666665	0.87169521	0.7394979	0.26710415
M281T1080	1E-18	1E-18	0.0454	1E-18	0.0144	0.02061291	1E-18	0.00326	0.00942433	1E-18	0.0024093	1E-18	1E-18	0.0183	0.0118	0.00301	0.0162	1E-18	1E-18	0.00708556	1E-18	0.01488954	1E-18
M263T90	1.96919159	1E-18	0.32396154	1E-18	0.01543008	1E-18	0.12452624	0.05609471	0.06838171	0.07109904	0.03272247	0.03651156	0.06347421	1E-18	0.01841793	1E-18	1E-18	1.02463689	0.04873725	1E-18	0.02125113	0.41740694	0.01537179
M62T229	329.419472	3.47906112	10.6841579	3.17237393	1.74076026	1.75516494	9.49559207	2.31534826	0.90533261	3.65414802	0.59413961	2.60932049	0.63807847	5.32339126	19.3812586	2.42424541	4.37251063	18.2617112	4.89961873	11.3578017	0.86645003	10.3374377	2.09067364
M241T487	62.9320956	3.05776833	23.4566312	3.45344239	3.34865593	4.42630206	21.4417498	3.12129655	0.34506805	5.39413603	1.04695323	0.40044248	0.70362723	2.2473417	7.235454	0.64937781	40.1133102	267.452843	0.16944404	3.48702747	1.45286114	5.75027342	7.15984409
M331T654	6.40778789	0.15801915	0.19144994	0.22851664	0.13367642	0.80939217	1.17913597	0.04935717	0.07984004	0.45894566	0.04003493	0.14053547	0.17252735	0.0283881	0.01832253	0.05098044	0.74262102	6.47419238	0.20797446	1.38286372	0.17050211	0.30810855	0.72507512
M223T667	0.4552308	0.32196662	4.31911752	0.11970742	4.41565059	2.48458769	5.37331314	1.74929991	0.16362838	5.03290851	0.25602672	0.41721396	1.01497399	0.18078055	42.9838582	0.13357411	6.21784113	55.1459767	0.35080722	9.88130105	1.60528486	2.533916	0.47539289
M385T893	1E-18	0.07759578	0.64623793	0.24038643	1E-18	0.50199735	1E-18	1E-18	0.20133916	1E-18	0.03870077	0.10657226	1E-18	0.32248244	1E-18	1E-18	1E-18	1E-18	0.21041264	1E-18	1E-18	1E-18	0.22640308
M135T1541	0.27449372	2.34607152	12.4593778	1E-18	2.95486894	2.8805686	5.15976108	0.59972637	1.15309012	1.33100437	0.27338657	1.31887305	1E-18	12.7980232	0.00764284	1E-18	1E-18	1E-18	1E-18	1.7226978	0.44292808	1.89832214	1.21108997
M122T344	8.95286532	0.67789476	2.42201813	0.62697655	2.73296073	0.84560885	3.15523907	0.23321096	0.42873758	1.66330733	0.12727767	0.26983536	0.46594975	1.13435647	0.5229288	0.45596478	0.9859779	21.8527188	0.88076247	1.51888268	0.55059633	2.58017198	1.19775263
M319T1007	5.85744822	0.24428795	1.38660009	0.121353	0.10578696	0.31196764	0.60474096	0.0715136	0.03832364	0.19478627	0.07494827	0.03453178	0.14818464	0.40072528	0.269568	0.02922882	0.47321731	0.7039354	0.1022451	0.09434723	0.05181339	0.30977093	0.2266796
M336T775	0.28677235	0.18256529	1E-18	0.26629231	1E-18	1.07411385	0.56263893	1E-18	0.05890446	0.02892129	0.03141633	1E-18	1E-18	0.04018067	1E-18	1E-18	0.02562524	1E-18	0.17675973	0.27357261	0.00583643	1E-18	0.30767943
M75T483	54.7204821	1.48844839	5.48351703	0.75177196	9.321823	2.46322027	2.5856197	0.34871534	0.57135174	4.17875708	0.19399711	0.73461192	2.33296512	4.8683924	1.88264968	0.28661571	3.69873292	19.8674279	1.24229056	2.31338919	0.59539103	2.42104137	0.70522408
M283T1467	1.68719891	0.05944524	0.43658791	0.03386865	0.09956601	0.21280691	0.03821568	0.03569933	0.03544579	0.04494622	0.01092546	0.01625701	1E-18	0.07084253	0.013	0.01060376	0.01788761	0.83083176	0.0185361	0.07940991	1E-18	0.04472157	1E-18
M305T577	1E-18	0.0303359	0.16848708	1E-18	1E-18	0.01785378	0.21182287	1E-18	1E-18	0.01300775	1E-18	1E-18	0.01143466	0.022262	1E-18	1E-18	1E-18	0.16684839	0.00616373	0.0318954	0.00394341	1E-18	0.05506033
M321T770	10.0078803	0.14360845	0.14021784	0.23613578	0.08802561	0.24040741	0.85559131	0.01535003	0.24124222	0.09473932	0.00248508	0.07270292	0.03627129	1E-18	0.2728525	0.03407871	0.02407208	0.85352103	0.52232694	0.89366531	0.00635874	0.03050301	0.37013218
M321T766	13.6163212	0.14359664	0.14023546	0.13024763	0.08804846	1.37705642	0.92327821	0.0153513	0.25968646	0.10840142	0.00248519	0.1505427	0.03626127	1E-18	0.30473367	0.0341043	0.0240701	1.08462299	0.3262486	1.0455907	0.00635722	0.03049575	0.44082598
M130T140	3.66776961	1E-18	0.62245327	0.08778473	0.26507707	0.54996187	0.06000188	0.36612149	0.02924855	0.0078966	0.01171761	1E-18	1E-18	0.70628406	0.60758005	0.02418615	1.02794583	1E-18	1E-18	0.0399693	0.0447396	0.0132977	1E-18
M120T59	56.5729143	0.55417952	1.77555875	0.54010172	0.82107951	0.5995506	1.52576002	0.43754862	0.15299987	2.08836641	0.14552046	1.2205397	0.09452155	2.23403811	0.74533047	0.09881511	1.38861719	6.0585496	0.74160024	0.64537847	0.48426561	1.10101298	0.48786987
M381T81	1E-18	1E-18	1E-18	0.0722	1E-18	1E-18	0.293	0.0189	1E-18	0.42452912	1E-18	0.82317403	0.02847813	1E-18	0.0125	1E-18	0.05003405	1.03541972	1E-18	0.28014335	0.22566604	0.109	0.87397234
M120T85	1.59359673	0.07152558	0.57813674	0.02581116	0.2201908	0.16254278	0.12665662	0.03526846	0.0305017	0.11847382	0.01468341	1E-18	0.03555088	0.02555084	0.07088404	0.01160968	0.07175511	1.62371205	0.01745587	0.28626487	0.0035981	0.14693697	0.18202379
M181T607	26.9846277	2.83689751	25.3834755	0.33650859	5.41828618	1.3915869	5.27281122	0.97307365	1.58663654	1.83612156	0.62552014	1.40676714	1.59416632	1.61934247	139.110029	0.07351743	2.31719022	632.044889	0.47923453	8.94345057	0.39113788	7.14213896	0.84692235
M165T933	11.6149552	0.44251054	2.60627426	0.26796659	0.83675132	0.829936	1.28865543	0.10427956	0.21831012	0.46315801	0.09921123	0.25621092	0.36300129	1.30493838	0.3733132	0.15009973	0.7260433	6.64631532	0.29997935	0.68426997	0.14461208	0.67002665	0.41898466
M133T138	86.3268989	0.300698	1.31701418	0.49250918	1.50806312	0.76918173	0.06017658	0.02430295	0.11612245	1.23630052	0.06647318	0.10875529	0.03652174	1.78432693	0.33801976	0.18008601	1.97851464	12.7514363	0.14149307	1.7865791	0.15733413	1.11200264	2.02736804
M179T87	1.01545173	0.02303985	0.07840688	0.01116743	0.0538753	0.3097786	0.0811577	0.0095881	1E-18	0.0653041	0.02331681	0.0117695	0.01677664	0.08358424	0.03476772	0.01891521	0.17409371	0.75061	0.01357259	0.0792571	0.0145299	0.09174121	0.08385581
M136T89	1E-18	0.04175005	0.33450193	0.00553459	0.10641048	0.10516661	0.05811342	0.06147909	0.02664164	0.06518409	0.01468294	1E-18	0.0212873	0.02810532	0.07499544	0.01878427	0.09452267	1E-18	1E-18	1E-18	0.01611933	0.08708057	0.07042427
M140T1518	2.07917068	0.04596204	0.10725901	0.01752735	0.01862437	0.09915591	0.08985204	0.02128277	0.00772244	0.03629405	0.00775667	0.01765702	0.03029605	0.04629249	0.02069367	0.02407352	0.01956302	0.2137194	0.02892619	1E-18	0.01170167	0.03514169	0.04631645
M221T89	1.29359766	0.05545757	0.36601431	0.0108928	0.06883702	0.03579232	0.24556103	0.01950192	0.0477422	0.03948614	0.02296522	0.02551858	0.01531759	0.03695825	0.11980336	1E-18	0.10814671	0.60051049	0.04576991	1E-18	0.01710232	0.22815344	1E-18
M80T296	1.10058424	0.04618421	0.11152223	0.02077376	0.02785782	0.10961108	0.10198161	0.01529372	0.01104629	0.02412775	0.00223489	0.01312267	0.02026658	0.03960139	0.01890978	0.0102613	0.05237891	0.35711504	0.00616115	0.06836159	0.01440801	0.03603021	0.01014095
M319T1574	3.549905	0.05862246	0.96907676	0.03207937	0.10454517	0.15477509	0.39523119	0.03617292	0.0550157	0.12895383	0.04643206	0.07120138	0.06453182	0.29146713	0.14595997	0.03189767	0.30295563	2.20542635	0.05652706	0.20305761	0.03169678	0.18864996	0.15160304
M361T518	0.29997419	0.13801178	1E-18	1E-18	0.0332	0.13846002	1E-18	1E-18	1E-18	1E-18	0.0623	0.0303	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.0338	0.01900944	1E-18	1E-18	0.04531086
M219T692	4.83429557	0.09098994	0.44310822	0.04708972	0.07288316	0.09827621	0.26696755	0.02129813	0.00327959	0.17758419	0.0131134	0.03633709	0.07438541	1E-18	2.19033194	0.10131258	0.10094891	1.75296149	0.03651071	0.59964795	0.04352339	0.09765744	0.0409957
M309T765	9.17226563	0.84010352	3.35726286	0.98018798	0.06642068	2.56558216	0.59752056	0.11981061	0.18644999	1.34725056	0.09678255	0.23308062	0.41292517	0.0206574	0.25041479	0.02227873	1.53286508	4.41253537	0.04179182	1.45185669	0.13409426	0.04913118	1.01976991
M297T982	1E-18	0.01800889	1E-18	1E-18	1E-18	0.28718864	0.15041967	1E-18	0.00568054	0.0332206	0.00569765	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.05275783	0.02398681	1E-18	0.01307168
M244T88	1E-18	1E-18	0.35862241	1E-18	0.02070028	0.03200124	0.26587418	0.01562636	1E-18	0.11776872	0.02154081	1E-18	1E-18	0.0487108	0.12711318	1E-18	1E-18	0.22293128	1E-18	1E-18	0.01933424	0.08123633	0.04137972
M135T85	2.66440481	0.09763499	10.4568177	0.04129164	0.08096714	0.13390044	0.03667025	0.01787353	0.18581643	0.05967726	0.08975263	0.0445444	0.06385527	4.21291074	0.07572548	0.01227677	0.86243883	1.17593949	0.49353433	0.11123268	0.03693981	0.02818946	0.10628782
M197T85	1.85912991	0.07846728	0.22154191	0.07352469	1E-18	0.05990473	0.56283277	0.07074665	0.00578521	0.09790813	0.01442649	0.05184621	0.08289253	0.1085778	1E-18	1E-18	0.09844636	1.25884282	1E-18	0.04984437	0.0147918	0.17789682	0.08238666
M165T587	6.31600897	0.08658807	1.73148238	0.01468847	0.1621755	0.32367894	0.46291672	0.06427321	0.08101698	0.22731586	0.07305589	0.16490244	0.19407493	0.28548122	0.19596318	0.1464134	0.22180736	2.19336987	0.02062981	0.55956901	0.07384838	0.19352371	0.38548101
M92T66	9.85519145	0.65015197	1.19952238	0.32594034	0.8495944	0.75995949	0.45884733	0.14728287	0.26246303	0.56281341	0.00658879	0.05064937	0.45903392	0.39837229	0.35066016	0.23496922	0.34860894	3.2778959	0.07215882	1.92485273	0.13192689	0.19316874	0.20522465
M367T654	0.32898391	0.184	0.05739334	0.15560787	1E-18	0.52779497	0.10241527	0.00374811	0.02166221	0.16328132	1E-18	0.10291501	0.00987144	1E-18	1E-18	1E-18	0.101	0.99923491	0.17161484	0.8754721	0.11297268	0.02670771	0.27660421
M251T568	6.18255143	0.22339776	0.74112452	0.21257842	0.37006521	0.61909544	0.79162275	0.12276816	0.01452234	0.34036815	0.0050631	0.04677759	0.26916834	1E-18	1.49886463	0.08296586	0.14677992	5.85555451	0.03953359	1.41509492	0.22384665	0.223922	0.26221223
M93T84	4.26526716	0.15216315	22.2644901	0.59537527	0.09166379	0.60465312	0.32941815	1.21864493	0.11806442	0.68172234	0.38942562	0.07651729	0.10069629	4.20457784	0.2406552	0.03612506	0.21877667	1.93143317	0.14444351	0.23678948	2.05308648	0.13638143	0.5691223
M116T426	20.8948328	1.92091512	57.4337934	6.30534242	1.65166808	3.85149003	3.94100102	1.52664293	1.19701453	2.26789263	0.5057992	0.28382017	0.9447969	1.75874083	1.77731137	0.45668843	1.76362404	31.2919689	0.68921249	1.88527468	0.43793651	3.4286331	2.27158139
M319T1420	1.92058855	0.06317877	0.55161212	0.029438	0.09258866	0.07557684	0.19725472	0.02543911	0.02960586	0.05347838	0.01582438	0.02196485	0.03799416	0.15981263	0.08857461	0.02339045	0.06511904	1.12568177	0.03918221	0.05561463	0.01861803	0.05996846	0.05934731
M379T791	5.93242794	1E-18	1.25	0.04891811	0.296	1E-18	0.0991	1E-18	0.03616371	0.09475002	1E-18	1E-18	0.08203333	1E-18	1E-18	0.01453447	0.42632952	3.3713985	1E-18	0.67018492	0.0517209	1E-18	0.244
M291T85	1.23	0.0492	0.507	0.0279	0.233	0.294	0.417	0.04388336	0.02648006	0.16853651	0.0862296	0.03468261	0.02603634	0.111	0.0787	0.0129	0.0956	3.84832962	0.0342	0.18863016	0.06703687	0.19	0.01571902
M310T89	1.20118516	0.05761969	0.67737691	0.02877958	0.06833007	0.10049308	0.2399267	0.03835102	0.03364364	0.10526869	0.04512074	0.03605655	0.03899814	0.12526455	0.06272442	0.02128389	0.08500756	0.7957649	0.03187566	0.07369513	0.0266948	0.09598961	0.05193593
M224T617	1E-18	1E-18	0.10481908	0.01388627	0.13840715	0.27389909	0.08008343	0.05189298	1E-18	0.17754529	1E-18	1E-18	1E-18	0.02278709	1.71362808	0.092686	0.1108836	1.29935414	0.0058345	0.38830643	0.09494901	0.03184242	0.03441084
M155T84	3.61508564	0.04239888	2.25212029	1E-18	0.11537533	0.03116831	0.23039951	0.02482229	0.16706226	0.03544673	0.02550447	0.03168132	0.08527221	0.44047386	0.14237146	0.03263495	0.14296212	0.58085079	0.03074344	0.16064182	0.00885962	0.12319467	0.02657064
M331T656	6.1474786	0.17976659	0.19146782	0.26107315	0.13362186	0.94189837	1.22015699	0.05335629	0.07981674	0.51083173	0.04628603	0.08836011	0.17258366	0.02839804	0.0183176	0.05095489	0.74270762	6.47419238	0.2307744	1.46339994	0.18098267	0.30799353	0.76420418
M80T737	4.16626389	0.14698474	0.78858618	0.07643644	0.21425101	0.28300325	0.49691923	0.05377062	0.08735075	0.15075106	0.03881712	0.06906735	0.09327023	0.38946152	0.18824882	0.04769512	0.27772677	2.23955071	0.03546259	0.26697994	0.0452207	0.20563667	0.1399733
M348T806	1.55876177	1E-18	1E-18	1E-18	0.01470675	1E-18	0.27327297	1E-18	1E-18	0.05417306	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.010474	1E-18	0.18990699
M365T617	2.18	0.032	0.06483649	0.16176981	0.074	0.87673522	0.14979657	0.02292612	0.10148241	0.29587189	0.00789	0.0785	0.07524907	1E-18	0.22631729	0.040111	0.284	2.34364942	0.099	0.71386801	0.12163698	0.13494554	0.45358469
M349T640	18.6734607	0.24385654	2.62617756	0.03126603	0.34651866	2.40509002	2.79665011	0.15743513	0.266	1.59457347	0.08176374	0.20059455	0.234	0.239	0.226	0.0107	0.99006581	7.9640263	0.34449669	2.00396612	0.255	0.38389111	0.96742399
M253T1576	1E-18	0.01037024	1E-18	0.00528298	0.05314997	0.05323855	0.06379661	1E-18	1E-18	0.04017332	1E-18	1E-18	0.01668158	1E-18	1E-18	1E-18	1E-18	0.30805989	1E-18	0.13658595	0.01139973	1E-18	0.02413924
M87T629	48.8144456	2.93896126	37.205433	2.08836812	1.96966259	0.53988211	5.27005432	0.18964587	0.86222418	1.60293361	0.1495824	1.10071015	0.63970178	2.41631336	1.07500322	1.21348589	1.71975854	47.6571732	1.07362071	1.4788763	0.55804823	1.5884222	1.44598873
M346T713	0.32062826	1E-18	1E-18	0.07644707	0.01985023	0.20040878	0.0389915	0.0029066	1E-18	0.01060164	0.03469088	0.00546981	1E-18	1E-18	0.03495734	0.003382	0.01728665	0.13633947	1E-18	0.06125787	1E-18	1E-18	0.17241444
M165T222	6.44531635	0.4098059	0.29235032	0.01699414	0.95398551	0.32654182	1.03688474	0.07999971	0.1114781	0.19985876	0.17134895	0.13069076	0.30976629	0.46150273	0.6669941	0.28931554	1.0225889	10.6256711	0.12183255	1.20074025	0.00802607	0.41474706	0.36475672
M369T728	40.6	0.493	5.95	1.45643591	1.69	1.55588587	3.31	0.40815749	0.07975781	0.91160215	0.31	0.48283139	0.53901269	0.0679	0.08047665	0.17617739	2.40885171	12.7078085	0.11203221	4.86612693	0.41301449	1.08401712	0.80374
M172T86	2.05637172	0.05152594	1E-18	0.04706114	0.17449875	0.533593	0.18875142	0.10936109	0.07004318	0.19339279	0.01259596	0.01103855	0.02246864	0.02172232	0.0239433	0.06685283	0.06351464	0.62160859	0.01445939	0.10277899	0.09154349	0.37532406	0.04860969
M213T89	2.52708949	0.0291295	3.55295316	1E-18	0.25715711	0.29693689	0.31420439	0.19463548	0.028031	0.33899271	0.04827205	0.02879429	0.05254865	0.30639707	0.24275874	0.03110999	0.06342632	0.58640628	0.01859214	0.13491896	0.03853967	0.27786306	0.0611165
M336T762	1.38802169	0.35023589	0.05335543	0.25790869	1E-18	0.15976522	0.83217076	1E-18	0.00565946	0.03282902	1E-18	1E-18	1E-18	0.02000553	1E-18	1E-18	0.02997396	1E-18	1E-18	0.12198194	0.00265941	1E-18	0.02459762
M236T91	0.63570718	0.04587616	1E-18	1E-18	1E-18	0.0185183	1E-18	0.06447585	1E-18	0.2130672	0.00810782	1E-18	1E-18	1E-18	0.02949451	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.09577967	0.11334636
M325T540	3.81975099	0.02648751	0.22593317	0.09857374	0.05034168	0.55899114	0.35647271	1E-18	1E-18	0.14015708	0.0254618	0.06973836	1E-18	1E-18	1E-18	0.01131989	1E-18	0.91354112	0.29018943	0.35510464	0.00482488	1E-18	0.14200909
M370T785	1E-18	0.05347739	1E-18	0.04013626	1E-18	1E-18	1E-18	1E-18	0.04621034	0.20202749	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.06159857	0.04529208	1E-18	1E-18	0.16218828
M297T610	1E-18	0.01463793	0.653	0.02882933	0.0498	0.08206571	0.629	0.0143	0.0931	0.02118395	0.01214068	0.0103	1E-18	0.177	1E-18	1E-18	1E-18	1E-18	0.0194	0.0268	0.02311487	1E-18	0.0142
M73T62	5.21220203	0.03736536	3.05124047	0.23288361	0.38398315	0.82186726	0.34183255	0.05744084	0.14134676	0.34284253	0.01843547	0.20124837	1E-18	0.6400278	0.42253007	0.01183657	0.24300801	0.87904346	0.02334943	0.07869248	0.0806998	0.16860258	0.63487406
M319T776	5.2681168	0.20737177	1.37291645	0.11654756	0.1813521	0.33491257	0.63281154	0.01718878	0.08622327	0.22001304	0.01600595	0.10323357	0.03114701	0.12627352	0.17436572	0.09238614	0.04129643	2.97619965	0.1259833	0.32149074	0.04321643	0.21654874	0.24509765
M292T738	1E-18	1E-18	1E-18	0.00531818	1E-18	1E-18	0.036649	0.00329	1E-18	0.01014435	1E-18	1E-18	1E-18	0.0184	1E-18	0.00299	1E-18	1E-18	0.00511	0.0195283	0.00307	1E-18	0.02902733
M137T161	17.6319818	0.11693477	2.23450049	0.75494714	0.68137853	0.31436579	1.13924521	0.18498084	0.07663887	0.87919375	0.090982	0.15769197	0.02499718	1.40784423	1.88512518	0.0990602	0.7679908	4.96769681	0.27969271	0.77397421	0.11796784	0.44662795	1.9528637
M371T777	1E-18	0.08951149	0.15606829	0.26343156	0.0153599	0.7340403	0.39778584	0.00960613	0.10285071	0.07750005	0.02934541	0.05111729	0.03901919	0.37069001	0.01069909	1E-18	0.08445268	0.62328481	0.09693621	1.24130984	0.05005717	0.01684259	0.18739281
M307T611	15.121635	1.36861589	1.70597006	0.58036458	0.31679171	2.58673392	4.24574235	0.10155997	0.23067278	1.25852362	0.05942521	0.08382277	0.16230483	0.11598652	0.01697803	0.07187914	0.83829999	9.24579138	0.18946505	2.94039833	0.29513955	0.25702252	0.76269458
M317T793	2.94248244	0.31567861	0.52917612	0.21024997	0.16794068	0.44903023	0.85848827	0.03716696	0.09531994	0.66642123	0.03960915	0.07438681	0.07241487	1E-18	0.07785018	0.01246809	0.39028057	2.74911371	0.1174793	1.57064056	0.12963352	0.15312759	0.42851643
M317T787	2.94248244	0.31567861	0.52917612	0.21024997	0.16794068	0.23077476	0.85848827	0.03716696	0.09531994	0.66642123	0.03960915	0.07438681	0.07241487	1E-18	0.04308677	0.01246809	0.39028057	2.74911371	0.1174793	1.57064056	0.12963352	0.15312759	0.42851643
M359T605	4.39284201	0.118	1E-18	0.09978385	1E-18	0.36364196	0.382	0.00453409	0.0291	0.11606011	0.01429777	1E-18	0.00716178	1E-18	1E-18	0.00916538	0.126	0.288	0.0418	0.02999991	0.03113108	1E-18	0.37332925
M169T60	1E-18	1E-18	0.17460084	0.02393537	0.04637853	0.23849301	0.09585241	0.03767993	0.0399096	0.09172613	1E-18	0.01528037	1E-18	0.15474043	0.11249397	0.0057126	0.06391965	0.14099303	0.00679231	1E-18	0.02118588	0.08541603	0.08937175
M159T89	0.68000664	0.03081833	1.25708897	1E-18	0.02555744	0.10799469	1E-18	0.08623313	0.02855079	0.06307103	0.00840218	0.01908826	0.0083811	0.2030752	0.07387922	0.01864758	0.06869152	0.35178819	0.08506044	1E-18	0.10002789	0.0633217	1E-18
M389T669	1E-18	0.24029073	0.06354553	0.11783569	1E-18	0.02375129	1E-18	1E-18	1E-18	0.05785122	1E-18	0.005275	0.00838551	0.0205	1E-18	0.04497055	0.08158386	0.55275273	1E-18	0.05478523	0.00365694	1E-18	0.19436841
M163T86	1E-18	0.06293525	2.4195686	0.04575278	0.07361125	0.06541555	0.15785972	0.07963271	0.02092	0.11978176	0.01266209	0.02422316	0.02207774	0.24890612	0.25486506	0.01998142	0.28572922	0.42269332	0.03500691	0.14330782	0.05835789	0.19999868	0.03952939
M135T1335	11.2773264	0.65425442	4.64818869	0.49706993	2.36977288	1.82814085	2.08553812	0.34864817	0.50369248	0.7852089	0.25493997	0.22226672	0.43962211	1.71456306	0.36851649	0.20668904	0.9331841	9.04644258	0.28211191	1.10466496	0.17048781	1.12874015	0.79502929
M111T86	0.31598858	0.50921701	0.46691817	0.03102232	0.12898623	0.32514528	0.30965599	0.1145342	0.05415053	0.14937501	0.06310839	0.01692722	0.08004253	0.44455082	0.2850293	0.05944171	0.37387908	3.00574113	0.08952049	0.3429908	0.13934649	0.13478549	0.09291478
M293T771	2.21443887	0.03807962	0.74102116	0.01383207	1E-18	0.56422627	0.07001619	1E-18	0.02432225	0.02912504	0.0048843	1E-18	0.01270174	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.2588642	1E-18	1E-18	0.03044736
M191T88	1.53008027	0.13022252	0.40474228	0.03359952	0.08436376	1.06690089	0.21275071	0.0234968	0.02856504	0.26375296	0.01137426	0.02680066	0.04078524	0.41111809	0.21141991	0.0237346	0.07016752	2.17941658	0.19711012	0.05190675	0.339942	0.06262641	0.18941967
M353T597	0.486	1E-18	1E-18	0.05004187	1E-18	0.29893456	1E-18	0.0038	1E-18	0.01396561	1E-18	1E-18	1E-18	1E-18	0.01019294	1E-18	1E-18	0.17229832	1E-18	1E-18	1E-18	1E-18	0.01169318
M165T1422	1.38867793	0.17264569	2.08895691	0.13291057	0.57315369	0.1832363	0.98488071	0.01517863	0.19252991	0.08966853	0.09892642	0.11418457	0.24019145	0.08246774	0.42468335	0.05259575	0.73510819	1.15843819	0.09531747	0.46476761	0.02018387	0.17116511	0.17584797
M221T82	1.48454788	0.05843909	3.73752467	0.01718281	0.09761313	0.10227756	0.22442031	0.00900305	0.06903805	0.06980788	0.04900936	0.06623587	0.04812252	0.14202391	0.18171283	0.00817779	0.15956715	0.55364607	0.02931731	0.07087623	0.02535261	0.14295563	0.06737902
M239T607	30.4229259	0.79169254	11.3050193	0.90481645	1.91747206	1.60856883	3.74149972	0.9555878	0.19779916	2.38384359	0.19655415	0.38384341	2.58386211	2.8861293	2.85667683	0.72522699	6.18948356	22.3375106	0.24928386	3.11785893	1.04657139	5.82310766	0.09214973
M385T963	1.06	0.00957517	1E-18	1E-18	0.27454661	1E-18	0.70838281	0.0996139	1E-18	0.32616158	1E-18	0.0227026	0.10871288	1E-18	1E-18	0.06773778	0.4716065	0.77363684	1E-18	0.24540607	0.22275686	0.49638132	0.03748046
M373T730	7.96266238	0.82744877	1.29302501	0.86048795	1.00292291	0.43461412	6.13856562	0.15695171	0.33340918	1.12726056	0.08248726	0.1379651	0.15389002	0.46128372	0.28712904	0.05697607	1.45141112	7.01837092	0.3159761	2.17057621	0.22236828	0.75535087	1.68336429
M155T1529	55.6013944	1.8999383	12.182551	0.8149824	3.0778963	2.47438097	4.49235035	0.75779837	0.85385946	1.45860659	0.45984219	0.73505128	1.06059786	4.51743826	1.84612484	0.92509356	2.41135215	22.1882584	0.51301618	1.90259006	0.31373857	1.89496105	1.57814963
M383T857	1E-18	0.09745805	0.44107975	0.07820358	1E-18	0.11796201	0.44914865	1E-18	0.0929901	0.04405588	0.05473304	0.02349885	0.06748627	0.14306544	1E-18	0.0257705	0.39211659	0.9978098	1E-18	0.43397917	0.02771732	1E-18	0.24410785
M367T759	0.91343009	0.01099897	1E-18	0.04179968	0.09899575	0.04609744	0.03398888	0.01139723	0.03499892	0.07332689	1E-18	1E-18	0.04377019	0.02160762	0.0184	1E-18	0.5694044	1.06110268	1E-18	0.43877088	0.05529515	0.01257068	1E-18
M183T656	10.4428699	0.31370639	3.13112999	0.54346656	0.72639593	1.23804954	1.94726128	0.22643926	0.40236349	1.08886725	0.14789459	0.50245237	0.64200407	0.75999209	1.98770828	0.19799264	1.30738596	14.1044935	0.47415352	2.70810801	0.41937046	0.8639802	1.09604281
M260T89	0.54944392	0.07800423	1E-18	0.0152658	1E-18	0.08342901	0.2176485	0.00390485	0.00572912	0.0187731	1E-18	1E-18	1E-18	1E-18	0.00959363	1E-18	1E-18	0.34075649	1E-18	0.130254	1E-18	1E-18	0.06078035
M697T1575	1E-18	0.15675457	0.9469978	0.0794033	0.05725129	1E-18	0.08283013	0.07932465	0.08702514	1E-18	0.01167529	1E-18	1E-18	1E-18	1E-18	1E-18	0.06523492	0.74814156	1E-18	1E-18	1E-18	1E-18	0.01015972
M371T723	38.1133796	2.97183989	6.47673349	3.18704072	3.87638888	0.20927792	22.0230583	0.63048589	0.03092078	4.21645275	0.35095534	0.61729236	0.76821807	1.78605367	0.12465974	0.25798419	5.46653711	25.2063565	1.39555823	8.36706359	0.87037813	2.83117166	5.88875978
M385T687	2.78	0.0976	1.16	0.52941945	0.0512	0.653	0.362	0.04564304	0.02979946	0.275	0.0394	0.0266	0.0789	0.102	0.08171795	0.0646	0.14	5.05	0.157	0.249	0.0829	0.2	0.07080135
M124T89	2.9824206	0.09331858	0.39318691	0.15313002	0.15243152	0.16048788	0.12133623	0.47594748	0.14251412	0.08387281	0.26134113	0.25292864	0.04777721	0.33273061	0.05460995	0.03214364	0.08284587	0.84238196	0.03412727	0.34513295	0.67840359	0.10702362	0.04164603
M319T817	0.69969129	0.01683001	1E-18	0.02997047	0.06852923	0.11449164	0.06413949	0.01392491	0.03739575	0.08288388	0.00519008	0.00720129	0.04838311	0.08889087	0.04147922	0.00957689	0.0735713	0.17420585	0.00500229	0.21796795	0.01416078	0.09809878	0.06904379
M331T759	1.06715867	0.04688944	0.12144806	0.16162385	0.0136069	0.43302631	0.68988131	0.00391531	0.06735004	0.13501654	1E-18	0.01890411	0.10205796	1E-18	0.00808086	0.04853806	0.21240534	1.73991216	0.05471723	0.36545622	0.05150657	1E-18	0.08328369
M165T1218	10.5589529	0.29960251	0.74907767	0.2886375	0.92782848	0.8126956	1.01925881	0.09201123	0.24381793	0.3127996	0.09330142	0.14819547	0.32288567	0.9707287	0.28027123	0.14886343	0.53084676	5.56805764	0.17740216	0.37118278	0.14487959	0.23520392	0.3599566
M341T682	5.45182477	0.03812565	0.62729063	0.18331486	0.05655274	0.3995195	0.42113278	0.02212327	0.04978955	0.17818479	0.01976832	0.09591274	0.07427702	0.02420859	0.04622542	0.04727423	0.1259589	0.37748877	0.08287705	1.02364904	0.0191291	0.07229236	0.38667743
M68T435	17.9029377	0.34859489	11.1015021	0.25429126	0.66204751	2.22666916	0.62819557	0.12805723	0.20112568	0.13045857	0.61153369	0.28258314	0.12078906	6.35586317	0.33498323	0.0813287	0.45471178	3.57153614	0.43472361	0.27887146	0.0445461	0.49532253	0.29363358
M131T91	2.27525006	0.07406062	4.6684341	0.02071286	0.09743952	0.81149839	0.24225558	0.11568309	0.02017687	0.02706113	0.21573065	0.02977514	0.04174999	0.25618938	0.61935868	0.07421638	0.21958328	1.85585241	0.19098745	0.06355333	0.03412157	0.29300912	0.17166147
M367T643	0.32893601	0.14598801	0.17628445	0.21863676	0.0485	0.91308034	0.18892018	0.00830336	0.05473669	0.38770301	0.00309	0.10961801	0.05679833	0.0224	1E-18	0.00387	0.13966643	1.25340834	0.11887784	1.52140057	0.18189801	0.15377429	0.4674448
M161T556	198.92522	0.19507597	47.3034532	4.43601608	8.9629526	4.37565456	11.4111028	1.01503933	3.0785046	1.4457658	0.516785	2.21635427	2.06118555	8.37265798	0.0317406	1.25705037	2.7287077	64.7879944	5.24778553	5.66268752	0.01301515	5.01542553	0.28798159
M223T88	1E-18	1E-18	0.19169883	1E-18	1E-18	0.04271289	1E-18	0.07441289	1E-18	0.04594881	0.03638898	1E-18	1E-18	0.09729423	0.06499102	1E-18	0.02797123	1E-18	0.03219847	0.01708213	1E-18	0.23722763	1E-18
M138T87	2.02624727	0.08831741	0.35046884	0.09299296	0.05709962	0.15384926	0.11509549	0.07728843	0.03025607	0.61455262	0.01600624	0.04120113	0.05739449	5.65992679	0.1735152	0.00777974	0.3754566	1.25904278	0.05256267	0.11939432	0.22051196	0.32836495	0.13755742
M319T91	0.77089791	0.01015209	1E-18	0.01228242	0.01721112	0.06948293	0.08236493	1E-18	0.01342006	0.01471303	0.01479614	1E-18	0.02598519	0.02527045	0.0459021	0.00318496	0.04054296	0.40484076	0.00743258	0.02152685	0.00712025	0.01842099	0.02664107
M287T656	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	1E-18	0.01103484	0.0156	0.00904	0.00268	0.00515668	0.0181	1E-18	1E-18	1E-18	1E-18	0.193	1E-18	0.0691485	0.00332522	1E-18	0.0259
M73T87	13.7628682	0.65748172	3.90532874	0.11320637	0.2165904	0.60938713	0.25756448	0.20915323	0.49977443	0.50927703	0.12399317	0.33351539	0.52024033	1.35998458	0.25211359	0.27022993	1.07229699	5.39794587	0.20330035	0.32545042	0.09436957	1.33043413	0.11595333
M227T89	6.98359636	0.03992619	1.41821858	0.05640875	0.05947599	0.70860194	0.86300698	0.21658383	0.31116063	0.10010643	0.01240403	0.13086804	0.10118545	0.20532938	0.0392783	0.01188821	0.13209176	0.82535484	0.63461803	0.14418222	0.08061383	0.08085302	0.04591731
M167T1576	1E-18	0.01462278	0.18437728	1E-18	0.0145035	1E-18	0.15562409	0.00718981	1E-18	0.01164327	1E-18	0.00261043	0.0079916	1E-18	1E-18	1E-18	1E-18	0.49551311	0.01044694	0.07729227	1E-18	0.03336959	1E-18
M84T1584	14.8969668	0.53239998	2.45987749	0.35795239	0.73861334	0.60826432	1.02782981	0.12720509	0.25128482	0.41975622	0.10012607	0.24496481	0.17435678	0.97364331	0.23828749	0.12957351	0.60106176	4.56119005	0.16857445	0.8931204	0.12085477	0.07593881	0.39791736
M333T78	1E-18	1E-18	1E-18	0.00673479	1E-18	1E-18	1E-18	1E-18	1E-18	0.04677625	1E-18	0.02252125	0.04372269	1E-18	1E-18	1E-18	0.02713877	1E-18	1E-18	0.09047271	1E-18	0.01894339	0.09430746
M59T575	2.01490432	1.47951383	8.13579568	0.33182296	0.55006718	3.1511039	5.38576293	0.63465321	0.38992533	1.41124282	0.53774867	0.44354477	0.74383126	3.69545288	0.39846504	0.36295674	2.0087984	26.8435521	0.926335	2.56401346	0.03083352	2.22296553	1.61296684
M115T570	3.53522817	0.58598569	3.42767256	0.43558815	0.89487382	1.95829528	2.26671781	0.14767996	0.36519254	0.62724338	0.12821779	0.27734531	0.37987223	0.5789254	0.49264796	0.18235673	0.51774283	10.8250338	0.3096532	0.7762753	0.21815015	0.55096252	0.61894192
M344T674	1.7668033	0.19960196	0.36183212	0.04472935	0.29466112	1.3050365	1.69839938	0.05814911	0.02370103	0.92280986	0.01086439	0.02941203	0.05403368	0.03356063	0.04168183	0.03808159	0.41693054	3.72154911	0.09937971	0.72957778	0.05309306	0.17880281	0.72181251
M308T725	5.64135512	0.2536966	0.23400883	0.22219671	0.05369805	3.71940828	1.13508351	0.01865804	0.00743502	0.36089881	0.04050941	0.13377777	0.03230239	1E-18	0.0170702	0.00313405	0.03985838	0.28647496	0.01240047	0.59472916	0.00757761	1E-18	1.09712776
M94T83	5.36638003	0.1433585	0.24291868	0.0317875	0.12143204	0.33959595	0.13909885	0.00611862	0.09922897	0.17882022	1E-18	0.02776903	0.09567505	0.52378694	0.12345013	0.09874185	0.18446704	2.58034047	0.01506431	0.303398	0.0370193	0.19484409	0.12855422
M671T764	1.63026326	0.03616806	0.40596613	0.0274119	0.12687987	0.12840685	0.04281335	0.01964897	0.02104203	0.01078205	0.0079014	0.00307454	1E-18	0.00761242	1E-18	0.00077789	0.00823414	0.09587075	0.00981833	1E-18	1E-18	0.03184233	0.00889232
M149T89	5.50517897	0.30176059	2.46801855	0.10712183	0.62336865	0.66792129	1.64577333	0.36963259	0.20815397	0.4948167	0.43088352	0.05118786	0.10441946	0.51599819	0.41902146	0.20988138	0.43823079	3.58031631	0.47002223	0.44034773	0.10500421	0.70048618	0.35603914
M163T494	2.10514253	0.05227501	0.4514571	0.0432192	0.36223463	0.45665366	0.33283323	0.02950978	0.17127471	0.14888255	0.03416281	0.14226652	0.11955503	0.59649652	0.56088525	0.05967804	0.08500051	1.9373553	0.28803602	0.48515149	0.03592901	0.13018876	0.1781052
M307T660	140.593085	16.0891239	16.5656547	6.65578787	5.66040783	0.21502691	87.2945144	1.39070988	0.07163345	26.0285741	1.68039233	2.27545612	1.8803745	1.72264076	0.84825196	0.47045099	6.45684701	74.8878775	2.47680785	26.4727197	2.1588256	6.67542792	20.9945227

II. Analysis of the exo-metabolome of an elicited alga

The surrounding seawater of elicited and control seaweeds was first analyzed by GC-MS from seawater samples collected at 1h, 4h, 12h and 24h after elicitation by GG. After data processing with Galaxy-W4M, the data matrix comprised a total of 1548 ions detected in samples, reduced to 296 selected ions after filtering. The results of the multivariate statistical analysis are represented in the PLS-DA in Fig 16a, which showed that, after 1h of elicitation, control and elicited algae seem to respond similarly, in contrast to elicited algae after 4h which seem to release different compounds from control algae. However, in samples collected later, after 12 and 24h of elicitation, control and elicited algae were not discriminated. The treatment (elicitation versus control) applied to the alga did not allow to discriminate their exo-metabolome (MVA.test, 999 permutations, NMC = 0.444, p-value = 0.317), whereas the sampling time significantly affected the bouquet of volatiles in the exo-metabolome of *L. digitata* (MVA.test, 999 permutations, NMC = 0.283, p-value = 0.001). Moreover, the sampling times were separated along the axis 1 (Fig 16b).



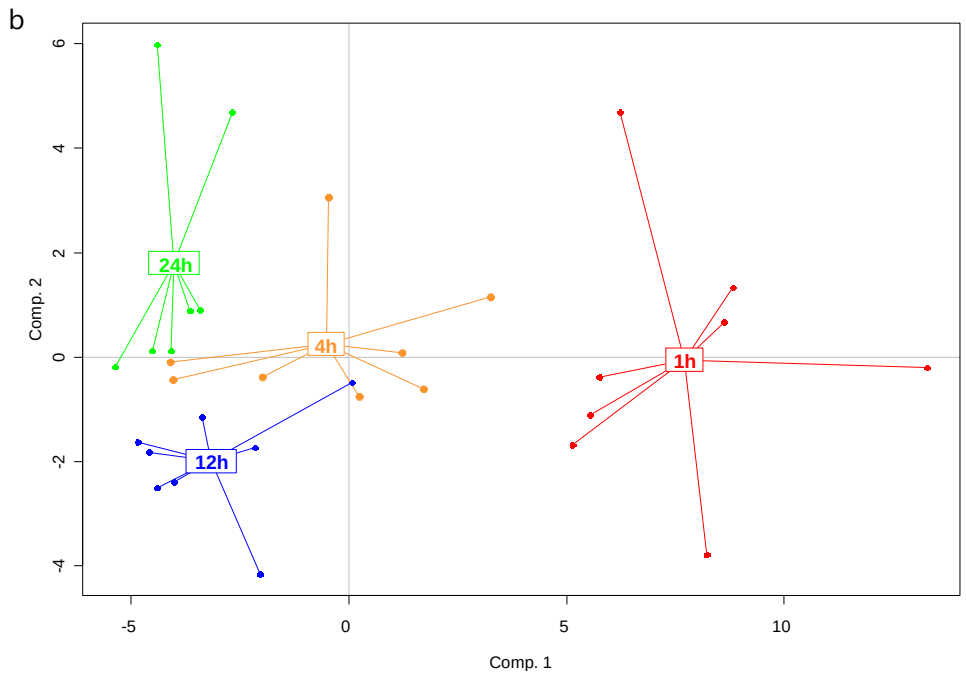


Figure 16. PLS-DA of the GC-MS analysis of the exo-metabolome of elicited (E) and control (C) *L. digitata* according to the sampling times (1h, 4h, 12h and 24h). Samples are represented after filtering of ions, finally obtaining 296 ions according to treatment and sampling time (a) and only according to sampling time (b).

The surrounding seawater of elicited and control *L. digitata* after different incubation times (1h, 4h, 12h, 24h) was then analyzed in LC-MS in both negative and positive modes. After 3 trials of analysis in positive mode, the chromatograms still showed a contaminating element all along the running time, making them non interpretable. However, metabolomic analyses in LC-MS in negative mode resulted in a matrix of intensities of ion peaks detected in each sample. After processing with Workflow4metabolomics, 678 ions were detected and the final data matrix contained 457 ions after filtering of extreme running times and isotopes. These data were subjected to statistical analyses with R software, with the packages pls and RVAideMemoire, allowing to obtain Partial Least Square – Discriminant Analysis (PLS-DA) and the Number of Misclassification (NMC) and the p-value associated. The PLS-DA in Fig 17a showed that if control and elicited algae sampled after 1h of elicitation did not seem to be separated, elicited algae after 4h seem to release different compounds from control algae. Like for GC-MS analyses, late sampling times (12h and 24h) could not allow to discriminate between control and elicitation treatments. When all sampling times were pooled, statistical analyses did not allow to conclude that the exo-metabolome was significantly different according to the treatment, either elicitation or control treatment (MVA.test, 999 permutations, NMC = 0.44688, p-value = 0.35). However, the PLS-DA showed in Fig 17b seems to discriminate the sampling times along axis 1, confirmed by multivariate statistical analyses which demonstrated that the composition of the exo-metabolome of an alga significantly depended on the sampling time in the experiment (MVA.test, 999 permutations, NMC = 0.34219, p-value = 0.001). Moreover, the number of misclassifications

(NMC) remained low, with less than 35% of variability which cannot be explained by the treatment and the sampling time.

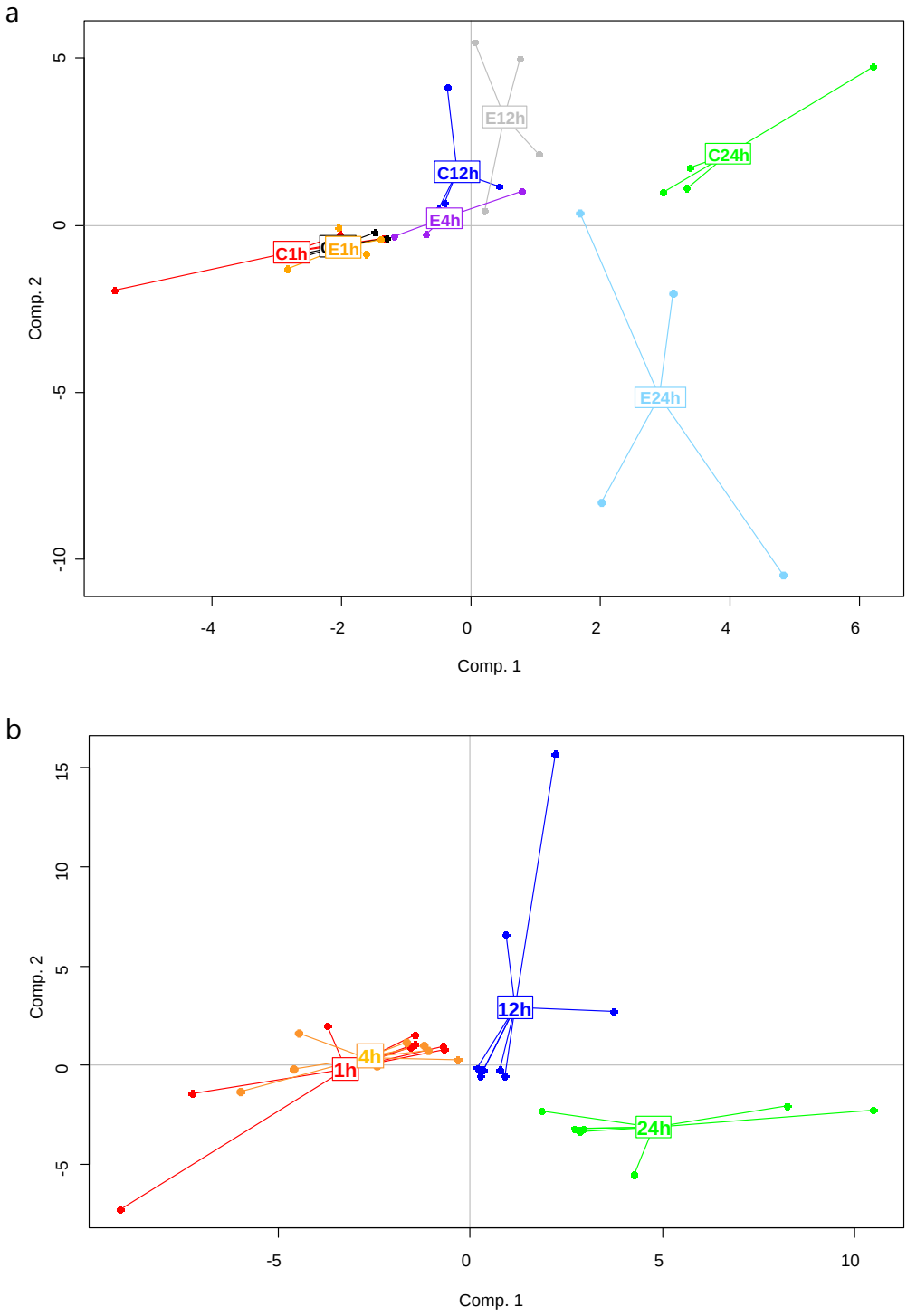


Figure 17. Partial Least Square - Discriminant Analysis (PLS-DA) of the LC-MS analysis in negative mode of the exo-metabolome of elicited (E) and control (C) *L. digitata* according to the sampling times (1h, 4h, 12h and 24h). Samples are represented after filtering of ions, finally obtaining 457 ions, according to treatment and sampling time (a) and only according to sampling time (b).

Some ions were selected as they were already identified as induced in response to biotic and abiotic stresses in brown algae, and mostly after copper stress in *L. digitata*. They could thus be annotated thanks to comparison of their m/z (mass/charge) ratio with the $[M-H]^{(-)}$ ions of metabolomic databases, as putative fatty acids and their oxidized derivatives (Fig 18).

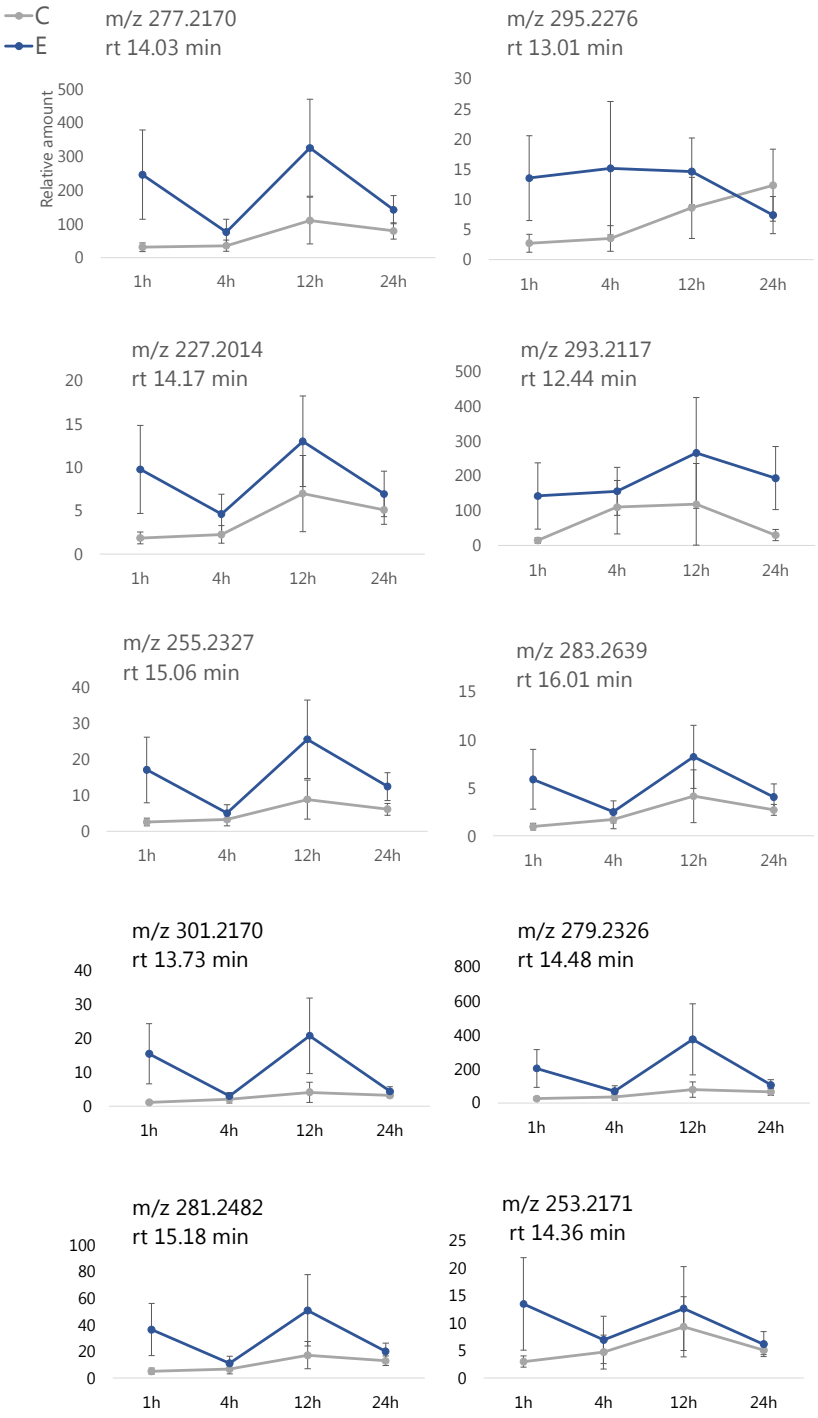


Figure 18. Relative amounts of some compounds annotated as fatty acids and their oxidized derivatives in surrounding seawater of elicited and control *L. digitata* plantlets, after 1h, 4h, 12h and 24h. The ions could be annotated as followed: 277.2170 m/z $[M-H]^{(-)}$ rt 14.03 min: $C_{18}H_{30}O_2$, linolenic acid ; 295.2276 m/z $[M-H]^{(-)}$ rt 13.01 min: $C_{18}H_{32}O_3$, hydroxyoctadecadienoic acid (HODE); 227.2014 m/z $[M-H]^{(-)}$ rt 14.17 min: $C_{14}H_{28}O_2$,

myristic acid; 293.2117 m/z $[M-H]^{(-)}$ rt 12.44 min: $C_{18}H_{30}O_3$, HOTrE ; 255.2327 m/z $[M-H]^{(-)}$ rt 15.06 min: $C_{16}H_{32}O_2$, palmitic acid; 283.2639 m/z $[M-H]^{(-)}$ rt 16.01 min: $C_{18}H_{36}O_2$, stearic acid ; 301.2170 m/z $[M-H]^{(-)}$ rt 13.73 min: $C_{20}H_{30}O_2$, eicosapentaenoic acid (EPA) ; 279.2326 m/z $[M-H]^{(-)}$ rt 14.48 min: $C_{18}H_{32}O_2$, linoleic acid ; 281.2482 m/z $[M-H]^{(-)}$ rt 15.18 min: $C_{18}H_{34}O_2$, oleic acid ; 253.2171 m/z $[M-H]^{(-)}$ rt 14.36 min: $C_{16}H_{30}O_2$, palmitoleic acid. Grey lines correspond to the control treatment and blue lines to the elicitation treatment. For each treatment at one sampling time, n=4. Statistical analyses performed between each pair of control and elicited algae at each sampling time did not show significant differences (Wilcoxon test, α=5%).

Except for the 295.2276 m/z $[M-H]^{(-)}$, rt 13.01 min, the other annotated ions tended to be emitted 1h after elicitation, then decreasing after 4h, increasing again at 12h and finally decreasing at 24h. Control algae emitted less quantities of these compounds all along the experiment. Moreover, the relative amounts of 277.2170 m/z $[M-H]^{(-)}$, rt 14.03 min , 279.2326 m/z $[M-H]^{(-)}$, rt 14.48 min and 293.2117 m/z $[M-H]^{(-)}$, rt 12.44 min putatively corresponding to linolenic acid, linoleic acid and the oxylipin HOTrE respectively, were until 20 times more abundant than the other compounds. The 295.2276 m/z $[M-H]^{(-)}$, rt 13.01 min remained in high amounts during at least 12h, then decreasing at 24h. According to statistical analyses performed on each control/elicited pair sample, relative amounts of these compounds were not significantly different between the two treatments at each sampling time (Wilcoxon test, α=0.05).

Discussion

1. A partial view of the exometabolome and putative signals emitted by an elicited alga

When metabolomic analyses were performed (3 years ago), the analytic technics that we used were not optimized for our samples. For example in GC-MS analysis, the sequence of sample injection did not comprise Quality Controls (QCs) which would have permitted a regular control of the quality of chromatograms and mass spectra all along the running time. Moreover, the tool Workflow4metabolomics is still under evolution, leading to different results obtained after metabolomic data processing between previous and current analyses. After LC-MS analysis of the exo-metabolome of an elicited alga, chromatograms obtained in the positive ionization mode presented an important contamination, making their interpretation not possible. This led to a lack of information on the composition of volatile compounds released in the medium. Thus, it should be interesting to make a new analysis in positive ionization mode, which would imply an optimization of the metabolomic analyses by LC-MS analysis, regarding the chemical gradient of elution. The results obtained in the positive mode would give supplementary information regarding, for instance, the content of the exo-metabolome in amino acids.

2. Changes over time of both endo- and exometabolome in response to multiple external factors

This work conducted on inducible chemical regulations in the kelps *L. digitata* and *L. spicata* in response to grazing allowed to get new information about the global changes of the endo- and the exo-metabolome of a grazed kelp, and about the response timing and the metabolic pathways that were activated upon attack. First, in contrast to analysis of the endo-metabolome which showed global different compositions in chemical compounds between grazed and ungrazed algae, the exo-metabolome was significantly different according to time. Results in both GC-MS analyses and in LC-MS in the negative mode allowed to discriminate samples collected early in time from those collected late, and the exo-metabolome seemed to be different between elicited and control algae after 4h of incubation. At late sampling times, the treatment effect could be hidden by the effect of the experimental design, which could for example cause a lack of oxygen or nutrients for the kelp from 12 h of incubation. Ions that discriminate the two treatments could be hidden by other ions that are predominantly influenced by time. Moreover, there is often an important background noise in metabolomic analyses, which can hide the direct effects of a treatment on algal endo- and exo-metabolome.

3. A potential induction of the oxylipin pathway

Based on metabolic profiling data, both internal algal tissue and surrounding seawater analyses showed an accumulation and release of potential free fatty acids and oxylipins. After LC-MS analyses of the exo-metabolome *of L. digitata* after elicitation, 8 free fatty acids and 2 oxylipins could be annotated. Interestingly, the relative amounts of all the fatty acids were high 1h and 12h after GG elicitation, whereas one oxylipin remained in high amounts until 12h before decreasing at 24h. However, the exact nature of these compounds was not demonstrated. Complementary investigations should thus be performed in order to validate or refute the identification of the fatty acids and oxylipins annotated in LC-MS in negative mode. For that purpose, it would be essential to make metabolomic analyses of the samples in LC-MS/MS added up to analysis of chemical standards of these targeted compounds.

Thus, in addition to being accumulated in algal tissues, this complementary work highlighted that kelps also released some of these free fatty acids and their oxidized derivatives into the seawater in response to elicitation. These compounds were already described as emitted in the surrounding seawater after 3h and 8h of copper stress applied to *L. digitata* (Ritter et al., 2008; Ritter et al., 2014). Lab and field experiments on *L. digitata* had also shown that aldehydes, which are oxidized derivatives of polyunsaturated fatty acids (PUFAs), were emitted during and 1h after low tide, and after elicitation for 1h by oligoguluronates (Goulitquer et al., 2009). The chemical compounds that were emitted in the seawater after elicitation could thereafter participate to a potential signaling for a neighboring kelp. In the chapter 2, we explore this hypothesis and the potential responses of a “warned” neighboring kelp to grazing. Further studies need also to be done to determine the nature of the chemical compounds that could induce defense responses in the neighboring kelps.

Chapter II

Distance signaling in kelps and defense against herbivores

Introduction

Besides local responses inside algal tissues, kelps also respond to grazing stress through the release of volatile and soluble compounds into the surrounding seawater. Later on, these compounds can potentially be perceived by herbivores or by neighboring algae. In brown algal communities, distance signaling could play crucial roles mostly for prevention of a danger, such as during herbivore attacks. For example, waterborne cues emitted upon grazing were shown to induce higher concentrations of phlorotannins in *Ascophyllum nodosum* tissues which were less palatable than ungrazed algae (Toth and Pavia, 2000). In *Fucus vesiculosus*, distance signaling induced defense responses of the neighboring *Fucus* individuals (Rohde et al., 2004). These studies thus showed that waterborne signals emitted by the challenged alga can serve as warning signals to induce production of defense compounds inside algal tissues of neighbor conspecifics. They can induce metabolic modifications in the alga which perceives some of these signals. For example, in *F. vesiculosus*, secondary metabolites such as terpenoids, polyphenols and fatty acids are produced against herbivory, which also trigger gene regulation namely involved in lipid metabolism and in defense and stress responses (Flöthe et al., 2014). The kelp *L. digitata* was shown to release volatile polyunsaturated aldehydes and oxylipins, which are oxidized derivatives of fatty acids, in the medium upon biotic and abiotic stresses (Ritter et al., 2008; Goulitquer et al., 2009).

It seems that the efficiency of these chemical modifications in algal defense against herbivore attacks are both species- and grazer-specific. For instance, *Laminaria japonica* exposed to grazing by *Littorina brevicula* was less palatable for conspecific grazers during a subsequent grazing, while previous exposure of kelps to the abalone *Haliotis discus* did not decrease the palatability for a subsequent grazing by *H. discus* (Molis et al., 2008). Similarly, grazed *A. nodosum* by *L. obtusata* were less susceptible to further grazing than ungrazed algae, which was not the case after grazing by *Idotea granulosa* (Pavia and Toth, 2000). One likely explanation for these differences lies in the degree of specialization between the grazer and its host. When two species are intimately associated in an ecologically specialized relationship, as the result of a co-evolution between the two species, the defense mechanisms either are inefficient because the herbivore has adapted to avoid or tolerate these compounds, or display other functions in the biotic interaction. As opposed to the terrestrial environment, most marine herbivores are generalists (Hay and Steinberg, 1992), with only a handful marine species involved in specialized associations (Hay, 2009). In these cases, it seems that induced chemical cues produced in response to grazing have complex effects on specialized grazers, including their attraction instead of their repellence as also described in plants (Kessler and Baldwin, 2001). For instance, the location of food or hosts by grazers or pathogens can be facilitated by compounds like DMS (Dimethylsulfide) produced by the algae (Pohnert et al., 2007), leading to behavioral changes of the herbivore that increases its ability to find and consume algal food sources (Steinke et al., 2006). Coleman et al., (2007) found that feeding of *L. obtusata* on *A. nodosum* induced the attraction of two

predator species that could feed on the herbivore. Thus, specialist and generalist herbivores should interact differently with their algal host and, conversely, algal responses should be different according to the degree of specialization of the herbivore.

In the context of kelp/herbivore interactions, we hypothesized that a grazed kelp could have an impact, through chemical distance signaling, on metabolism regulations of neighboring algae, possibly leading to protection against grazers. Thus, in this chapter, we tested whether these chemical signals could have a role in algal defense against grazers through activation of metabolic pathways. Moreover, field observations on *L. spicata* revealed high amounts of *S. scurra* grazers on a single individual, while some neighbors could be free of grazers. We then hypothesized that grazing by herbivores could trigger metabolic modifications in grazed algal tissues and in the neighboring kelps, which could then modify herbivore behavior towards these kelps. Thus, we tested the effects of distance chemical signals on a subsequent grazing by specialist herbivores compared with a grazing by generalist herbivores. The chemical signals were generated either by elicitation with oligoguluronates as they were shown to activate innate immunity metabolism and priming effect (Küpper et al., 2002; Cosse et al., 2009; Thomas et al., 2011), or by a previous grazing, as we have shown in Chapter I that this induces metabolism changes after 48 h. We hypothesized that the chemical signal is then transmitted by co-incubation between the sender and the receiving kelp individuals. Finally, the effects of these signals were assessed by monitoring the evolution over time of algal endo-metabolome, by measurement of the algal consumption by specialist and generalist herbivores, and by testing the modifications of herbivore behavior remotely.

The first part of the chapter is dedicated to the article which deals with ecological and chemical modifications in kelps upon grazing stress. The first purpose of this article was to determine the effects of chemical signals originating from a neighboring grazed kelp on herbivore behavior, through monitoring of algal consumption by grazers. Then, the effects of these chemical signals were investigated on algal metabolic regulations in *L. spicata* through metabolomic approaches. Then, the second part of this chapter aims at studying the effects of different algal grazing treatments on herbivore behavior by monitoring their attraction towards grazed or elicited kelps, or their receiving neighbors. In this part, not only specialist herbivores but also generalists were used to test their food preferences, through choice experiments for healthy kelps, elicited or grazed, co-incubated with a grazed or an elicited kelp, or using wounded stipes in the lab and in the field. First, elicited and healthy *L. digitata* were tested for preference of the generalist herbivore *Haliotis tuberculata* (abalones). Secondly, grazed *L. spicata* and co-incubated of grazed kelps were presented as food choices to the generalist herbivore *Tegula atra*. Then, attraction of *L. spicata*'s specialist herbivore *S. scurra* was tested for its preference for natural wounds on *L. spicata* stipes. Finally, a preliminary *in situ* experiment tested the attraction of *S. scurra* for artificial or natural wounds on *L. spicata*'s stipes.

I. Ecological and chemical changes of *Lessonia spicata* to grazing by specialist herbivores

General outline

In order to determine the effects of grazing stress on kelp defense responses, we performed non choice bioassays on *L. spicata* with its specialist herbivore *S. scurra* and metabolic profiling on the kelp submitted to different treatments. We tested first the effects of a direct grazing and a co-incubation with a grazed kelp on responses of *L. spicata* to a subsequent grazing. We compared the effects of a direct grazing treatment by *S. scurra* and a chemical elicitation treatment by oligoguluronates on algal consumption during a subsequent grazing by *S. scurra*. Results showed that Grazing but not Elicitation treatment influenced consumption by herbivores with a higher consumption of kelps that were previously grazed, than controls or elicited kelps. This difference was, however, significant only at the end of austral summer, but not during spring, suggesting a strong effect of seasonality on algal physiological response to grazing stress.

In order to investigate algal metabolic regulations in algae co-incubated with grazed kelps, algal metabolic profiling was performed after an indirect grazing of *L. spicata* by *S. scurra* compared to direct grazed kelps, according to time. Here we showed that the endo-metabolome of a grazed *L. spicata* was different from the one of its co-incubated neighbor, and from its respective control without any grazer. Moreover, a co-incubated kelp with a control alga without grazer showed a different metabolome from a co-incubated kelp with a grazed alga. Among these compounds, potential fatty acids and oxylipins could be found as induced after direct and indirect grazing treatments over the 2-day grazing. Thus, metabolite profiling analyses suggested that the kelp co-incubated with a grazed alga would perceive and integrate a soluble signal released into the seawater by the grazed alga. In the neighboring kelp, metabolic regulations seem to occur, especially regarding the fatty acid pathway and their oxidation.

Finally, this article allowed to conclude that responses of grazed kelp strongly depended on the season, in terms of weight gain and defense against grazers, in contrast to elicited kelps which were not subjected to these changes. In addition, algal consumption after Grazing treatments and metabolome analyses were in agreement. Direct grazing and co-incubation with a grazed kelp showed different grazing levels as well as different metabolomes. Therefore, direct grazing modified the metabolome of the kelp, then changing the one of its neighbor, and this could participate to defense reactions of the kelp.

Article

Ecological and chemical changes of *Lessonia spicata* to grazing by specialist herbivores

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Article in preparation

Ecological and chemical changes of *Lessonia spicata* to grazing by specialist herbivores

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Abstract

In response to grazing by herbivores, marine macroalgae have developed chemical defenses. A part of these chemical compounds are produced and kept into algal tissues, while others are emitted in the surrounding seawater, constituting a distance signaling. Moreover, grazing by herbivores affect regulations of transcription in algae and modifications in metabolic pathways. In brown algal kelps, we have shown systemic responses in the brown alga *Laminaria digitata* upon defense elicitation and demonstrated a priming effect, induced by chemical cues present in seawater surrounding a kelp bed and/or released from neighboring brown algae. Such distant chemical signaling should have a major ecological role in structuring marine algal and herbivore communities. However, ecological impacts of this chemical signaling on kelp/herbivore interactions, as well as the nature of the chemical compounds produced into algal tissues remain unknown. Thus, in the context of kelp/herbivore interactions, we studied defense of *Lessonia spicata* against its specialist grazer *Scurria scurra*, present along Chilean costs by means of bio-assays, and, simultaneously, we explored modifications of the algal metabolism by metabolomics approaches. We found that algal defense against grazers depends significantly on the season, especially between March and November. In March, *L. spicata* previously grazed were more eaten by grazers, thus less capable of defending themselves, compared with the end of the year. Moreover, metabolomics studies showed that the endo-metabolome of a grazed kelp was different from its neighbor, as well as from a naive kelp. These results should help to understand biochemical mechanisms in the ecological context of kelp defense against herbivores.

Introduction

Kelp populations form complex ecosystems, including communities of micro- and macroscopic organisms living, reproducing and/or feeding in this environment. They are often subject to grazing by herbivores which live with them in the same ecosystem. Because they are sessile, kelps have to cope with these biotic stresses by setting up defenses against grazing by herbivores. It is now well known that chemical defense plays a crucial role in algae/herbivore interactions at different levels: directly and locally against grazers, systemically in the whole alga, and indirectly via interplant signaling. These chemical defenses may involve different classes of molecules. Among the best studies is the case of phlorotannins. Brown macroalgae, for instance, can defend directly against grazers by their specific ability to produce and store phlorotannins (Hay and Fenical, 1988). These defense chemical compounds were shown to be induced after grazing of *A. nodosum* by different *Littorina* species (Pavia and Toth, 2000) and *Idotea baltica* (Haavisto et al., 2010), then negatively affecting a subsequent grazing (Toth et al., 2007) by conspecifics and decreasing reproduction of grazers (Toth et al., 2005) or algal palatability (Haavisto et al., 2010). It was also found that algal defense responses were triggered by distance signaling via waterborne compounds. Indeed, after perception of waterborne cues by the alga *A. nodosum* induced after grazing by *I. baltica* or *Littorina obtusata*, the kelps contained higher concentrations of phlorotannins and were less palatable than control ungrazed algae (Toth and Pavia, 2000).

These studies show that waterborne signals emitted by the challenged alga can serve as warning signals to induce production of defense compounds inside algal tissues of neighbor conspecifics. Similar defense distance signaling was found in the brown alga *Fucus vesiculosus*, where grazing by *I. baltica* induced the release of a signal that triggered defense responses in neighboring *Fucus* individuals (Rohde et al., 2004) and in *A. nodosum* which uses waterborne signals for higher production of defense compounds (Toth and Pavia, 2000). Diatoms are also able to defend against grazing by copepods, by producing and emitting a wide range of polyunsaturated aldehydes, leading to a decrease of grazer reproduction (Pohnert, 2005). It is now well known that modification of the algal chemistry can change rapidly the susceptibility of algae to grazer consumption (Aguilera, 2011) and probably food preferences of herbivores, only a few hours after grazer consumption, and then disappear when grazing challenge ends (Aguilera, 2011).

A few studies so far have investigated the actual regulations of algal metabolism induced by distant signaling in response to grazer attacks. In *Fucus vesiculosus*, secondary metabolites such as terpenoids, polyphenols and fatty acids are produced against herbivory (Wahl et al., 2011). It was also shown to result in gene expression regulations, including up-regulation of genes involved in lipid metabolism, carbohydrate metabolism, intracellular trafficking, and defense and stress response (Flöthe et al., 2014). In *Laminaria digitata*, Küpper et al. (2001) first demonstrated that this brown alga

recognizes and reacts in response to application of cell wall polysaccharides from kelp, named as oligoguluronates (GG). Another study showed that these chemical elicitors were able to trigger a higher protection of the kelp against further pathogen infections (Küpper et al., 2002). However, unlike bacterial lipopolysaccharides elicitors, GG elicitors are not able to induce release of fatty acids and accumulation of oxylipins in *L. digitata* (Küpper et al., 2006). Later, Goulitquer et al. (2009) found that, in response to different biotic and abiotic stresses, the kelp *L. digitata* releases volatile polyunsaturated aldehydes (PUAs) and oxylipins in the medium. These compounds may thus participate to a chemical signaling between kelps, as *L. digitata* was found to perceive waterborne cues present around chemically elicited neighboring plants, then changing early responses in metabolism as well as induction of genes (Thomas et al., 2011), also after 24-hour time elicitation by oligoguluronates (Cosse et al., 2009).

It seems, however, that thes chemical responses to grazing are grazer specific. Indeed, Pavia and Toth (2000) found that *A. nodosum* plants grazed by *L. obtusata* were less susceptible to further grazing than ungrazed algae, unlike when grazed by *I. granulosa* which had no effect on secondary attacks. Fresh *Laminaria japonica* exposed to *Littorina brevicula* were less palatable during a subsequent grazing than when previously consumed by the abalone *Haliotis discus* (Molis et al., 2008). One likely explanation for these differences lies in the specificity of the association between the grazer and its host. When two species are intimately associated in an ecologically specialized relationship, as the result of a co-evolution between the two species, the defense mechanisms either are inefficient because the herbivore has adapted to avoid or tolerate these compounds, or display other functions in the biotic interaction. For instance, (Coleman et al., 2007) found that feeding of *L. obtusata* on *A. nodosum* induced the attraction of two predator species that could feed on the herbivore. . Location of hosts by grazers or pathogens can be facilitated by compounds like DMS (Dimethylsulfide) produced by the algal host (Pohnert et al., 2007), leading to behavioral changes of the herbivore that increases its ability to find and consume algal food sources (Steinke et al., 2006).

In this study, chemical and ecological approaches were combined to investigate the nature of a chemical signaling between kelps in response to grazing by herbivores, and to test the effects of this signaling on metabolic regulations occurring into algal tissues and on herbivore deterrence. We used the kelp/herbivore couple *Lessonia spicata* and *Scurria scurra,* originated from Chile. *Lessonia spicata* (Suhr) Santelices (González et al., 2012) lives on the low intertidal of wave-exposed rocky shores of central and southern Chile where it is a ecologically and economically important resource (Vásquez, 2008). The kelp hosts a specialized herbivore *Scurria scurra* Lesson (Patellogastropoda, Gastropoda) that lives within its holdfast and at the basal part of the stipes (Santelices et al., 1980). *S. scurra* has a restricted range of host species: it can be found on the bull kelp *Durvillaea antartica,* but this interaction opportunistic as it depends on the relative abundance of the host. On the contrary, its association with

L. spicata is independent of host relative abundance, indicative of its selectivity of this host species (Meynard, 2014). *S. scurra* lives and feeds in deep scars reaching stipe's medullary tissue, and causing sever deformation and depigmentation of the stipe. Only one limpet per scar and one scar per stipe are generally observed (Muñoz and Santelices, 1989). However, superficial grazing scars associated to *S. scurra* are frequently observed on upper parts of the stipes and on the fronds. Grazing is thus expected to trigger defense reactions from the kelp, in response to tissue damaging. This grazing effect on the host is, however, unclear. It is thought that this grazer could have a positive effect on *L. spicata* survival by pruning stipes, thus reducing drag force and preventing whole individual dislodgement by wave action (Santelices et al., 1980). Even if the costs and benefits of this biotic interaction have not yet been characterized, it is expected that the scars are sources of strong cellular stress and likely release different kinds of chemical compounds.

As kelp forests represent considerable food sources for marine herbivores, algae have to set up efficient chemical defenses. However, since *S. scurra* specifically feeds on *L. spicata*, it is expected to overcome algal defense compounds. The nature of these compounds and their role in distant signaling are unknown, as well as their actual effects on the herbivore's feeding behavior. Therefore, this study aimed at characterizing metabolic regulations in such grazed kelps, and assess whether these could lead to a better defense against subsequent grazing. Furthermore, the putative effect of distant signaling on neighbor individuals was investigated for both the metabolic changes and the grazing activity of *S. scurra*.

Materials and methods

Algal and herbivore collections

The kelp *Lessonia spicata* and its specialized herbivore *Scurria scurra* were sampled from rocky shores of Las Cruces (33°30'09"S : 71°37'59"O), Central Chile, between July 2014 and December 2015. Grazers were softly removed from basal parts of stipes during low tide, and kept cooled for transportation to the laboratory (no more than 1h). Then, they were fasted before the experiment, in a tank with constant seawater flow during 48 hours, at ambient temperature. Kelp harvesting consisted of collecting juvenile kelps. The whole *L. spicata* plantlets were sampled: one holdfast with all the stipes growing on it, with a maximum length of 20-50cm. Plantlets were stored in a tank with running seawater and air flow during one to two days for acclimation away from potential chemical signals of adult individuals in the natural stand.

General laboratory experimental set up

The experiments were divided into two sequential phases: treatment phase and bio-assay by *S. scurra* grazing. For the treatment phase, the experimental unit consisted in two *L. spicata* plantlets placed in a

40 L-aquarium with constant seawater and air supplies. One of them was submitted to a stressful treatment, either chemical elicitation by oligoguluronates or direct grazing by *S. scurra*, and the other kelp was co-incubated with the treated one. In parallel, a control unit consisted in 2 plantlets, one being manipulated in the same way, but without any elicitor or grazer, and the other one co-incubated without any further manipulation. The co-incubation of the treated individual with its neighbor (or the respective controls) lasted 24 or 48 hours, according to the type of treatment (see below for details). For the bio-assay, one frond of each kelp (for the treated kelps, the frond exposed to the stressful treatment) was cut at the base of the frond, and then placed individually in separate plastic boxes of 6L with constant seawater flow for four days, together with two *S. scurra* grazers put on the frond. Each frond was blotted on paper towel to remove liquid water from surface and weighted before the bio-assay and after two and four days of grazing by *S. scurra*. In addition, the length and width of all grazers used for the bio-assay were measured, to calculate algal consumption standardized by grazers' size. Ten replicates of this experimental unit were set for each treatment, i.e. grazing or elicitation.

As the grazing assay was based on algal consumption, we have analyzed the effects of the experimental set-up and sampling time on algal weight change, to account for non-feeding-related changes in wet mass (i.e. autogenic changes). In the treatment phase of the experiment, two kelps were placed in an aquarium and treated following the same protocol (Grazing and Elicitation). Fronds were then cut, blotted and weighted, and put separately in 6L-boxes without any grazers. These fronds were then weighted after two and four days in seawater. Each experiment was independently run three times for each type of treatment.

Grazing experiment

Two individuals of *S. scurra* were first deposited on one frond of 10 *L. spicata* plantlets kept in a net, to avoid herbivore escaping. Kelps in nets were named DG (direct grazed), whereas the other was named IG (indirect grazed, e.g. not directly in contact with the grazer, but co-incubated with the DG alga). The controls used for each treatment were named C_{DG} (Control direct grazed) and C_{IG} (Control indirect grazed) respectively. After 48 hours of co-incubation, the most grazed frond of DG and one frond of IG, C_{DG} and C_{IG} plantlets were selected for the bio-assay.

Elicitation experiment

For chemical elicitation, alginate oligoguluronates (poly-alpha-1,4-L-guluronic acid blocks, GG blocks) were prepared by acid hydrolysis according to Haug et al. (1974), using sodium alginate from *Laminaria digitata* stipes (Danisco, Landerneau, France). The homopolymeric blocks of GG were used as an elicitor at a final concentration of 150 $\mu g.mL^{-1}$ as described in Küpper et al. (2001). Elicitation was done by placing, during 1 hour, one frond of the directly elicited individual (E) in a 50 mL-Falcon tube filled with 150 $\mu g.mL^{-1}$ GG dissolved in seawater. The Falcon tube was adjacent to the aquarium so the integrity of the individual was kept, and no more than 5 cm of frond tissue was exposed to air, between

the aquarium and the tube. The control of Elicitation treatment was processed similarly, but the Falcon tube was filled with seawater only. These treated fronds (E and C_E) were then rinsed with seawater and replaced in the aquarium. In August, seawater was renewed one hour after elicitation and let in the aquarium for 24 hours. In November, plantlets were placed in aquarium with constant water supply. Fronds elicited in GG solution (E) or immersed in seawater (C_E), and fronds from the co-incubated kelps (IE and C_{IE} respectively for Indirectly Elicited and Control indirectly elicited) were cut at the stipe, blotted and weighted for the bio-assay.

Metabolite profiling and data analysis

The kinetics of metabolism regulations associated with kelp responses to herbivory for directly grazed and co-incubated individuals was analyzed from separate experiments. Grazing by *S. scurra* was permitted during 6, 12, 24 and 48 hours, and two fragments per kelp were then separately frozen in liquid nitrogen and kept at −80°C for analysis. In parallel, similar set up was applied to control plantlets. Three replicates were taken per condition at each sampling time.

For each treatment (C_{DG}, DG, C_{IG}, IG) and sampling time (6, 12, 24 and 48h), metabolic extracts were obtained from 100 mg of frozen algal powder with 1 mL MeOH:H_2O (8:2), as described in Ritter et al. (2014). In each sample, 1.25 µg.mL^{-1} 12-OH-lauric acid was added as an internal standard for the negative mode analysis. Aliquots of 50 µL of each extract were used for metabolomic analyses. They were first separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an Electro Spray Ionization (ESI) source running on the negative ionization mode, as described in Ritter et al. (2014). Metabolic samples were analyzed using a 1.9 µm Thermo Hypersil Gold C18 column (100 x 2.1 mm) and the mobile phase (0.1% acetic acid in water–acetonitrile) was programmed from 100 : 0 to 5 : 95 acetonitrile–water (v : v). Xcalibur 2.1 software was used for instrument control and data acquisition.

Mass spectra data were processed by XCMS on the online version (Galaxy-Workflow4metabolomics, Giacomoni et al., 2014), after conversion of raw spectra to MzXML format. Data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples necessary at 0.7. For correction of retention time, the obiwarp method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 10. Fillpeaks step was used with the chrom filling method. Finally, annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. The final table contained ions abundances in samples, which corresponded to peak areas.

Data given by CAMERA contained 678 ions. Normalization were done first by dividing integrated areas by the highest one of the same sample, and then by dividing the areas of one ion by the highest in all the samples. This would allow to compare the relative amount of one ion in different samples. After manual elimination of background and redundant (isotope) ions, the final table of data contained 514 ions.

Statistical analyses

The algal weight gain was evaluated according to the season and to the treatment applied to the kelp using 1-way ANOVAs followed by Student-Newman-Keuls (SNK) post-hoc tests for comparisons of the mean weight gain. Algal consumption by herbivores during bio-assays after Grazing treatment was analyzed using Kruskal-Wallis test for comparison of mean consumption between seasons when normal distribution was not respected, or 2-ways ANOVAs and SNK tests to compare the mean consumption according to season and treatment, when appropriate. For the Elicitation experiment, algal consumption variations were considered separately in March and in November. Data were then analyzed using 1-way ANOVAs followed by SNK tests for comparisons of mean consumption depending on the Elicitation treatment. Statistical analyses of data were carried out using R.
Metabolomic analyses were performed using Partial Least Squares - Discriminant Analysis (PLS-DA), followed by extraction of the most discriminant ions. Where possible, annotation of ions was made from previous studies (Ritter et al., 2008). The free softwares R and SIMCA were used to perform statistical analyses.

Results

Algal weight gain after Grazing treatment

The lab experiments were conducted in different periods of the year. Figure 1A shows the percentage of weight gain of *L. spicata* in March and in November, in all samples from both Elicitation and Grazing treatments. It shows that, after a Grazing treatment (for DG and IG as well as for C_{DG} and C_{IG} treatments), the rate of weight gain is significantly higher in November than in March ($F=4.46$, $p=0.04$). Weight increase was higher in November than in March after the first two days following Grazing treatment, and this difference remained constant during at least 2 days of the experiment ($F=4.3478$, $p=0.042$). During this November experiment, weight gain was significantly higher for C_{IG} treated kelps compared to the other treated fronds after two days ($F=5.90$, $p=0.01$) as well as after four days (data not shown, 1-way ANOVA; $F=3.70$, $p=0.04$). However, for the Elicitation treatment, there was no significant difference of algal weight gain between March and November, after two days (Fig 1A; $F=0.13$, $p=0.72$) as well as after four days ($F= 1.03$, $p=0.32$).

Algal consumption after Grazing treatment

The real consumption of algae by grazers was evaluated by weighting kelps after two days and four days of bio-assay corrected by the specific algal weight gain of the corresponding season, according to the treatment (Grazing or Elicitation). Algal consumptions were corrected using the formula: M2-r*M2/100 in which M2 is the weighted mass of the alga after 2 or 4 days of bio-assay, and r is the rate of weight gain of the frond. r = 0.9% in March and 1.7% in December after two days of bio-assay; r = 2.1% in March and 3.5% in December after four days.

Whatever the treatment applied to the kelps and the season, there was no significant differences in *S. scurra* consumption rate during the first 2 days of grazing (2-ways ANOVA, see Table 1A). However, after four days of bio-assay, DG treated fronds lost higher amounts of biomass due to grazing compared to any other treatment (Fig 2), and this was statistically significant in March only (2-ways ANOVA, see Table 1B). There was no significant differences in consumption rate of the co-incubated fronds. There was a significant effect of season, with a higher biomass loss in March than in December.

Elicitation treatment has no effect on algal consumption

Season did not have a significant effect on algal weight gain after Elicitation treatment. Therefore, frond weights were corrected by r = 2.1% after two days and r = 2.9% after four days. There was higher herbivore consumption during the August experiment than in November, however the experiment was made in slightly different ways in August and in November. Therefore, this comparison between seasons must be considered with caution. In both seasons, no effect of elicitation, either direct (E) or indirect (IE), was detected on herbivore consumption rate. The same pattern was observed after two days of bio-assay (Fig 3), for both March (1-way ANOVA; F=0.28, p=0.84) as well as November (1-way ANOVA; F=0.49, p=0.69), and after 4 days of bio-assay (Fig S1), (F=0.37, p=0.77 in March; F=0.81, p=0.50 in November).

Metabolic changes after direct and indirect grazing

Global PLS-DA, including data of 514 selected ions from all the samples analyzed in negative ionization mode, did not discriminate any Grazing treatment at any sampling time (Fig 4A). However, pairwise comparison showed that grazed kelps (DG) were clearly discriminated from controls without grazer (C_{DG}; Fig 4B). The same pattern was observed when comparing indirect-grazed kelps (IG) with the respective controls (C_{IG}; Fig 4C), as well as when comparing co-incubated algae (IG) with directly grazed (DG; Fig 4D), evidencing that different metabolic regulations occurred after the different Grazing treatments.

When focusing on the kinetics, some ions which discriminated the four Grazing treatments, in particular compounds like fatty acids and oxylipins, known to be generated in response to an oxidative stress (Ritter et al., 2014). Their relative amounts (obtained after normalization) are represented in Figure 5 in

the different Grazing-treated kelps, during the four sampling times. Eicosapentaenoic acid (EPA) was mostly induced in grazed kelps from 24h of grazing, and its relative amount increases until at least 48h compared to control kelps. The oxylipin 13- hydroxy-9Z,11E-octadecadienoic acid (13-HODE) was mainly produced in C_{DG} and DG kelps after 6h of grazing. After 24h, IG kelps produced the highest amount of 13-HODE, two times more than its control C_{IG}. At 48h, the amount of this compound decreased in IG kelps, more than three times less than in the DG and C_{IG} kelps. Two phytoprostanes (Fig 5 and S2) were also produced in higher amount in the DG kelps after 6h of grazing, and one of maintained a constant relative amount from 12 to 48h of grazing, while the other dropped at 48h. On the contrary, IG kelps had a lower induction of one phytoprostane (Fig 5) at 6h of grazing, but then reached higher relative amount as in DG kelps, and finally decreased at 48h. Co-incubation with control kelps also induced production of this phytoprostane after 12h. Prostaglandin J (Fig 5) was induced in all Grazing-treated kelps after 6h, and then decreased after 24h, except in IG kelps at this sampling time. The same pattern was observed for prostaglandin B (Fig S2), except that its relative amount remained high until 24h in DG kelps.

Discussion

Seasonality of algal weight gain

Besides grazing stress, *Lessonia spicata* lives in a highly stressful environment, the low intertidal, submitted to strong variation in temperature, brightness or salinity, which influence its physiology as well as its metabolism. Here we demonstrate that season has a significant effect on algal weight gain after a Grazing treatment. In central and northern Chile, coastal environments are under the influence of upwelling events, which are stronger during austral spring and early summer (i.e. between September and January; Vasquez et al., 1998; Blanco et al., 2001; Vega et al., 2005). Upwelling brings a massive supply of nutrients and cools down seawater's temperature nearshore, strongly stimulating vegetative growth in kelps (Vásquez et al., 1998). Up to 3.5% of weight increase after 4 days of laboratory experiment suggests that the experimental set-up

In November, weight increase was higher for C_{IG} kelps than any other Grazing-treated kelps, which can be interpreted as the consequence of a lower stress induced by the treatment but also the experimental manipulation. Indeed, this was the least manipulated treatment, with no influence of co-incubation of a treated kelp. DG and C_{DG} were comparable in that both were placed in nets, and showed very similar weight gain. On the contrary, IG kelps, co-incubated with DG individuals, had the lowest registered weight gain (although the average value did not differ statistically from the remaining treatments). This result suggests some kind of effect of the hypothesized distant chemical signaling produced by DG kelps.

Interestingly, these differences were not observed during Elicitation experiments. First, the absence of differences among treatments of algal weight gain suggests that Elicitation did not cause any strong physiological effect on the kelps. Second, algal weight gain remained constant during upwelling and non-upwelling seasons for Elicitation experiments, as opposed to Grazing experiments. This strongly suggests that Grazing did cause some stress, and that this stress had more negative effects on growth rate during the non-upwelling season. The great similarity of weight gain between November Grazing experiments and Elicitation experiments (any season) might be interpreted as a higher amount of resources during the upwelling season, allowing grazed kelps (either directly or indirectly through co-incubation) to cope with that level of stress, and maintain their growth rate.

Algal consumption after direct grazing or elicitation

We also showed that algal consumption by *S. scurra* varied according to the different Grazing treatments but not according to Elicitation treatments. This was already observed in the brown alga *Ascophyllum nodosum*, where mechanical damages showed no induction of defenses against herbivores, nor significant changes in phlorotannin levels in algae, compared with grazing by *Littorina obtusata* (Pavia and Toth, 2000). In *Fucus vesiculosus*, direct feeding of *I. baltica* and *L. littorea* induced chemical defence in *F. vesiculosus*, while simulated herbivory (i.e. mechanical damage) did not trigger algal defense (Rohde et al., 2004). Similarly, Coleman et al. (2007), in a choice experiment, demonstrated that artificially damaged *A. nodosum* and naïve fronds were equally attractive to the grazer *Lipophrys pholis*, while grazed algae were clearly more attractive than artificially damaged ones. Thus, it seems that algae are able to discriminate between artificial damage and grazing, and induce specific responses that modify the behavior of the grazer. Our experiments show that *L. spicata* also has this capacity, and that chemical elicitation by oligosaccharides is not an artificial substitute to elicitors of responses to natural grazing by herbivores.

Grazing induced changes in herbivore consumption rate: DG fronds were significantly more consumed than other Grazing-treated fronds after 4 days of bio-assay. This pattern seems contrary to what is expected under the establishment of defense mechanisms. There are actually several examples of positive feedback of grazing rate. Herbivore-induced *A. nodosum* were significantly more attractive to *L. pholis* grazers (Coleman et al., 2007). At least two mechanisms could be hypothesized to explain this pattern. One is that the kelp is not able to cope with the stress caused by the grazing, leading to uncontrolled molecular responses during the oxidative burst. Such kind of response has been evidenced for some abiotic stresses, like heavy metal contamination. For instance, oxidative stress caused by copper contamination in *L. berteroana*, a sister species of *L. spicata*, causes an uncontrolled lipoperoxide accumulation, leading to cell damage and dysfunction (Contreras et al., 2009). Such cellular deregulation is likely limiting the seaweed's capacity to efficiently activate defense mechanisms, providing an opportunity for the herbivores to consume unprotected tissues. An alternative hypothesis

is that the herbivores are actually able to sense the chemical compounds released by the algae during grazing, inducing some kind of behavioral change. Our experiments were conducted with *S. scurra* which is a specialist herbivore selectively using *L. spicata* for shelter and food (Meynard, 2014). The behavior of this herbivore includes digging hole on stipes and holdfasts, and even though the limpets are able to move along stipes and fronds, they always go back to holes during low tide. Therefore, they probably have the capacity to locate the holes, which are grazed tissues, by sensing the chemical cues produced by the holes. Moreover, because specialist herbivores are expected to have the capacity to overwhelm the repellent effect of defense compounds, the increase in consumption rate might actually be a strategy to compensate the loss of food quality. Pavia and Toth (2000) found that phlorotannin levels inside *A. nodosum* tissues increased only after grazing by the fucoid specialist herbivore *L. obtusata*, but not after grazing by a generalist grazer. In this case, increase in toxic compounds in the tissues is associated with an increase in consumption rate, probably as a compensatory process. Therefore, the increase in consumption rate of *S. scurra* is not indicative of a failure of defense mechanisms. Rather, it shows that the herbivore senses the state of the algae, and modifies his feeding behavior accordingly.

Interestingly, the increase in consumption rate was statistically significant only in March, which is when upwelling is weaker. We have shown that growth rate is reduced in March. Altogether, this could suggest that *L. spicata* is less able to produce defense compounds against grazers than in upwelling periods. This is in agreement with the study of Molis et al. (2006) which shows that season has an effect on induced defenses in *Ecklonia* against *Littorina* after direct grazing or co-incubation with a grazed kelp. The study made by Rohde and Wahl (2008) showed that grazing by *Idotea* follows a seasonal pattern, with a peak of grazing in summer, then decreasing in early winter. Seasonal variations in chemical composition of kelps could also explain changes of responses against herbivores depending on the period of the year (Schiener et al., 2014). Such hypothesis of seasonal regulation of defense mechanisms would however require further experimental testing to confirm this pattern on *L. spicata*.

Consumption of co-incubated *L. spicata* with grazed or chemically elicited algae

Grazing was not significantly affected by co-incubation with treated (e.g. grazing or elicitation treatment; IG and IE) *L. spicata*. However, the lowest registered values of grazing were observed in kelps co-incubated with DG individuals in November's Grazing experiment. This tendency seems to support the hypothesis that priming exists and increases the protection of the kelps against grazing. Thomas et al. (2011) showed that the kelp *Laminaria digitata* was able to induce more rapidly specific gene regulations when co-incubated with elicited algae, and that they had a different pattern of oxidative burst and volatile emissions. More recently, distant signaling among *A. nodosum* was evidenced with the herbivore *L. obtusata*, which had a lower consumption rate on primed seaweeds (Flöthe and Molis, 2013). Rohde et al. (2004) demonstrated that grazing by *I. baltica* on *F. vesiculosus* released water-

borne cues that triggered induced defenses in neighboring algae, as previously found in *A. nodosum* in response to grazing by *L. obtusata* (Toth and Pavia, 2000).

The fact that we observe a tendency of decreased grazing in IG fronds only in November further reinforces the idea that upwelling, by allowing a higher primary productivity, could also improve protection against different stresses, including grazing.

Metabolic profiling of Grazing-treated *L. spicata*

Grazing differences observed in bio-assays after a Grazing treatment can be associated with changes in algal endo-metabolome. Kelps previously exposed to a direct grazing had a different global metabolome than their respective control, as well as when compared to co-incubated kelps. Actually, the observation of three different metabolic profiles matches the observation of 3 groups of treatments affecting grazing rates (i.e. increase rate in DG, decreased rate in IG, as compared to all the remaining treatments and controls). Changes in metabolite profiles were also showed in the red alga *Gracilaria vermiculophylla* after grazing (Nylund et al., 2011; Rempt et al., 2012) and mechanical wounding (Nylund et al., 2011), with a high increase of prostaglandins compared with the species *G. chilensis* (Rempt et al., 2012). In red algae, Bouarab et al. (2004) found out some chemical compounds produced during challenging by defense elicitation, like polyunsaturated fatty acids, prostaglandins as well as oxylipins which confer higher resistance to the alga when challenged by a green algal endophyte. Moreover, Ritter et al. (2008) found that prostaglandins were produced after 8 hours of exposure to copper in *L. digitata,* as well as high concentrations of 13-HODE, and a wide range of EPA derivatives upon copper stress. 13-HODE was also found in the kelp *L. digitata* after challenge with lipopolysaccharides (LPS) from bacteria (Küpper et al., 2006) and in the red alga *Chondrus crispus* after exposure to *A. operculata* elicitors (Bouarab et al., 2004). EPA was already shown to be retained in damaged cells (Leflaive and Ten-Hage, 2009) and linked to polyunsaturated aldehydes (PUAs) released in the medium via lipoxygenases (Fontana et al., 2007) to prevent further attack around the organisms. In previous studies, EPA have been shown to be released in high amounts by diatoms upon cell damage, and highly toxic for crustacean herbivores (Jüttner, 2005). Galloway et al. (2012) and van Ginneken et al. (2011) showed that brown and red algae shared a basic common abundance of EPA, including *Laminaria hyperborea*. More recently, phytoprostanes were found in a wide variety of macroalgae (Barbosa et al., 2015). Other compounds like phlorotannins were also identified in brown algae (Toth and Pavia, 2000) in response to grazing, but it was suggested that they were not the sole compounds responsible for the anti-herbivory activity.

Conclusion

Thus, algal defenses in response to herbivores depends on the period of the year. Indeed, during upwelling season in Chile (September - January), when nutrients and light are widely available for kelps, growth and weight gain of kelps are more important than in the rest of the year. Kelps may then defend more efficiently against herbivores in this season. On the contrary, non-upwelling season conditions would not allow previously grazed kelps to defend efficiently, leading to weakening of the kelp and invasion of *S. scurra* herbivores on one *L. spicata* plant. In upwelling season, these directly grazed kelps could warn their neighbors via waterborne cues, which might decrease consumption of these neighbors during a subsequent grazing. However, these mechanisms do not seem to be occurring under chemical Elicitation. Indeed, whatever the season, direct and indirect Elicitation do not seem to modify algal weight gain, nor defense responses against grazers. This might suggest that the kelp is able to discriminate between different kinds of tissue damage by activating different metabolic pathways, and that the herbivore is sensitive to the specific metabolic status of the kelp.

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Figure legends

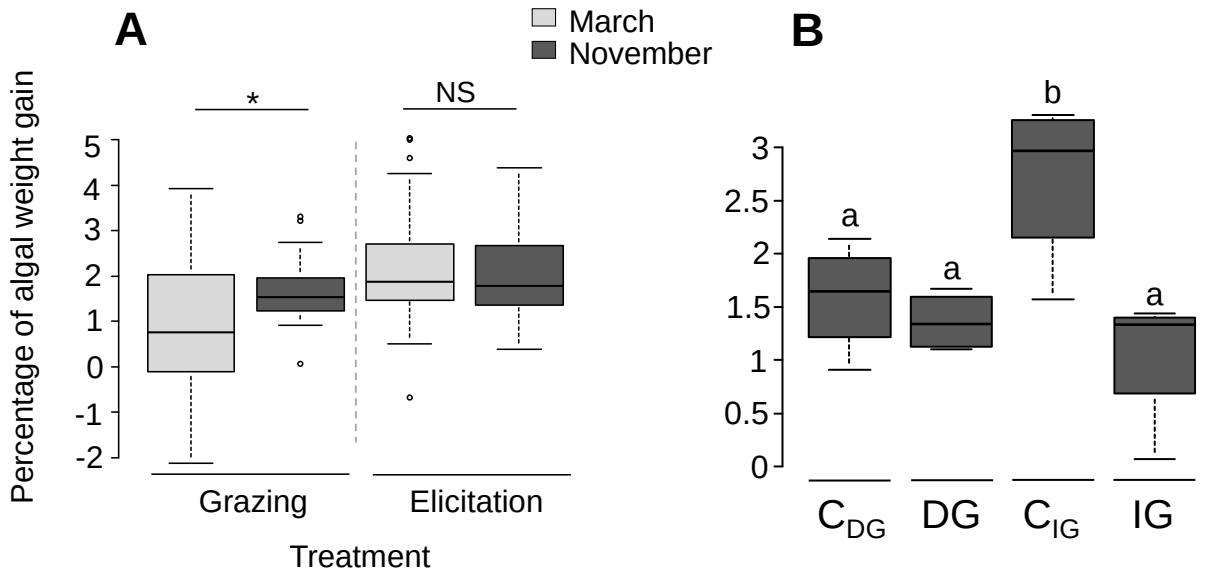

Fig. 1. Percentage of weight gain of *L. spicata* A) Weight gain in March and in November, following Grazing or Elicitation treatment. Significance of mean difference is indicated: *p<0.05, NS = p>0.05, as determined from 1-way ANOVA tests. Grazing experiment, n=40 in March, ==16 in November; Elicitation experiment, n=36 in March, n=15 in November. B) Weight gain of kelps exposed to the different Grazing treatments C_{DG}, DG, C_{IG}, IG in November; n=4 for each treatment. Letters shared in common between the groups indicate no significant difference (ANOVA, p<0.05; SNK test).

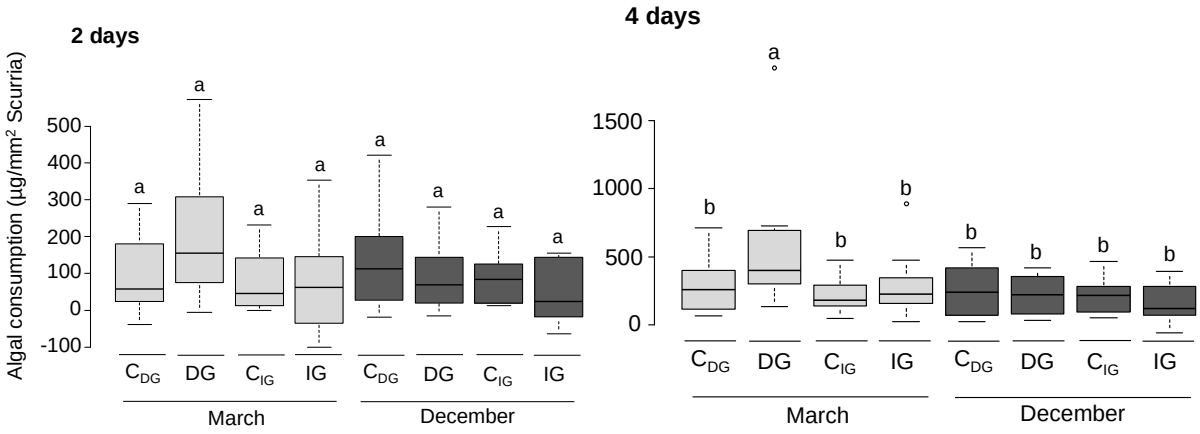

Fig. 2. Algal consumption of *L. spicata* by *S. scurra* after Grazing treatment in March (light grey) and in December (dark grey) after two and four days of bio-assay. DG: Direct Grazing (one frond is directly exposed to grazing by *S. scurra*); C_{DG}: Control Direct Grazing (a frond treated as DG but without any grazer); IG: Indirect Grazing (co-incubated with the DG frond); C_{IG}: Control Indirect Grazing (co-incubated with the C_{DG} frond, without grazer). March and December; n = 10 per treatment. Letters shared in common between the groups indicate no significant difference (2-ways ANOVA, $p<0.05$, and SNK post-hoc test).

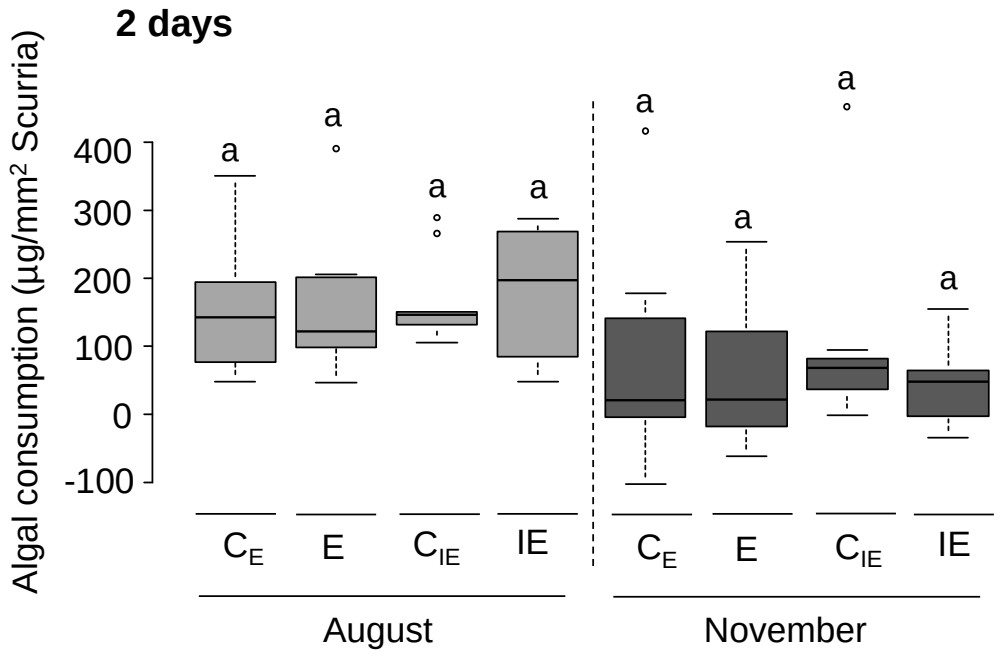

Fig. 3. Algal consumption of *L. spicata* by *S. scurra* after elicitation treatment in August (middle grey) and in November (dark grey), after 2 days of grazing, according to the different Elicitation treatments: E: Elicitation (one frond is directly exposed to elicitation by oligoalginates); C_E: Control Elicitation (a frond treated as E but without elicitation); IE: Indirect Elicitation (co-incubated with the direct elicited alga); C_{IE}: Control Indirect Elicitation (co-incubated with the control kelp, without elicitation). August: C_E, n=9; E, n=9; C_{IE}, n=10; IE, n=10; November: C_E, n=10; E, n=10; C_{IE}, n=10; IE, n=9.

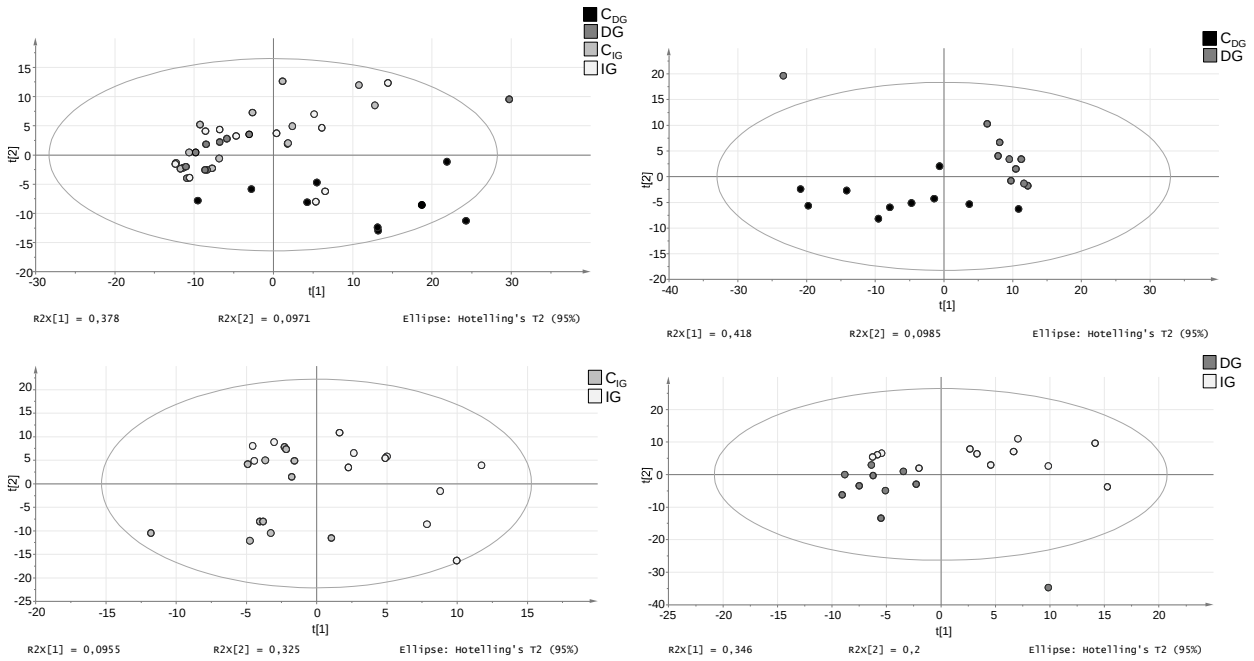

Fig. 4. Results of partial least squares discriminant analysis (PLS-DA) carried out for the compounds detected in the Grazing experiment. The plot represents the variations of 514 monoisotopic peaks quantified by UPLC-MS in negative ion mode in algal samples after 6h, 12h, 24h and 48h of Grazing treatments C_{DG} (black spots), DG (dark grey sports), C_{IG} (light grey spots) and IG (white spots).

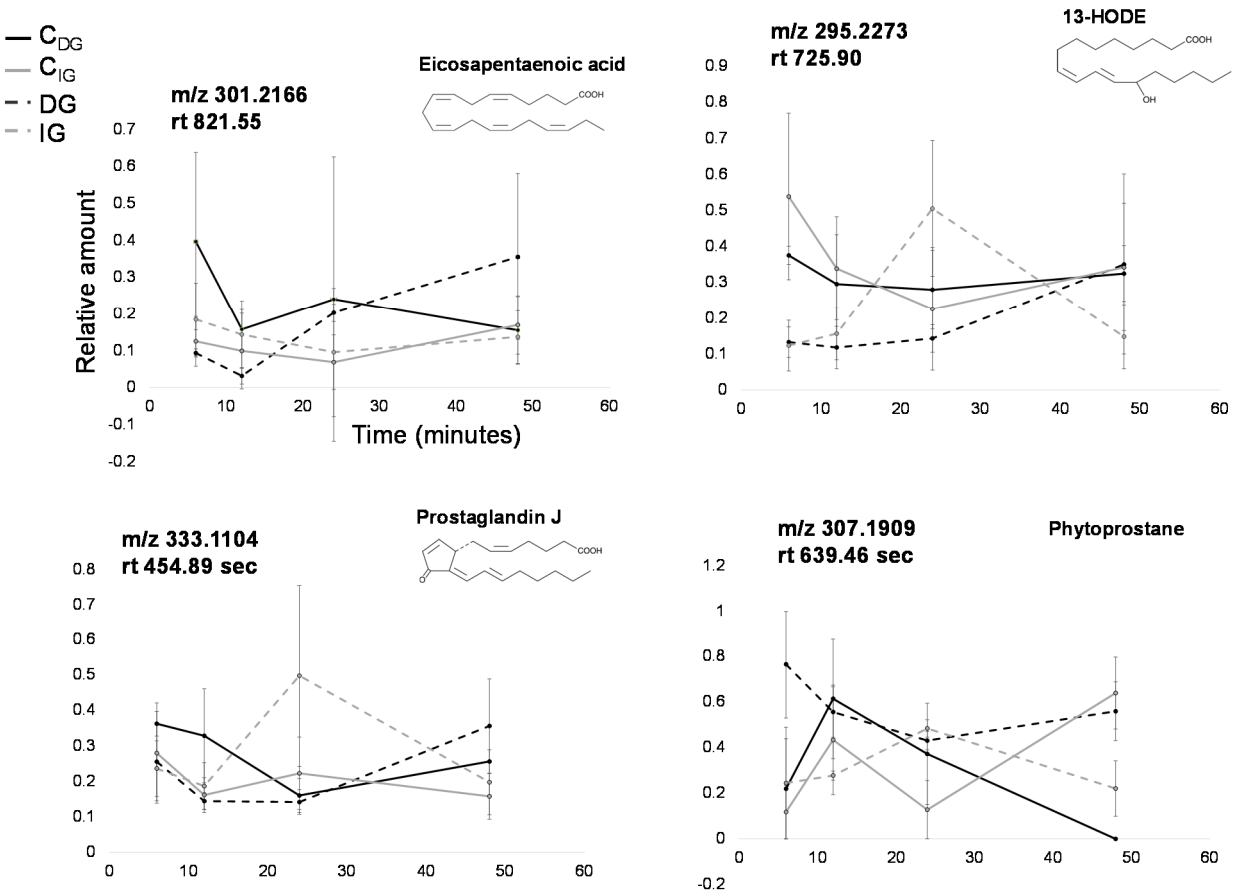

Fig. 5. Relative amounts of four annotated compounds from the endometabolome of *L. spicata* over 48 hours, according to the treatments C_{DG} (black), DG (dark grey), C_{IG} (middle grey) and IG (light grey). Putative corresponding compounds are indicated for each selected ion. They were annotated as eicosapentaenoic acid (EPA), 13-HODE, prostaglandin J and a phytoprostane. C_{DG}: 6h: n=3, 12h: n=2, 24: n=3, 48h: n=2; DG: 6h: n=2, 12h: n=3, 24: n=2, 48h: n=3; C_{IG}: 6h: n=3, 12h: n=3, 24: n=3, 48h: n=3; IG: 6h: n=3, 12h: n=3, 24: n=3, 48h: n=3. Means ± se.

Table 1. ANOVA results of season and treatment effects on algal consumption after Grazing treatment after 2 days (A) and 4 days of grazing (B).

A

Source	Df	Sum	MS	F	P
Season	1	10963	10963	0.8294	0.3655
Treatment	3	85599	28533	2.1586	0.1003
Season*Treatment	3	55244	18415	1.3931	0.2519
Residuals	72	951703	13218		

B

Source	Df	Sum	MS	F	P
Season	1	344831	344831	5.7549	0.01903
Treatment	3	388993	129664	2.164	0.09969
Season*Treatment	3	367141	122380	2.0424	0.11553
Residuals	72	4314174	59919		

Supporting information

S1 Fig. Algal consumption of *L. spicata* by *S. scurra* after elicitation treatment in August and in November, after 4 days of grazing, according to the different Elicitation treatments E, C_E, IE and C_{IE}.

S2 Fig. Relative amounts of two ions annotated as one phytoprostane and prostaglandin B over 48 hours, according to the treatments C_{DG}, DG, C_{IG} and IG.

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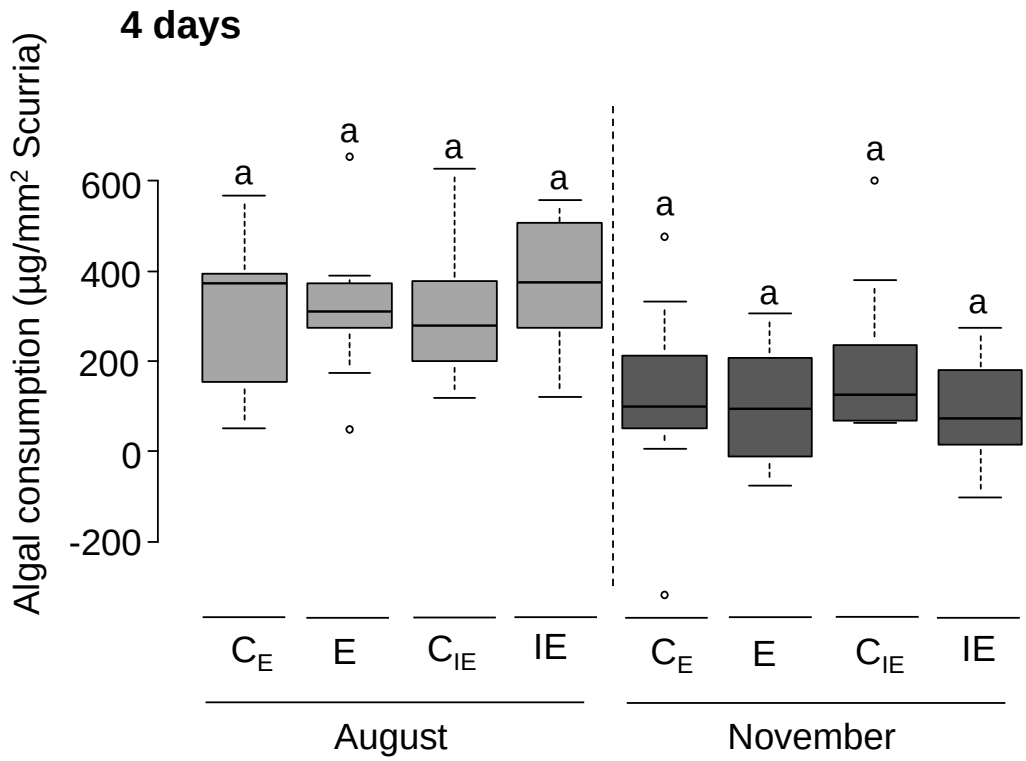
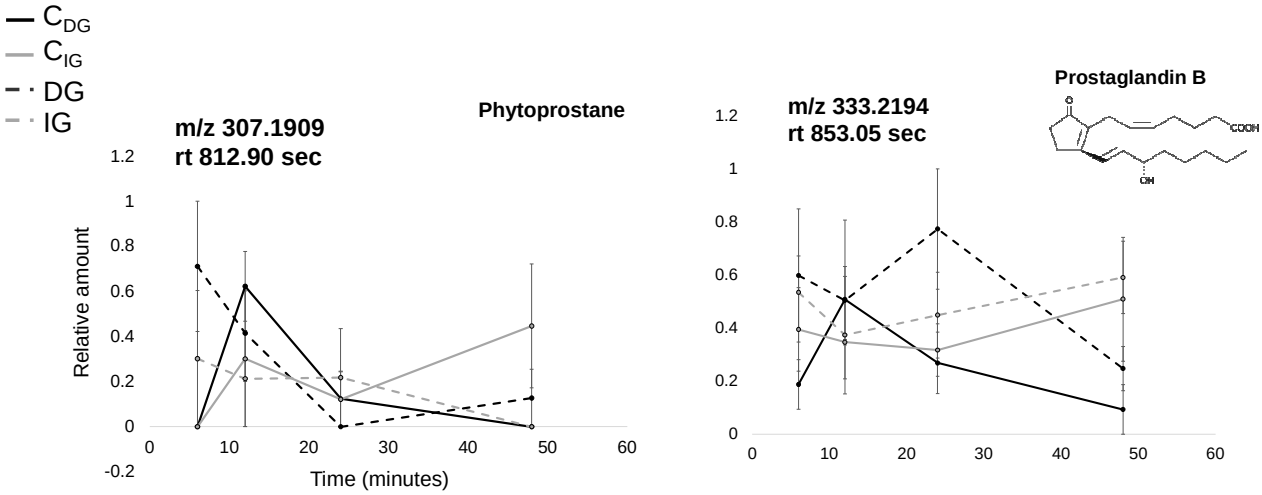
Supporting information



S1 Fig. Algal consumption of *L. spicata* by *S. scurra* after elicitation treatment in August (middle grey) and in November (dark grey), after 4 days of grazing, according to the different Elicitation treatments: E: Elicitation (one frond is directly exposed to elicitation by oligoalginates); C_E: Control Elicitation (a frond treated as E but without elicitation); IE: Indirect Elicitation (co-incubated with the direct elicited alga); C_{IE}: Control Indirect Elicitation (co-incubated with the control kelp, without elicitation). August: C_E, n=9; E, n=9; C_{IE}, n=10; IE, n=10; November: C_E, n=10; E, n=10; C_{IE}, n=10; IE, n=9.



S2 Fig. Relative amounts of two ions annotated as one phytoprostane and prostaglandin B over 48 hours, according to the treatments C_{DG} (black), DG (dark grey), C_{IG} (middle grey) and IG (light grey). C_{DG}: 6h: n=3, 12h: n=2, 24: n=3, 48h: n=2; DG: 6h: n=2, 12h: n=3, 24: n=2, 48h: n=3; C_{IG}: 6h: n=3, 12h: n=3, 24: n=3, 48h: n=3; IG: 6h: n=3, 12h: n=3, 24: n=3, 48h: n=3. Means ± se.

II. Impact of defense-induced kelp compounds on herbivore feeding behavior

1. Effects of oligoguluronate elicitation of *L. digitata* on algal consumption by a generalist herbivore

The choice test with *L. digitata* and *H. tuberculata* allowed to evaluate herbivore feeding behavior through the measurement of algal consumption by abalones. Algal mass after 2 and 4 days of grazing was subtracted to the initial algal mass in each feeder, constituting the consumption for each feeder. As two feeders of the same treatment were placed in each tank, the consumptions of the two feeders containing the same algal treatment in the same tank were added. Algal consumption according to the treatment applied to the kelps, represented in Fig 19, showed that elicited kelps tend to be more consumed by abalones than control kelps, although the difference was marginally significant (t-test, t = -2.27, df = 8, p-value = 0.053).

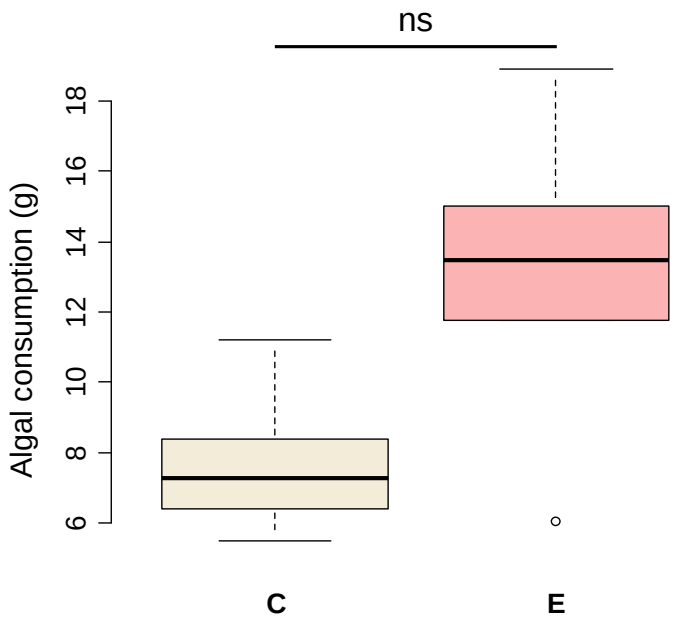


Figure 19. Consumption of control and elicited *L. digitata* by abalones for 4 days. ns indicates that consumption between the algal treatments is not significantly different (t-test, α=5%). n=5.

2. Effects of direct and indirect grazing of *L. spicata* on attraction of a generalist herbivores

These experiments were implemented as part of Youssef Yacine's internship. Two-choice tests were performed, using two algal food sources to assess herbivore attraction for one of the two choices. Results of choices of the herbivore *T. atra* between two algal treatments are shown in Table 4 and Fig 20. Treatment (A) corresponds to 36 h of grazing with a mean of 1.2 *T. atra* individuals per frond and 24 h of co-incubation between DG and IG kelps, while treatment (B) corresponds to 24 h of grazing with a mean of 2.5 *T. atra* individuals per frond and 24 h of co-incubation between DG and IG kelps. Table 4 presents the number of choices for each option and the p-value associated with the multinomial tests performed on each experiment. The non-choice option was included in statistical tests (multinomial tests). Surprisingly, *T. atra* significantly moved more often towards the compartment with no alga (None), than the one with healthy algae (C_{DG}). They also preferred previously grazed algae by *T. atra* with treatment $^{(B)}$ than the corresponding controls (C_{DG}) and than co-incubated algae with grazed kelps (C_{IG}) with treatment $^{(B)}$. However, these differences were not found after a previous grazing by *S. scurra* and after co-incubation with kelps grazed by *S. scurra.*

It is possible to conclude from these choice experiments that there are chemical communications among the kelp and the seaweed, and that these cues modify the behavior of the generalist herbivore *T. atra*. Interestingly, the communication among kelps modifies the signal perceived by the herbivore, with a clear attraction by grazed individuals, and a possible repulsion by primed and control individuals. And finally, the communication seems herbivore-dependent, as the experiment conducted on the specialized herbivore *S. scurra* did not induce any choice for further grazing.

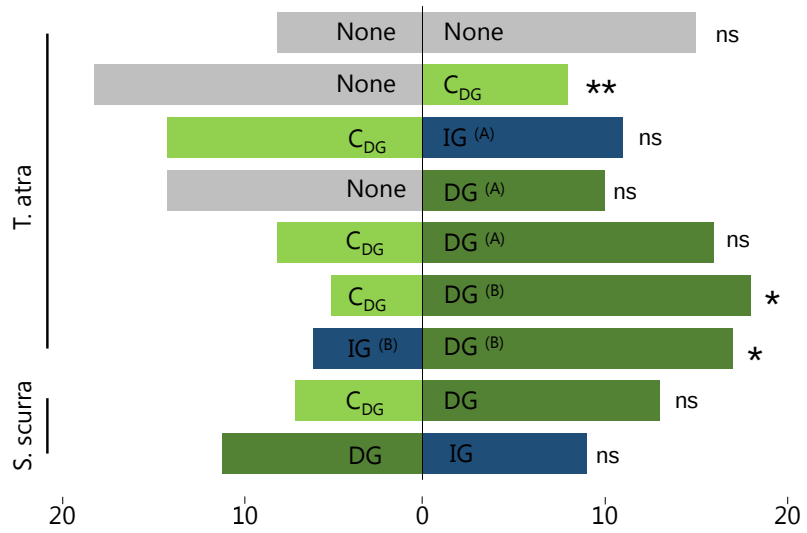


Figure 20. Results of the two-choice test between *L. spicata* and *T. atra* or *S. scurra* for either nothing (none) or seaweeds treated as Control Direct Grazed (CDG, a frond treated as DG but without any grazer), Direct Grazed (DG, one frond is directly exposed to grazing) or Indirect Grazed (IG, co-incubated with the DG frond). For the first choice tests, grazing and co-incubation treatments were performed using *T. atra* and for the lasts, *S. scurra* were used. Treatment (A) corresponds to 36 h of grazing with a mean of 1.2 *T. atra* individuals per frond and 24 h of co-incubation between DG and IG kelps, while treatment (B) corresponds to 24 h of grazing with a mean of 2.5 *T. atra* individuals per frond and 24 h of co-incubation between DG and IG kelps. Statistical analyses were performed using a multinomial test which considered 3 choices: choice A, choice B or non-choice. Significance of mean difference is indicated: **p<0.01, *p<0.05, NS = p>0.05, as determined from multinomial tests. n=30.

Table 4. Choices of *T. atra* or *S. scurra* between either nothing (none) or *L. spicata* treated as C_{DG}, DG or IG. Treatment (A) corresponds to 36 h of grazing with a mean of 1.2 *T. atra* individuals per frond and 24 h of co-incubation between DG and IG kelps, while treatment (B) corresponds to 24 h of grazing with a mean of 2.5 *T. atra* individuals per frond and 24 h of co-incubation between DG and IG kelps. Each experiment was repeated 30 times, using a new herbivore for each choice test. Statistical analyses were performed using multinomial tests which tested the choices between choice A, choice B and non-choice.

Choice A	Choice B	Number of choice A	Number of choice B	Number of non choice	Multinomial test (p-value)	
None	None	8	15	7	0.185	
None	C_{DG}	18	8	4	0.0068	**
C_{DG}	IG (A)	14	11	5	0.1117	
None	DG (A)	14	10	6	0.219	
C_{DG}	DG (A)	8	16	6	0.0724	
C_{DG}	DG (B)	5	18	7	0.0103	*
IG (B)	DG (B)	6	17	7	0.0331	*
C_{DG}	DG	7	13	10	0.4292	
DG	IG	11	9	10	973	

3. Effects of natural wounds on *L. spicata* stipes on *S. scurra* attraction

As *S. scurra* inhabit the wounds that they form in natural conditions, our hypothesis was that they could be more attracted to the wound through chemical compounds coming from this damaged site. We collected wounded and healthy *L. spicata* stipes in the field, placed one *S. scurra* individual in the middle of the stipe and monitor their movement along the stipe in the lab. On wounded stipes, *S. scurra* individuals could choose between the wounding site and other locations on the stipe of *L. spicata*, while healthy stipes were used as controls to test left or right preference. Position of *S. scurra* on the stipe was noted after 30 min, 1 h and 16 h observation. Despite the low number of replicates ranging from 10 (30 min and 16 h observation) to 20 (1 h observation), herbivores did not preferentially choose the wounding site rather than other parts of the stipe (Fig. 21). Moreover, it seems that their preference for one side of a healthy stipe increased with time.

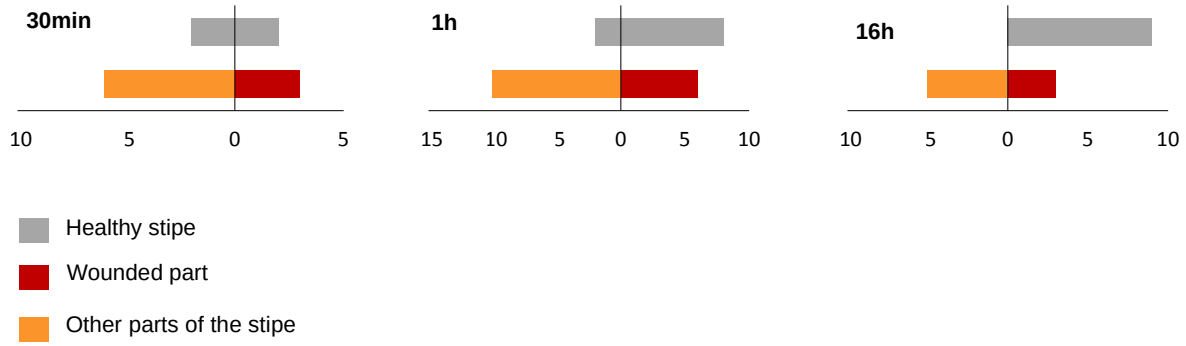


Figure 21. Number of observations for choices of *S. scurra* on *L. spicata* stipes. Choices on wounded stipes could be made between the wounded site and other parts of the stipe, while healthy stipes were used as controls, to test left or right preference. The herbivore was placed in the middle of the stipe and its position was noted after 30 min (n=10), 1 h (n=20) and 16 h (n=10).

4. *In situ* comparison of natural and artificial wounding effects on *L. spicata* stipes on *S. scurra* attraction

This experiment was performed as an extension of the previous one but in the field, directly involving natural conditions. Our hypothesis was that natural wounds would emit chemical compounds allowing herbivores to recognize their habitat and thus attracting them, while artificially damaged stipes would not be perceived in this way and would not be attractive for the grazer. This *in situ* experiment is a preliminary test of the attractive effect of a wound on *S. scurra* behavior, which implied a monitoring of herbivore position in wounds on *L. spicata*. Five individuals of *S. scurra* were removed from their hole during low tide, and repositioned on the kelp, either in the same hole, on an artificial wound, on the stipe close to the holdfast, or on the stipe close to the frond. Results presented in Tables 5 and 6

showed that there was no clear attraction pattern of *S. scurra* by artificial or natural wounds on *L. spicata* stipes. Observations of *S. scurra* in their wound on *L. spicata* stipes (F) showed that they all stayed in the wound during 5 days. Herbivores that were removed and put back in their wound generally stayed in their wound, suggesting no grubbing-up effect on herbivore behavior (C). After artificially wound the stipes, a natural colonization by *S. scurra* did not seem to occur, as no herbivores were found after 1 and 5 days (B) and a grazer placed on an artificial wound did not stay in this place (A). Moreover, when a grazer was placed outside its wound on the same stipe (D) or on a neighboring stipe (E), it generally did not go back to this wound after 1 day, but one grazer could return to the hole after 5 days. We observed that removing individuals of *S. scurra* during low tide turned them unable of reattaching rapidly to kelp surface. It is likely that they require some kind of acclimation in running seawater before being manipulated. However, besides these experimental limitations, it seems that natural holes (i.e. caused by *S. scurra* themselves) are more attractive than artificial wounds. Further experiment should examine more carefully this phenomenon, as well as the determinants of the choice of a place on a stipe to dig a new hole.

Table 5. *In situ* treatments of *L. spicata* tested on attraction of *S. scurra*.

A	**Attraction of an artificial wounding 24h after wounding generation**
	5 *L. spicata* with artificial wounding made at D0
	1 *S. scurra* added on the wounding at D1
B	**Control artificial wounding**
	5 *L. spicata* with artificial wounding made at D0
	No addition of *S. scurra*
C	**Control of grubbing-up effect**
	5 *L. spicata* with natural wounding and 1 *S. scurra* into the wounding
	Removing and putting back the herbivore in the wounding
D	**Attraction of the natural wounding**
	5 *L. spicata* with natural wounding and 1 *S. scurra* into the wounding
	Removing and putting the herbivore outside the wounding, on the same stipe
E	**Natural recolonization**
	5 *L. spicata* with natural wounding and 1 *S. scurra* into the wounding
	Removing and putting the herbivore on a neighboring stipe on the same *L. spicata* individual
F	**Observation of S. scurra in its wounding**
	5 *L. spicata* without treatment

Table 6. Results of the in situ experiment after 1 and 5 days, for each treatment applied to the kelps.

	Day 1	**Day 5**
A	nothing	nothing
	on the same stipe outside the wounding	basis of the stipe
	nothing	nothing
	on the holdfast	on the holdfast
	nothing	nothing
B	nothing	nothing
	nothing	nothing
	nothing	nothing
	nothing	nothing
	nothing	nothing
C	into the wounding	*L. spicata* individual not found
	into the wounding	into the wounding
	2 *S. scurra* into the wounding	into the wounding
	into the wounding	into the wounding
	nothing	nothing
D	basis of the stipe	basis of the stipe
	nothing	into the wounding
	into the wounding	into the wounding
	nothing	nothing
	nothing	nothing
E	nothing	into the wounding
	basis of the neighboring stipe	*L. spicata* individual not found
	nothing	nothing
	nothing	basis of the stipe
	L. spicata individual not found	nothing
F	into the wounding	into the wounding
	into the wounding	into the wounding
	into the wounding	into the wounding
	into the wounding	into the wounding
	into the wounding	into the wounding

Discussion

1. Constraints in ecology experiments

All the experiments of this chapter share the common constraint of biological variability and influence of uncontrolled environmental conditions usually faced in ecology experiments. For preliminary and exploratory experiments (experiments c) and d)), the low number of replicates generally did not allow to draw solid conclusions, as statistical tests could not be applied. The *in situ* experiment on attraction of *S. scurra* by *L. spicata*'s wounds was performed on only 5 individuals of *L. spicata* per treatment. Moreover, as this experiment was performed in the field, many parameters could not be controlled, such as the effects of the tide and the waves or the ambient temperature, and the likely requirements of high tide conditions. This experiment should thus be repeated several times in order to get a better estimation of the effects of algal wounds on herbivore attraction. In non-choice feeding assays, the algal biological variability was added to the season effect, but mostly by the biological variability due to the herbivore (genetic variability, age, appetite, biological rhythm possibly influenced by the rhythm of tides,…) and especially to the interaction between the herbivore and its algal host. Thus, biological replicates have to be numerous in order to explicitly take into account the interaction of these parameters on the effects of direct and indirect grazing, and algal-derived distance signaling.

If results showed that *T. atra* was clearly attracted to already grazed *L. spicata* by conspecifics for 2 days, but lab and field experiments testing *S. scurra* attraction for natural or artificial wounds on kelp stipes did not give evident conclusions. These experiments did not show a clear attraction of *S. scurra* for artificial and natural wounds. *S. scurra* did not seem to recognize a natural wound, but seemed to stay in the wound that it shaped itself. The chemical compounds of the grazed tissues inside the wound could thus be very localized and internalized without being diffused in the surrounding environment. This hypothesis could explain the absence of natural recolonization of artificial wounds during the experiment, as well as the absence of new holes in stipes already colonized by a *S. scurra*.

2. Effects of direct and indirect grazing on consumption by grazers and on algal metabolic regulations

Despite this variability, some conclusions can be noted on deterrence of *L. spicata* on *S. scurra* and the metabolic regulations into algal tissues. Results on consumption of *L. spicata* by herbivores in non-choice bio-assays showed that both the algal physiology and the activity of grazers were different according to the season. Indeed, algal weight gain was higher in November while consumption by herbivores increased in March. This seasonal effect could be explained by the upwelling occurring between September and January, which provide high amounts of nutrients and fresh water are provided to the surface, possibly leading to a higher growth rate and resistance to herbivores compared with the

non-upwelling season (from February to August). Moreover, algal resistance can also be modified, as the chemical composition of algal tissues was already shown to change according to the period of the year.

Analyses of the endo-metabolome of *L. spicata* allowed to discriminate a grazed alga from its co-incubated neighbor, and from an ungrazed healthy alga over 48h. Thus, metabolic modifications occur not only in algal tissues upon grazing but also, and differently, after co-incubation with a grazed alga. Further studies allowed to annotate some compounds as fatty acids and oxylipins, suggesting that the fatty acid metabolism was activated and regulated upon grazing and co-incubation with a grazed kelp, and that the co-incubated kelp could possibly perceive a chemical signal from the grazed kelp. However, these annotation hypotheses still have to be verified through LC-MS/MS analyses and comparison with chemical standards.

3. Effects of defense-induced kelp compounds on herbivore feeding behavior

Complementary experiments on herbivore preference upon grazing stress in kelps could highlight that previously grazed or elicited algae were preferred for generalist herbivores compared to ungrazed or control algae, for both *L. digitata* and *L. spicata*. This result first showed that herbivores were able to perceive different signals coming from stressed algae and control algae, then choosing the stressed kelps. These chemical compounds emitted following a stress (elicitation or grazing) could therefore allow herbivores to better locate an edible food source, i.e. previously consumed. Our results were also consistent with previous observations on *A. nodosum* and *L. japonica* which responded differently according to the herbivore, changing their metabolic and ecological responses (Pavia and Toth, 2000; Molis et al., 2008). Our results could appear inconsistent with previous ones performed on the Fucale *F. vesiculosus*. In this case, two-choice feeding assays showed that ungrazed *F. vesiculosus* were significantly preferred to grazed algae by the generalist isopod *Idotea baltica* and by *Littorina obtusata*, but only after 9 and 15 days, respectively, of previous grazing (Flöthe et al., 2014). Feeding bio-assays showed that previously grazed *F. vesiculosus* were less palatable compared with non-grazed controls. However, the timing of our experimental set-up was significantly shorter, with a previous grazing period lasting only 2 days. Interestingly, in similar conditions (3 days of previous grazing), both *I. baltica* and *L. obtusata* showed a slight preference towards fresh grazed *Fucus* (Flöthe et al., 2014). Thus, responses to grazing are not universal as they strongly depend on the herbivore and algal species, and also vary according to time and season. There is still a lack of studies in order to understand the effect of each parameter which can favor or deter the herbivore.

Our two-choice bio-assay also suggested that the generalist herbivores *T. atra* preferred previously grazed *L. spicata* compared to their co-incubated neighbors. This observation could be

explained by the stronger recognition of freshly grazed algae by herbivores, letting the intact neighboring algae (indirect grazed) imperceptible for them. Another hypothesis would be the deterrent perception of the indirect grazed kelps by the herbivore, leading herbivores to choose grazed kelps. However, according to our results on metabolic regulation upon grazing pressure (Chapter I and II), a short grazing period (up to 2 days) was sufficient to induce *de novo* biosynthesis of compounds, such as fatty acids and oxylipins, that could be attractive for neighboring herbivores. On the contrary, a longer grazing stress (more than one week) would lead to the production of deterrent compounds and anti-herbivory protection, as shown in *Fucus* (Flöthe et al., 2014). This early attractive effect could also depend on the type of herbivore: *T. atra* seemed to recognize kelps that were grazed by its own species, while not discriminating kelps grazed by the specialist herbivore *S. scurra*. This specific recognition could be linked to a chemical recognition of grazed *L. spicata* according to the herbivore species or the relationship between each herbivore species and this algal species. Thus, depending on the fact that the herbivore is either generalist or specialist of *L. spicata*, on the algal species (for instance between Laminariales and Fucales) and on the timing of algal induction, the chemical signature of compounds emitted by a direct grazed or an indirect grazed kelp could be different.

Chapter III

Aldehyde-based signaling and defense of kelps against herbivores

Introduction

In response to grazing by herbivores, Eukaryotes evolved defense mechanisms of innate immunity. These comprise the recognition of elicitors through innate receptors, followed by a transient oxidative burst, involving the activation of the membrane-associated enzyme NADPH oxidase and a rapid apoplastic production and accumulation of reactive oxygen species (ROS) like O^{2-} and H_2O_2 in the surrounding environment (Conrath et al., 2002). These first steps of innate immunity, also present in algae, allow recognition between self (or Microbe-Induced Molecular Patterns; MIMPs) and non-self (or Microbe-Associated Molecular Patterns; MAMPs) and lead to defense responses (Weinberger, 2007). In the kelp *L. digitata*, specific derived products from alginates, the oligoguluronates (GG), constitute MIMPs and were shown to trigger an oxidative burst including a release of H_2O_2, which had anti-microbial effects (Küpper et al., 2001). Oligoguluronates were also shown to induce a protection of the kelp from infection by the endophyte *Laminariocolax tomentosoides* within 7 days, when kelps were previously elicited (Küpper et al., 2002).

Moreover, mechanisms of innate immunity in Eukaryotes include the release of free fatty acids and their oxidized derivatives, named oxylipins. Brown macroalgae produce oxylipins from C18 and C20 fatty acids, which could be involved in algal defense (Potin et al., 2002; Pohnert, 2004). Treatment of *L. digitata* thalli with lipopolysaccharides (LPS; cell wall extracts from bacteria) from *Salmonella abortus equi* was shown to induce the production of free fatty acids and oxylipins (Küpper et al., 2006). Moreover, jasmonic acid was described in vascular plants after derivatization of C18 and C20 oxylipins. In an evolutionary point of view, it is likely that jasmonate signaling have evolved between the primary and the secondary endosymbiotic events and is therefore considered as a putative common ancestral trait of photosynthetic Eukaryotes (Bouarab et al., 2004). Most of studies on biosynthetic pathways of the fatty acid and their oxidized derivatives and their involvement in algal defense against herbivores were conducted in microalgae belonging to the Stramenopiles lineage, the diatoms. Several aldehydes, produced enzymatically by lipoxygenases (LOX) and hydroperoxide lyases (HPL), were found to decrease the reproduction success and egg viability of copepods that grazed on diatoms (Pohnert, 2004). In macroalgae, fatty acid derivatives originating from the oxylipin pathway were shown to have attracting roles as sexual pheromones in brown algae (Pohnert and Boland, 2002), as well as defensive roles in red algae against epiphytes (Lion et al., 2006), endophytes (Bouarab et al., 2004) and herbivores (Rempt et al., 2012).

In the kelp *Laminaria angustata*, aldehydes are also produced enzymatically. C6 aldehydes are derived either from C18 or from C20 fatty acid and C9 aldehydes are exclusively formed from the C20 fatty acid, arachidonic acid (Boonprab et al., 2003a; Boonprab et al., 2003b). In *L. digitata*, Küpper et al. (2006) found that free fatty acids and oxylipins were produced upon application of LPS, and it was then demonstrated that a wide range of aldehydes were emitted by this kelp upon elicitation by

oligoguluronates (Goulitquer et al., 2009; Thomas et al., 2011) and that copper stress also induced a strong release of fatty acids, oxylipins and amino acids (Goulitquer et al., 2009; Ritter et al., 2014). Moreover, chemical analyses conducted *in situ* above kelp beds revealed emissions of aldehydes reaching a peak during and after low tide (Goulitquer et al., 2009).

In this chapter, we assessed the roles of aldehyde-based signaling in the context of kelp/herbivore interactions. We investigated the effects of aldehyde signals on both algal consumption by herbivores and on algal metabolism. The first part of this chapter is devoted to the article, which deals with the effects of aldehyde signaling on the interaction between the kelp *L. digitata* and its specialist herbivore *P. pellucida*. We evaluated their effects on algal consumption by their grazers, then on algal physiology by measurement of the oxidative burst and finally the metabolic regulations that they induce in algal tissues through metabolomic approaches. The second part of the chapter consists in complementary studies which evaluated the effects of aldehyde-based signaling on the interaction between the kelp *L. spicata* and the generalist herbivore *T. atra*.

I. Exploring the role of aldehyde-based signaling during kelps/herbivores interactions

General outline

In order to investigate the effects of aldehyde signaling during kelp/herbivore interactions, we applied combined approaches of metabolomics and bio-assays with the kelp *L. digitata* and its specialist herbivore *P. pellucida*. We first evaluated the effects of incubation of laboratory-grown algae in aldehydes on algal consumption by their grazers. Then, we assessed their impacts on algal physiology by measurement of the oxidative burst. We finally analyzed the metabolic regulations that they induced in algal tissues over 24 h after incubation. Results showed that aldehyde incubation induced different effects on later algal consumption by herbivores according to chemical nature, i.e. 4-HHE (C6-aldehyde), 4-HNE (C9-aldehyde) or dodecadienal (C12-aldehyde), and concentration of aldehyde, from 1 µg.mL^{-1} to 10 ng.mL^{-1}. This suggests that aldehyde perception could rapidly affect algal metabolism through different regulation pathways. Moreover, these aldehydes induced metabolic modifications in algal tissues compared to control algae, especially a production of FFAs and their oxidized derivatives, oxylipins. These modifications occurred differently according to time over one day, between early and late time periods. Thus, the results sugget that the perception of specific aldehydes could have a major ecological role on the interactions between kelps and their herbivores.

Article

Exploring the role of aldehyde-based signaling during kelps/herbivores interactions

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Abstract

In the terrestrial environment, volatile organic compounds, such as aldehydes, are emitted during defense responses by plants against herbivores and perceived by neighboring plants as warning distance signals, leading to priming. In the marine environment, similar distance signaling, based on water-borne cues, also exists during interactions between macroalgae and herbivores, but their biological and ecological roles remain unclear. In brown algae, chemical elicitation or grazing induces regulations of transcription and metabolic pathways, as well as emission of chemical compounds like aldehydes. In laboratory, upon stress conditions, the brown alga *Laminaria digitata* emits a wide range of volatile aldehydes, but their biological roles as potential defense signals remain unknown in this alga in response to grazing. In this context, bioassays using the limpet *Patella pellucida* and *L. digitata* from North-Atlantic Brittany are used for determining the effects of algal incubation with 4-hydroxyhexenal (4-HHE), 4-hydroxynonenal (4-HNE) and dodecadienal on algal consumption by grazers. Simultaneously, we have developed metabolomics approaches to study algal metabolic modifications after treatments of *L. digitata* with these aldehydes. The results displayed that, unlike treatment of the kelps with 4-HNE or dodecadienal, treatment with 4-HHE decreases algal consumption by herbivores, only at 100 $ng.mL^{-1}$. Moreover, we showed that algal metabolome is modified according to the type of aldehyde, especially concerning fatty acid degradation pathways. As kelp beds constitute complex ecosystems with roles of habitat and food source for marine herbivores, the perception of specific aldehydes could have a major ecological role on the structuration of marine kelps/grazers communities.

Introduction

In response to pathogen or herbivore attacks, land plants developed capabilities to set up defenses, locally at the point of the infection or of grazing pressure but also at distance by the emission and perception of volatile chemical signals either by the other parts of the plant or by neighboring plants. Other photosynthetic eukaryotic lineages, including red and brown algae, share ancestral innate immunity mechanisms with the Plant lineage, such as the recognition of biotic challenges by detecting Microbe-Induced Molecular Patterns (MIMPs) or Microbe-Associated Molecular Patterns (MAMPs) (Weinberger, 2007). This detection triggers an oxidative burst, corresponding to the activation of the plasma membrane-associated NADPH oxidase and the accumulation of reactive oxygen species (ROS) in the surrounding environment (Conrath et al., 2002), that has also been detected in animals (Torres and Dangl, 2005) and brown algae, such as *Laminaria digitata* in response to oligoguluronates elicitation (Küpper et al., 2001). In this species, the oxidative burst has shown anti-microbial roles, and is involved in the establishment of infection resistance against endophytes (Küpper et al., 2002).

In Eukaryotes, another conserved trait in innate immunity processes is the accumulation and release of fatty acids and oxylipins during defense responses. In land plants, the generation of oxidized fatty acid derivatives proceeds from oxygenation of free polyunsaturated fatty acids (PUFAs) mostly by intervention of lipoxygenases (LOXs), which generate hydroperoxides, until production of aldehydes by hydroperoxide lyases (HPLs) (Feussner and Wasternack, 2002). The derivatives of C18 (octadecanoids) and C16 (hexadecanoids) fatty acids such as salicylic acid, jasmonic acid and ethylene are known as defense hormones involved in intra- or interplant distance signaling (Fu and Dong, 2013; Meng and Zhang, 2013). Marine macroalgae were found to generate C20 and C18 PUFAs and oxidized derivatives during innate immunity responses (Potin et al., 2002; Bouarab et al., 2004). In response to bacterial MIMPs, the kelp *L. digitata* induced a rapid release of FFAs with a simultaneous accumulation of oxidized derivatives of linolenic (C18:2) and eicosapentaenoic acids (C20:5), and oxylipins like 13-hydroxyoctadecatrienoic acid (13-HOTrE) and 15-hydroxyeicosapentaenoic acid (15-HEPE) (Küpper et al., 2006). In this species, the fatty acids linolenic acid (C18:3) and arachidonic acid (C20:4) and the methyl jasmonate (MeJA) have also shown to induce an oxidative burst and also a production of number of FFAs, leading to a protection against algal endophytes (Küpper et al., 2009). In *L. angustata*, aldehydes and hydroperoxides were found constitutively present into algal tissues, such as C9- and C6-aldehydes (Boonprab et al., 2003a; Boonprab et al., 2003b; Boonprab et al., 2006), whereas diverse C6-, C7-, C8- and C9-aldehydes, such as 4-HHE and dodecadienal, were highly released in the surrounding seawater following elicitation by oligoguluronates or in response to oxidative copper stress (Goulitquer et al., 2009; Thomas et al., 2011). During low tides, aldehyde amounts into the seawater and in the air drastically increased above kelps beds, highlighting specific emissions of C6- and C9-aldehydes (Goulitquer et al., 2009) and water-borne cues present in seawater surrounding kelps at low tides have shown to later modify defense responses upon elicitation in young *L. digitata* (Thomas et al., 2011). In

diatoms, poly-unsaturated aldehydes (PUAs) biosynthesized from cell membrane-derived free fatty acids have shown to have a strong biological effect on their herbivores, the copepods (Miralto et al., 1999; Pohnert and Boland, 2002; Ianora et al., 2003).

Kelp beds are the natural sources of a wide range of aldehydes, for which the ecological and biological roles have been hardly explored. In this context, we questioned their potential roles as distance defense signals during interactions between kelps and their herbivores. Thus, we investigated the effects on algal protection against herbivores of three specific aldehydes, namely 4-hydroxy-hexenal (4-HHE), 4-hydroxy-nonenal (4-HNE) and dodecadienal that were previously shown to be strongly induced after diverse abiotic and biotic stresses in *L. digitata* (Goulitquer et al., 2009). In this purpose, we monitored the effects of algal incubation in aldehydes on subsequent consumption of *L. digitata* by its specialist herbivore *Patella pellucida*. We also studied the impacts on algal physiology and metabolism, by following oxidative responses and by analyzing the modifications of the algal endometabolome via metabolic profiling approaches.

Materials and methods

Algal material and herbivore sampling

All experiments were performed using young macroscopic diploid sporophytes of *Laminaria digitata*. Unialgal cultures of *L. digitata* sporophytes were obtained from random crosses of gametophyte batches. The latter were yielded in the laboratory from mature wild sporophytes collected in the same population. Two weeks after crossing, the sporophytes were transferred to 10 L flasks and grown until they reached a size of about 3 to 4 cm, in Provasoli Enriched Seawater (PES) culture media prepared with natural filtered seawater (FSW), collected off shore of Roscoff, as described in Thomas et al. (2011). Algal cultures were illuminated with daylight-type fluorescent lamps at an irradiance of 25 $\mu E.m^{-2}.s^{-1}$ for 10 h per day and kept at 13±1°C.

The blue-rayed limpets *Patella pellucida* of 3-4 mm length were collected either at low tide or by diving, on *L. digitata* fronds. In laboratory, grazers were kept in an aquarium, and fed on *L. digitata* fronds until bioassay. Running seawater was constantly supplied and chilled at 15±1°C, with constant air bubbling in half-light conditions.

Aldehyde and elicitation treatments

Stock solutions of (±)-4-hydroxy-2E-hexenal (4-HHE; 10 $mg.mL^{-1}$) and (±)-4-hydroxy-2E-nonenal (4-HNE; 10 $mg.mL^{-1}$) from Interchim were prepared in EtOH 100% and the solution of 2,4-Dodecadienal (20 $mg.mL^{-1}$) from Sigma was dissolved in distilled water. For one aldehyde exposure, *L. digitata* plantlets were incubated in 50 mL (4 individuals) or in 70 mL (8 individuals) of FSW for 4h at

13°C under continuous agitation. 4-HHE (H treatment), 4-HNE (N treatment), Dodecadienal (D treatment) were added to the plantlets after dilution in 10 µL of EtOH 70%, to obtain a final concentration of 10 ng.mL^{-1} to 1 µg.mL^{-1} according to the aldehyde and the experiment. The control algae were treated in the same way, by adding only EtOH 70% in filtered seawater.

Alginate oligoguluronates (poly-alpha-1,4-L-guluronic acid blocks, GG blocks) were prepared by acid hydrolysis according to Haug et al. (1974), using sodium alginate from *Laminaria digitata* stipes (Danisco, Landerneau, France). Elicitation treatment was performed by placing one *L. digitata* plantlet individually in 30 mL FSW with GG at a final concentration of 150 µg.mL^{-1} for 1 h (E treatment) at 13°C under agitation. Its control was treated equally, with seawater instead of GG solution.

For metabolomic analyses, each aldehyde treatment (H, N, D) was performed at 100 ng.mL^{-1} with 12 plantlets incubated together during 4 h, rinsed and put individually in 30 mL FSW under agitation. Four plantlets (corresponding to 4 pseudo-replicates) were frozen in liquid nitrogen after 4 h incubation in aldehydes (4 h time), and 4 h (8 h time) and 24 h (28 h time) after incubation in FSW. For elicitation treatment (E), the individual elicited and control plantlets were rinsed after 1 h, put in 30 mL FSW under agitation and freeze-dried after 3 h (4 h time) or after 24 h (28 h time).

Before bioassays with herbivores, algal treatments were performed using 8 plantlets incubated together with an aldehyde at different concentrations for 4h, or with GG solution at 150 µg.mL^{-1} for 1 h. Treated and control algae were then rinsed twice in FSW and placed individually in 30 mL fresh FSW for 24 h under agitation.

Hydrogen peroxide measurements

The concentration of hydrogen peroxide in the medium around algae was determined using the luminol chemiluminescence method (Glazener et al., 1991) with a Tristar luminometer (EG&G Berthold, Bad Wild-bach, Germany). During Elicitation (E) and Aldehyde treatments (H, N, D) performed at 100 ng.mL^{-1}, 150 µL of seawater were taken for one measurement, just before incubation in aldehyde or in GG, and then every 5 min during 1 h after addition of aldehyde or GG solutions. Aliquots were put in 96-well microplates (Lumitrac 200, White, Greiner Bio-One). In the luminometer, 50 µl of 20 U.mL^{-1} horse- radish peroxidase (Boehringer Mannheim, Meylan, France), dissolved in pH 7.8 phosphate buffer, and 100 µl of 0.3 M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) solution were added to the sample. Chemiluminescence was recorded immediately after the last injection with a signal integration time of 0.55 s. The concentration of H_2O_2 in nmol.g.FW^{-1} was calculated for each sample based on a standard calibration curve. The H_2O_2 production was estimated by integrating the total amount of H_2O_2 monitored over one hour. The experiment was repeated 4 times for each treatment and 1-way ANOVA was performed, followed by Student-Newman-Keuls (SNK) post-hoc tests for comparisons of the mean production, using the free software R.

Bioassays of algal consumption by grazers

For this 'no-choice' feeding based bioassay, 5 *L. digitata* plantlets were submitted to grazing by *P. pellucida*, while the 3 others were used to control autogenic changes of mass during experiment. Six *P. pellucida* were placed on one plantlet in 50 mL filtered seawater at 13°C for 6 days. Algal mass was measured at the beginning of bioassay before consumption, 3 days and 6 days after exposition to grazing or to nothing for control algae. The experiment was repeated 25 times for GG treated algae, and 5, 10 or 15 times according to the type of aldehyde and to the concentration. The data were analyzed using Kruskal-Wallis tests for comparison of mean consumption between the different treatments, for each aldehyde treatment, using the free software R.

Metabolomic analyses and metabolic profiling of FFAs and their oxygenated derivatives

For each algal treatment (H, N, D, E) and sampling time (4, 8 and 28h), metabolic extracts were obtained from 100 mg of frozen algal powder with 1 mL MeOH:H_2O (8:2), as described in Ritter et al. (in preparation). In each sample, 1.25 µg.mL^{-1} 12-OH-lauric acid was added as an internal standard for analysis in the negative mode. To separate FFAs and their derivatives from polar compounds, samples were first dried with liquid nitrogen at 40°C and metabolic extraction was made with 200 µL of Ethyl acetate and 200 µL H_2O. The ethyl acetate phase was recovered and dried with liquid nitrogen at 40°C. For metabolomic analyses, 90 µL EtOH were added to each sample and aliquots of 50 µl of each extract were used for analyses. Chemical compounds were first separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an Electro Spray Ionization (ESI) source running on the negative ionization mode, as described in Ritter et al. (2014). Metabolic samples were analyzed using a 1.9 µm Thermo Hypersil Gold C18 column (100 x 2.1 mm) and the mobile phase A was composed of 0.1% acetic acid in H_2O, and mobile phase B was 0.1% acetic acid in acetonitrile. The gradient, starting at 0% B, consisted of an initial hold at 60% mobile phase B from 1 to 3 min, followed by a gradient to 95% B in 12 min and a hold for 4 min, followed by re-equilibration for 4 min at 0% B, for a total run time of 23 min. Xcalibur 2.1 software was used for instrument control and data acquisition.

Mass spectra data were processed by XCMS on the online version (Galaxy-Workflow4metabolomics ; Giacomoni et al., 2014), after conversion of raw spectra to mzXML format. Data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples necessary at 0.7. For correction of retention time, the obiwarp method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 20, and 10 for the third group step. Fillpeaks step was used with the chrom filling method. Finally,

annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. The final table contained ion relative abundances in each sample, which corresponded to peak areas normalized by the standard peak area in the same sample. Multivariable statistical analyses were performed using Partial Least Squares - Discriminant Analysis (PLS-DA), and Number of MisClassifications (NMC) and p-value of PLS-DA were obtained, using R. Then, extraction of the 10 most discriminant ions using Variable Importance in Projection (VIP) and putative annotation of some of those ions were made based on previous studies (Ritter et al., 2008).

For the chemical identification of selected compounds, complementary analyses were carried out on the mass spectrometry facility of Corsaire-Laberca (Nantes), using a high performance liquid chromatography (HPLC) system (Ultimate® 3000 Dionex, Thermo Fisher Scientific). In this purpose, the following chemical standards from Interchim were used: jasmonic acid, 9(S)-hydroperoxyoctadecatrienoic acid (9(S)-HpOTrE), 13(S)-hydroperoxyoctadecatrienoic acid (13(S)-HpOTrE), 20-hydroxy-leukotriene B4 and stearidonic acid.The chromatographic separation was performed on Hypersil Gold C18 column (2.1 mm × 100 mm, 1.9 µm particle size, Thermo Fisher Scientific). Mobile phase consisted in water (A) and acetonitrile (B) both containing 0.1 % acetic acid (A). The used elution gradient (A:B, v/v) was as follows: 100:0 from 0 to 1.0 min; 40:60 at 3 min; 5:95 at 15 min for 4 min then 100:0 for 4 min. The injected volume was 5 µL, the flow rate was 350 $\mu L.min^{-1}$ and the temperature of the column was maintained at 35 °C. The LC system was coupled to a Hybrid Quadrupole-Orbitrap Mass Spectrometer (Q Exactive™, Thermo Fisher Scientific) with a heated electrospray ionization source (HESI). Nitrogen was used as sheath gas and auxiliary gas at flow-rates of 55 and 10 a.u (arbitrary units), respectively. The ion transfer tube temperature was set at 350°C, the vaporizer temperature at 300°C and the electrospray voltage was set at 3.0 kV in positive mode and -3.0 kV in negative with a s-lens RF level of 50%. For fullMS acquisition a polarity switching ion mode (positive/negative) with a mass range of m/z 65-975 at a mass resolving power of 70 000 Full Width Half Maximum (FWHM at 200 m/z) are used. The automatic gain control target and maximum injection time were 1e6 counts and 100 ms, respectively. The normalized collision energy (NCE) used for targeted MS/MS and data dependent MS/MS modes was 35. Each target m/z was monitored with a 40 seconds window (retention time ± 20 seconds), 2-amu isolation window (target m/z ± 1 amu). For injection, the extract was diluted by 2 in ACN:H_2O (50:50) and 5 µL were injected the same day on the LC-HRMS system.

Results

Aldehyde incubation showed different effects on later algal consumption by herbivores according to chemical nature and concentration

The effects of aldehydes on algal defense responses against herbivores were first assessed by measuring consumption of *L. digitata* tissues by *P. pellucida* during 3 days (Fig 1), following a 4-hour-incubation in different concentrations of aldehydes and 24 hours in FSW. Oligoguluronate (GG) elicitation of algae was tested in similar conditions. Consumption of elicited algae was higher than consumption of control algae, but not significantly (W = 248, p-value = 0.22). Dodecadienal increased significantly consumption by herbivores at 100 $ng.mL^{-1}$ compared to control (W = 24, p-value = 0.02). 4-HNE did not induce significant changes in consumption by herbivores at 1 $\mu g.mL^{-1}$ (W = 14, p-value = 0.84) and 100 $ng.mL^{-1}$ (W = 17, p-value = 0.42). However, effects of 4-HHE on algal consumption depended on the concentration of this aldehyde applied to the alga. Over 500 $ng.mL^{-1}$, 4-HHE had no significant effect on algal consumption by *P. pellucida* (W = 22, p-value = 0.06 and W = 17, p-value = 0.42 for 1 $\mu g.mL^{-1}$ and 500 $ng.mL^{-1}$ respectively). Interestingly, at the concentration of 100 $ng.mL^{-1}$, 4-HHE decreased significantly grazing by herbivores (Wilcoxon test: W = 52, p-value = 0.01). At lower concentrations, 4-HHE had no effect, neither at 50 $ng.mL^{-1}$ (W = 13, p-value = 1), nor at 10 $ng.mL^{-1}$ (W = 46, p-value = 0.79).

Aldehydes do not trigger an oxidative burst in L. digitata plantlets

As already established for *L. digitata* (Küpper et al., 2001), the amount of hydrogen peroxide (H_2O_2) was significantly increased over 60 minutes, in seawater surrounding *L. digitata* culture plantlets, after elicitation by oligoguluronates (GG), compared to control, showing the occurrence of a rapid oxidative burst. On the contrary, no significant difference of H_2O_2 accumulation was recorded in surrounding seawater of algae after addition of one of the three aldehydes, compared to control (Fig 2; ANOVA F-value = 19.98, p value < 0.001, SNK test: C=H=N=D<E). Elicitation by GG triggered H_2O_2 production of 3 to 4 times more than that measured in control *L. digitata* or treated with 4-HHE, 4-HNE or dodecadienal.

Aldehyde treatments induced metabolic modifications in algal tissues compared to controls

Partial least squares discriminant analyses (PLS-DA) were carried out on the 177 ions detected by LC-MS analyses (Fig 3). The statistical post-tests were not conclusive at each sampling time (PLS-DA: 4h: NMC = 0.69, p-value = 0.18; 8h: NMC = 0.82, p-value = 0.76; 28h: NMC = 0.78, p-value = 0.51.). This could reflect a high biological variability of the metabolic responses, avoiding to statistically discriminate the different treatments from each other. However, some general patterns could be highlighted. At 4h, the dispersion of the 4 replicates was higher for the algae treated by aldehydes, than those elicited or controls, suggesting more variability in metabolic profiles. At 4h, 8h and 28h, the

aldehyde-treated samples were clearly separated from the control points, even if some were close to each other or with the elicited samples, suggesting distinct metabolic profiles in elicited and aldehydes-treated algae compared to controls at each sampling time (Fig 3).

Aldehydes triggered production of FFAs and their oxidized derivatives

Among the ten most discriminant ions between different treatments at each sampling time, five ions were selected, because annotated as putative FFAs or their oxidized derivatives. Three of these *in silico* annotations were confirmed by further LC-MS/MS analyses, i.e. the stearidonic acid (m/z 275.2 rt 603 sec), a jasmonic acid (JA) derivative compound (raw formula of $C_{12}H_{18}O_3$, m/z 209.12 rt 364 sec) and a putative eicosapentaenoic acid (m/z 301.22 rt 679 sec). However, the two other ions (m/z 351.22 rt 346 sec and m/z 309.2 rt 403 sec) supposed to belong to oxylipins were not confirmed to be (13)-HOTrE and 20-hydroxy-leukotrien B4.

Figure 4 presents the relative amount of these 5 ions in *L. digitata*, along the time-course, according to the different treatments. Globally, 4-hour incubation in 4-HNE and 4-HHE led to a strong increase of their production 4 hours after incubation. This rapid induction was less pronounced for the dodecadienal and elicitation treatments, lasted up to 8h for the 4-HHE treatment, and was followed by a relative decrease for the 4-HNE and dodecadienal treatments. After 28h, the algae submitted to the dodecadienal treatment showed a stronger production peak for these compounds. More specifically, all the samples treated with the 3 aldehydes contained two to over four times more of the unknown compound m/z 351.22 compared to a control kelp at 4 hours, whereas its relative amount tended to be down-regulated after 8 or 28 hours. The stearidonic acid was rapidly more produced after 4-hour incubation in 4-HNE and 4-HHE than without treatment, and after 8 hours following the dodecadienal treatment. After 28 hours, the relative amount of this FFA was similar to that of the control algae, unlike in elicited algae where it reached twice more than the control level. For the 4-HHE treatment, a higher production of JA derivative was observed after 8 hours compared to other treatments. The relative amount of the unknown ion m/z 309.2 was similar or slightly increased in aldehyde-treated algae compared to control algae after 4 h. However after 28 h, its relative amount and that of the eicosapentaenoic acid were, respectively, 3 and 3.5 times more important after incubation in dodecadienal than after no incubation or after elicitation.

Discussion

Aldehyde perception rapidly affected algal metabolism through different regulation pathways

Here, we demonstrated that 4-HNE, 4-HHE and dodecadienal, applied at 100 $mg.mL^{-1}$, did not induce an oxidative burst, as observed using oligoguluronate elicitors in *L. digitata*. Thus, we could hypothesize that the NADPH oxidase is not activated in response to these compounds and that they do

not act as exogenous elicitors like oligoguluronates (Küpper et al., 2001). This also suggests that these C6-, C9- and C12-aldehydes did not trigger strong oxidative responses as the level of H_2O_2 in surrounding seawater was similar between aldehyde-treated and control algae. Reactive oxygen species (ROS) have already been shown to play roles in algal defense against pathogens. In red algae, ROS emitted were shown to have an antibacterial effect on *Gracilaria conferta* epiflora after oligoagar elicitation (Weinberger and Friedlander, 2000). In *L. digitata*, the oxidative response is also an essential element of natural resistance against bacterial degraders and the oligoguluronate-triggered oxidative burst was shown to significantly increase resistance against infection by endophytic pathogens (Küpper et al., 2002). Altogether our results suggest that the 4-HHE-induced protection against herbivores do not involve NAPPH-activation pathway nor ROS emission.

Following incubation with 4-HNE, 4-HHE and dodecadienal, we have investigated the changes of the algal endometabolome during a time-course up to one day. The comparison of metabolic profiles based on 177 detected ions did not allow to strongly discriminate the different treatments (incubation in aldehydes, control or elicitation) whatever the sampling time. However, in the PLS-DA the metabolic profiles of the control algae were not grouped with those of aldehyde-treated neither with those of elicited *L. digitata*, at 4, 8 and 28 hours. For elicitation, it has already been shown that GG application specifically induced metabolic regulation, as shown by the massive release of halogenated volatile compounds and aldehydes in the medium surrounding *L. digitata* one hour after elicitation with GGs (Thomas et al., 2011). Similarly, the differences observed in metabolic profiles of the aldehyde-treated algae are likely to correspond to different metabolic regulations. All the three aldehydes tested are therefore perceived as signals by *L. digitata* and seem to be integrated to induce production of metabolites at different timeframe depending on their chemical nature.

We could follow the profiles of some compounds that were already known as induced in response to abiotic and biotic stresses (Ritter et al., 2008; Goulitquer et al., 2009). Interestingly, both aldehydes 4-HNE and 4-HHE strongly induced an early production of the 5 annotated compounds compared with control and elicited algae after 4h or 8h after treatment, whereas the dodecadienal's incubation showed their increases later (28h after algal treatment). The chemical nature of the annotated ions has been validated by LC-MS/MS analyses for stearidonic acid and a jasmonic acid derivative, suggesting the activation of FFA and oxylipin pathway by these three aldehydes. These results are consistent with the study of Bouarab et al. (2004) which demonstrated that elicitation with MAMPs or methyl jasmonate activated the metabolism of C20 and C18 PUFAs in the red alga *C. crispus*, generating prostaglandins and jasmonates. Similarly, Küpper et al. (2006) found that MAMPs and MIMPs (LPS and GG respectively) were shown to induce an increased release of C18 and C20 FFAs in *L. digitata* within 30 min. However, unlike LPS, GG did not induce a significant accumulation of some oxidized derivatives of linolenic and eicosapentaenoic acid after one hour, suggesting that oligoguluronate perception would activate restricted oxylipin biosynthetic pathways. Interestingly, the aldehyde 4-HHE applied on *L.*

digitata at the concentration of 1 µg.mL^{-1} was already shown to significantly increase the production of the oxylipin 13-(HOTrE) after 24 hours (Goulitquer et al., 2009). Moreover, the aldehyde dodecadienal induced a stronger release of stearidonic acid compared to other aldehydes. This fatty acid was already shown to be strongly produced in the red alga *C. crispus* in response to infection by the endophyte *A. operculata*, concomitantly with arachidonic acid and linolenic acid (Bouarab et al., 2004). Altogether our results suggest that 4-HHE, 4-HNE and dodecadienal could trigger the production of their own precursors by activation of different metabolic pathways.

Aldehydes showed antagonist effects on algal consumption according to their chemical nature

According to the nature of the aldehyde, *L. digitata* responded differently to a subsequent grazing by *P. pellucida*. Dodecadienal increased significantly the consumption by herbivores while 4-HHE decreased it when applied at 100 ng.mL^{-1}, and had no effect. The metabolic regulation observed in response to the aldehyde incubation suggested that dodecadienal, 4-HNE and 4-HHE were perceived by the kelp, and they seemed to induce different metabolic pathways in the kelp. If 4-HNE did not trigger protective responses towards herbivores, it could be not sufficient to activate defense responses against grazers. The different effect of 4-HNE and 4-HHE could also be due to the chemical properties of the compounds, such as solubility. Indeed, challenging of *L. digitata* with oligoguluronates or copper stress showed a strong release of 4-HHE mostly in the water phase, while 4-HNE was rapidly transferred in the air phase (Goulitquer et al., 2009), related to a lower amount in the water phase. Dodecadienal was released in an equivalent way between the two phases (Goulitquer et al., 2009), but showed an antagonist effect than 4-HHE on algal consumption by grazers. This should be linked to the activation of particular metabolic pathways, as illustrated by the specific induction patterns of the five selected metabolite markers.

The optimal concentration of aldehydes for observation of algal protection was 100 ng.mL^{-1} of 4-HHE and 4-HNE. However, this concentration is substantially higher than aldehyde amounts that are released in the environment or by *L. digitata* following stress in laboratory experiments. Indeed, measured concentrations after 1 h elicitation in the laboratory was around 0.2 ng.mL^{-1} 4-HHE, 2.0 ng.mL^{-1} 4-HNE and 4.0 ng.mL^{-1} dodecadienal. In the field, the highest concentrations of aldehydes measured over kelp beds were around 0.015 ng.mL^{-1} 4-HHE 1 h after low tide and 0.03 ng.mL^{-1} 4-HNE 30 min after low tide (Goulitquer et al., 2009). This high difference in aldehyde concentrations could be explained, for instance, as these aldehydes may be emitted by numerous kelps in the same area, then perceived by the exposed neighbors, leading to efficient algal defenses against herbivores. We can also hypothesize that these compounds could be included in a blend of volatiles, involving a wide range of chemical compounds which could act synergistically on algal responses against grazing. The ratio of concentrations between the different compounds in the blend could also be crucial for the perception of the warning signal by neighboring kelps.

Conclusion

In *L. digitata*, the incubation in different aldehydes modified algal consumption by herbivores and algal chemical profiles, suggesting their involvement as defense signals during kelp/herbivore interactions. Interestingly, according to their chemical nature, aldehyde signals have antagonist effects on protection against grazers, and seem to induce different metabolic pathways. For instance 4-HHE increased the resistance of the kelp within 1 day against the specialist herbivore *P. pellucida* and induced early production of fatty acids and oxidized derivatives after aldehyde application. At the contrary dodecadienal appeared to favor algal consumption, and a later induction of fatty acid metabolism. Further studies should be conducted to decipher the molecular mechanisms induced by these different aldehydes and evaluate their effects on generalist herbivores. This could lead to a better understanding of the aldehyde-based signals as defense chemical cues in the marine environment.

Figure legends

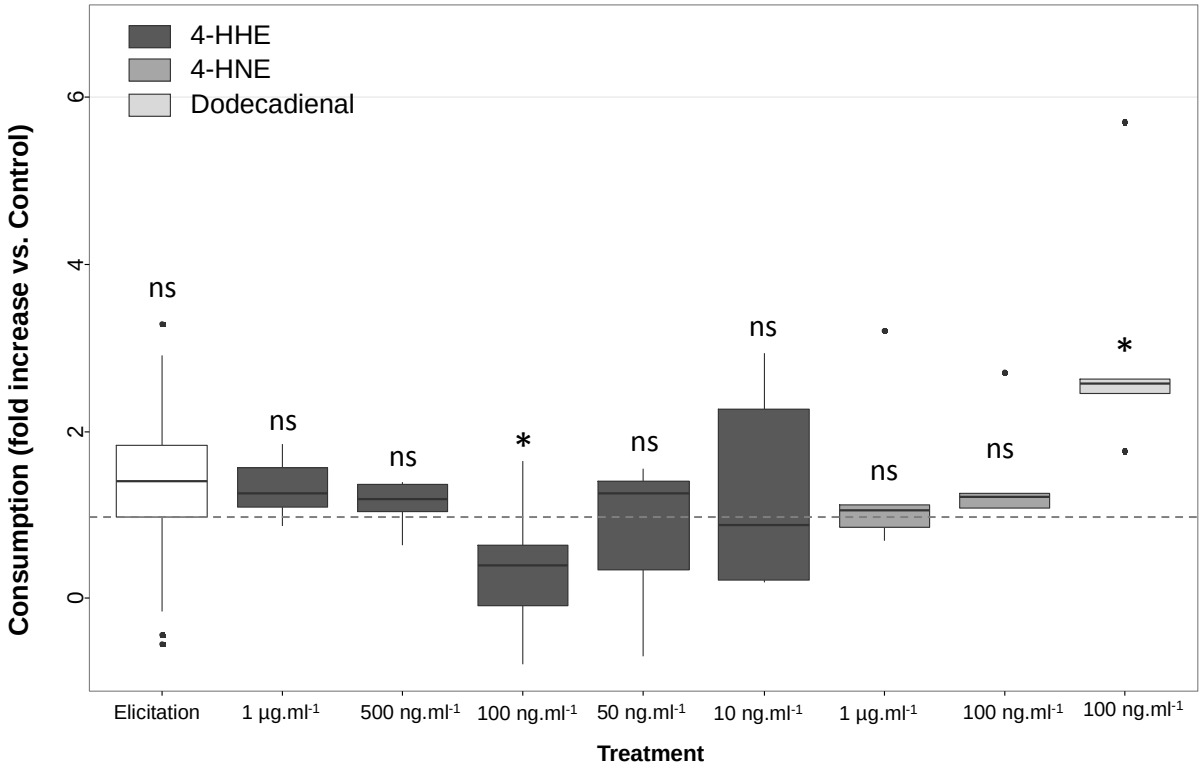

Fig 1. Effects of different treatments on consumption of *L. digitata* by *P. pellucida* grazers after 3 days. One day before putting in contact with herbivores, the algae were incubated either with GG for 1h (Elicited) or with different aldehydes for 4h, i.e. 4-HHE, 4-HNE or dodecadienal. Results are expressed in algal consumption (measured by fresh mass differences) relative to control (without treatments of algae). Elicited: 150 µg.mL^{-1} (n=25). Aldehydes were applied on algae at the following concentrations: 4-HHE 1 µg.ml^{-1} (n=5), 500 ng.ml^{-1} (n=5), 100 ng.ml^{-1} (n=15), 50 ng.ml^{-1} (n=5) and 10 ng.ml^{-1} (n=10); 4-HNE 1 µg.ml^{-1} (n=5) and 100 ng.ml^{-1} (n=5); dodecadienal 100 ng.ml^{-1} (n=5); Wilcoxon tests were performed for each treatment, to compare the consumption of treated algae with the consumption of the control algae (without treatment). * significantly different from the control; ns: non significantly different from the control.

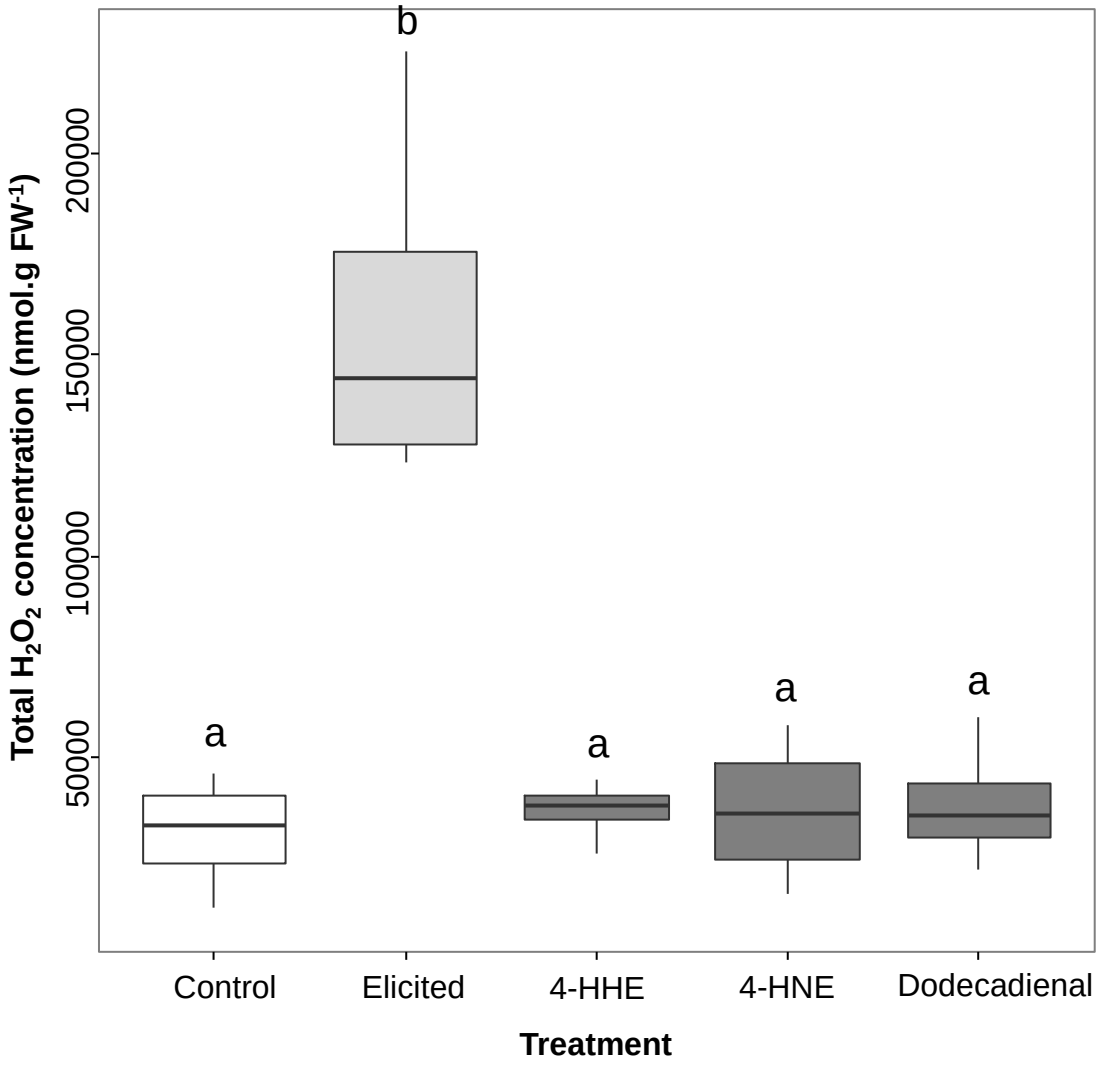

Fig 2. H_2O_2 production over 60 minutes in algal surrounding seawater after application of oligoguluronates at 150 µg.ml^{-1} or of 4-HHE, 4-HNE or dodecadienal at 100 ng.ml^{-1}. After checking normality, ANOVA analysis was performed: F-value = 19.976, p-value = 7.165e-06, SNK test: C=H=N=D<E (n=4). Letters above the error bars indicate groups that are significantly different ($P < 0.05$).

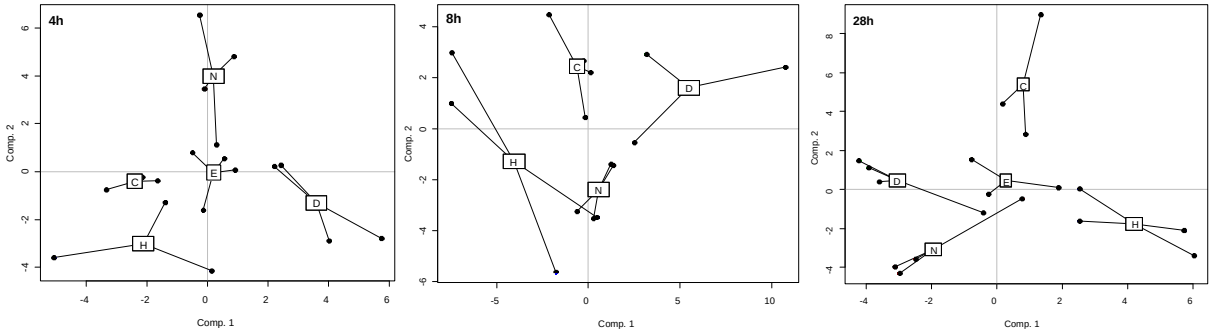

Fig 3. PLS-DA of the metabolic profiling based on 177 ions, from the endo-metabolome of *L. digitata* sampled at 4h, 8h and 28h after incubation in aldehydes 4-HHE (H), 4-HNE (N) or dodecadienal (D). Aldehyde treatments were compared with a control without treatment (C) and an elicited alga (E). MVA test, 999 permutations 4h: NMC = 0.69, p-value = 0.18; 8h: NMC = 0.82, p-value = 0.76; 28h: NMC = 0.78, p-value = 0.51.

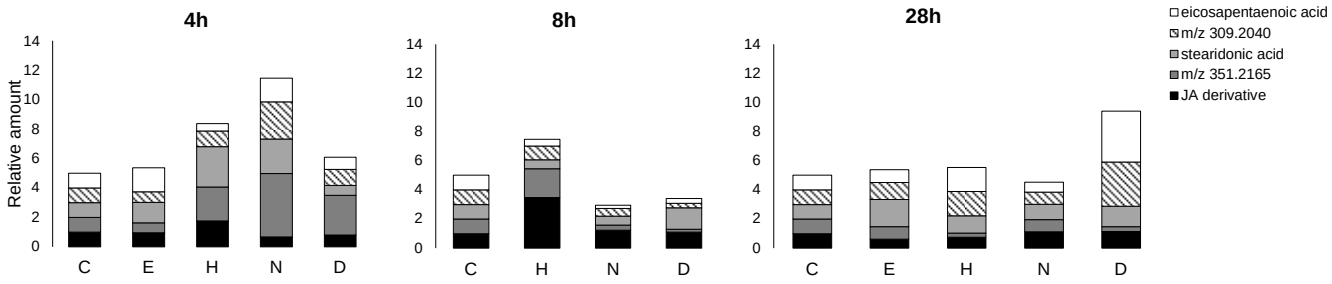

Figure 4. Relative amount of five compounds in *L. digitata* sampled after 4h, 8h and 28h of incubation in aldehydes 4-HHE (H), 4-HNE (N) or dodecadienal (D). Results are compared with a control without treatment (C) and an elicited alga (E). For each compound, the control level was set to a relative unit of 1 to express the fold-variation in the other conditions (absolute concentration values are provided in Tables S1). Three compounds were identified by LC-MS/MS analyses as stearidonic acid (m/z 275.2 rt 603 sec), jasmonic acid derivative (raw formula of $C_{12}H_{18}O_{3,}$ m/z 209.12 rt 364 sec), putative eicosapentaenoic acid (m/z 301.22 rt 679 sec).

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II. Aldehyde-based signaling in the interaction between *L. spicata* and the generalist herbivore *T. atra*

The results of this experiment were obtained by Y. Yacine in Las Cruces. Algal consumption of *L. spicata* was measured after 1 day and 2 days of grazing by *T. atra*, subsequently to treatment with either the aldehyde 4-HHE or the 4-HNE, or after a co-incubation with grazed *L. spicata* fronds. Two independent experiments were performed using application of aldehydes at 100 ng.mL^{-1}, and consumption of control kelps was not significantly different between the two experiments after 1 day of grazing (Wilcoxon test, W=28, p-value = 0.72) and after 2 days (W=38, p-value = 0.57). Results of these experiments were thus grouped in a single dataset. Fig. 22 shows the results of algal consumption after 1 day of grazing after the different treatments (algal consumption after 2 days of grazing is not presented as the same results were obtained). Incubation of *L. spicata* in the 4-HHE and in the 4-HNE at 10 ng.mL^{-1} did not induce any tendency of algal consumption by grazers, whereas treatment with the aldehydes at 100 ng.mL^{-1} tends to decrease the consumption, although not significantly. Co-incubation with grazed kelps, considered as a reference for distance signaling treatment, did not induce any modifications of algal consumption in comparison to control algae.

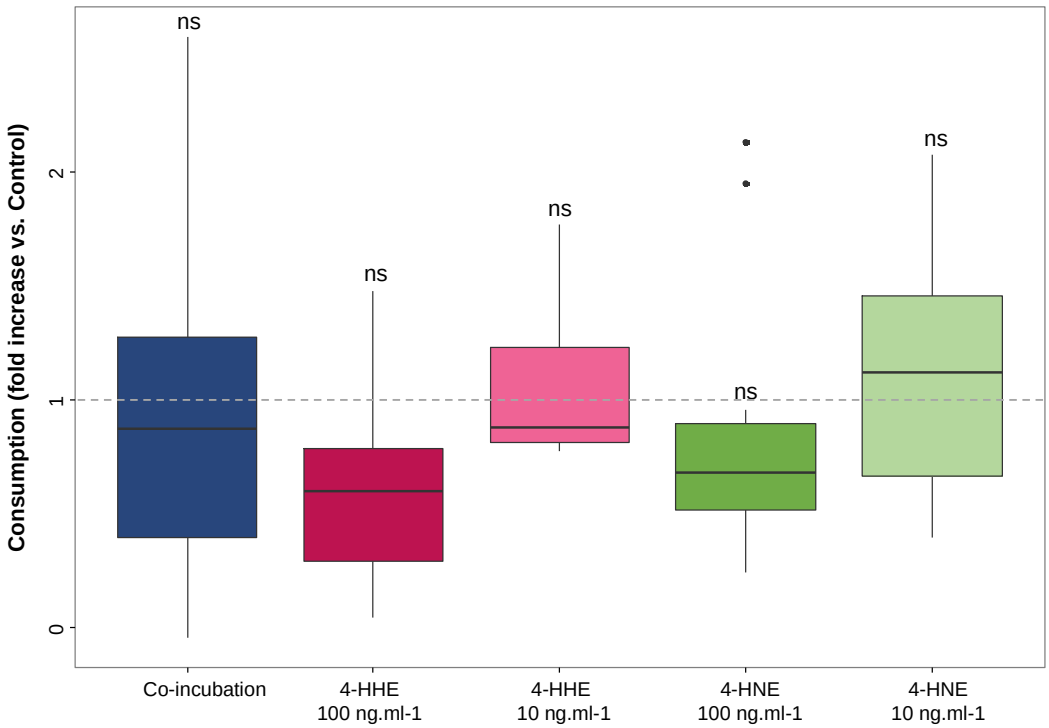

Figure 22. Ratios of algal consumption after one day by *T. atra* of treated *L. spicata* either with 4-hydroxyhexenal (4-HHE) or 4-hydroxynonenal (4-HNE) or co-incubated with a grazed kelp (co-incubation) over control algae (without treatment). ns indicates no significant difference between the treated kelps and the control (Wilcoxon test, α=0.05). n=8.

Discussion

1. Aldehydes have antagonist effects on algal protection against herbivores

Investigations on the effects of aldehydes on algal consumption by both generalist and specialist herbivores showed that kelps seemed to respond differently according to the chemical nature and concentration of the aldehyde. Whereas no effect was observed with 4-HNE, *L. digitata* consumption by *P. pellucida* was significantly reduced after application of 100 ng.mL^{-1} 4-HHE, but significantly increased after application of dodecadienal. In the kelp *L. spicata*, the tendencies were similar with the decrease of the consumption by *T. atra* after an application of 4-HHE and 4-HNE on the algae compared with control algae without treatment. Secondly, the aldehyde concentration applied on the kelp appeared to influence the effect on a subsequent grazing. Indeed, only the 100 ng.mL^{-1} concentration of 4-HHE applied on *L. digitata* significantly decreased algal consumption, while lower and higher concentrations did not seem to have any effect on *P. pellucida*'s feeding behavior. Similarly, the concentration of 100 ng.mL^{-1} was effective to decrease consumption of *L. spicata* with both aldehydes 4-HHE and 4-HNE. Thus, these results obtained in two distant kelp species suggest that 4-HHE could play a common role in aldehyde signaling and defense against generalist and specialist herbivores. 4-HNE could also be involved in similar defense processes, suggesting that they could both act on the same metabolic pathways. On the contrary, dodecadienal that was shown to favor the consumption by herbivores is likely to induce other metabolic pathways. Altogether these results suggest that as in plants and diatoms, aldehyde-based signaling occurs in kelps and could have a role during interactions with herbivores. Specific aldehydes are likely to interfere with the regulation of distinct metabolic pathways, leading to antagonist physiological responses, such as protection or attraction of herbivores.

2. Aldehydes are inducers of algal metabolic regulations

Studies on the metabolism changes in algal tissues in response to the three aldehydes showed that these compounds did not induce an oxidative burst, suggesting that they act downstream the activation of the NADPH oxidase. Indeed, the metabolic profiles of *L. digitata* were clearly modified after 4-hour incubation with aldehydes, compared to control. This support the hypothesis of both perception and biological integration of the aldehydes by the algae as chemical signals. More specifically, we showed that they triggered the production of polyunsaturated fatty acids and oxylipins, with different timeframes according to the aldehyde: 4-HHE and 4-HNE induced a rapid increase (between 4 and 8 h after incubation in aldehydes), while dodecadienal triggered it later (1 day after incubation). Thus, the increased algal consumption after dodecadienal application could be explained by an induction or a repression of some metabolic pathways, for instance involved in production of fatty acids and their derivatives, in contrast to 4-HNE and 4-HHE which could stimulate defense pathways. However, other

chemical compounds could act on algal defenses, constituting a complex blends in which some compounds such as 4-HHE and 4-HNE would act synergistically for a better defense of the kelp.

3. Could aldehyde-based signaling be efficient in the natural environment?

The optimal concentration of aldehydes for observation of effects on algal protection was 100 ng.mL^{-1} for 4-HHE and 4-HNE. However, this concentration is substantially higher than aldehyde amounts that found in the environment and previously measured during laboratory experiments. Indeed, measured concentrations after 1 h elicitation in the laboratory was around 0.2 ng.mL^{-1} 4-HHE, 2.0 ng.mL^{-1} 4-HNE and 4.0 ng.mL^{-1} dodecadienal. In the field, the highest concentrations of aldehydes measured over kelp beds were around 0.015 ng.mL^{-1} 4-HHE 1 h after low tide and 0.03 ng.mL^{-1} 4-HNE 30 min after low tide (Goulitquer et al., 2009). In our experiments, the lowest concentration (10 ng/ml^{-1}) did not show any effect. This high difference (around 1 to 5000 times more) between aldehyde concentrations *in situ* and that showing a biological effect on kelp protection in the laboratory could be explained, for instance, if these aldehydes would be emitted by several kelps in a same area, highly increasing local concentrations, that could be perceived by neighbors, leading to efficient algal defenses against herbivores. We can also hypothesize that these compounds could be part of a blend of volatiles, involving a wide range of chemical compounds likely including other aldehydes, which could act synergistically on algal responses against grazing. In the light of our results, this complex blend could include the aldehyde 4-HHE for a better protection of *L. digitata* against *P. pellucida*. In addition to the nature of the chemical compounds, the ratio of concentrations between the different compounds in the blend could also be crucial for the perception of the warning signal by neighboring kelps and in efficient chemical signaling during defenses against herbivores.

Conclusions and perspectives

1. Two complementary approaches in chemical ecology: metabolomics and bio-assays

Metabolic profiling approaches are powerful global methods for comparing metabolic states of algae submitted to different treatments over time. These methods allowed to highlight global metabolic regulations that were set up in kelps in response not only to direct grazing stress but also to indirect grazing stress. We found that kelps regulated their inner metabolism locally in the wounding area and after co-incubation with a grazed neighbor, as well as their exo-metabolome, after perception of exogenous elicitors. The metabolic regulations included changes in their relative amounts in fatty acids and their oxidized derivatives, and in their amino acid content during the first two days. The transient modification in fatty acid algal content could also be linked to the observed herbivore attraction and could participate to the perception of a remote food source, although other chemical cues could be involved in attraction behavior of herbivores. Moreover, these endo- and exo-metabolome varied over time, at least within two days. More specifically, we chose to target compounds deriving from the fatty acid pathway, hypothesizing that this metabolism could be involved in a potential distant communication inside and outside algal tissues, as already suggested by several previous studies in the laboratory. A metabolomic global approach could present some limits at various levels. The data acquisition and their quality depend strongly on the initial quality of the biological samples, which is highly determined by the extraction method. Indeed, in order to get a view as large as possible of a metabolome, we usually performed extraction techniques using the mix MeOH/H_2O which allowed an extraction of a wide range of compounds. However, this often caused troubles during metabolomic analyses, as too many ions were detected and could thus hide the interesting information, i.e. the differentially-induced compounds, explaining physiological modifications. Optimizations of the extraction method as well as of the analytical techniques are thus crucial for chromatograms and mass spectra of reasonable qualities. Moreover, data processing was performed using the Workflow4metabolomics platform, which is a bioinformatic tool, thus still under evolution, comprising more and more precise functions for a better adequacy to the data. This has to be taken into account by regularly updating functions to optimize the analysis. Beyond the global metabolomic analyses, it is worthwhile to underline that the final annotation of ions, is often a bottleneck, as the available chemical databases still contain a limited number of marine chemical compounds, especially from algae. Most of actual knowledge is still sparse and mainly available through previous studies or through collaborations with other labs working in marine research domain.

The complementary approach of metabolomics in chemical ecology is the setting up of bio-assays for testing the biological or ecological role of identified chemical compounds. Thanks to non-choice and multiple choice bio-assays developed and carried out during this thesis project, we first showed that recently grazed kelps were more attractive and subsequently more consumed by generalist and specialist herbivores than control kelps without treatments and their co-incubated neighbors. However, some global conclusions could not be drawn, as numerous uncontrolled factors interfered with

our observations. For instance, we showed that the season had a strong effect on algal physiology and on its consumption by grazers. The experimental setups have thus to be improved in order to take into account this side effect, for example by conducting several bioassays in parallel at the same period of the year. The experimental design of co-incubation of two kelps could be performed using a grazed frond in a box upstream to a naïve frond, which will only receive the water flow from the grazed alga. This would avoid the negative stress effect of the net on the grazed kelp and its reciprocal effect on its neighbor. These external factors are even stronger in *in situ* experiments, influenced for instance by the effects of the waves, reducing the relevance of the results. Increasing the number of replicates thus plays a central role in relevant bio-assay experiments, due to the various hardly controllable external factors.

Combining metabolic profiling and ecological approaches, another prospect of this research could focus on a bio-guided fractionation of the soluble chemical compounds emitted by a kelp having a strong effect on protection against grazing. Successive filtration of the complex blend of emitted compounds associated with bio-assays of their effects on algal consumption could lead to the identification of the chemical families acting on kelp/herbivore interactions.

2. Grazed kelps seem to better attract grazers and be more consumed

Unexpectedly, we observed that challenging kelps by grazing for 2 days or defense elicitation by oligoguluronates generally stimulated a subsequent grazing by herbivores and also attracted them. In *L. spicata*, we showed that, only in summer, the kelp could gain weight and be less consumed when it was previously grazed by conspecific herbivores. This season effect was interpreted as the occurrence of up-welling on Chilean coasts in this season (between September and January) which could provide nutrients to the water surface, leading to higher algal growth and potentially better defenses against aggressors. On the contrary, during the rest of the year, the kelps could be less provided in nutrients, thus containing less energy for growth and defense. However, the effect of a previous grazing on subsequent algal consumption was once observed after a previous grazing, but, in that case, not after elicitation by oligoguluronates. Kelps could therefore be more attractive for herbivores only after grazing. This could suggest that a natural grazing by herbivores and a simulated grazing by oligoguluronate would induce different defense mechanisms in kelp tissues upon a subsequent grazing, implying activation or repression of different metabolic pathways according to the treatment (grazing or elicitation). In *L. digitata*, specialist as well as generalist herbivores seem to consume preferentially kelps for which defenses were elicited 24 hours before, leading to regulations of metabolism. In the case of *L. spicata*, in some conditions, the grazing stress over two days is likely to induce the release of specific attractive compounds at the surface of the thallus or in seawater, while chemical elicitation did not. Therefore, kelps have the capacity to actively respond to herbivore damage, but the actual triggers and metabolic pathways activated during this process might differ among species/season/ecosystem. It

can be hypothesized that herbivores could perceive distance compounds coming from these challenged algae, detected as degradation products of the kelp, and thus as an edible food source. Interestingly, this observation has been made in the two kelp species, suggesting that this attraction trait could be common in kelps, either inherited from the common ancestor, or originating from evolutionary convergence. The direct effects of fatty acids and oxylipins on feeding by herbivores could be tested through incorporation of pure or mixed compounds in artificial food, given to generalist and specialist grazers. For further investigating the attraction of herbivores in a natural environment, *in situ* experiments could also be performed on *L. digitata* in order to test the natural recolonization of grazers in a calm environment compared with a medium constantly under influence of waves.

Moreover, it seemed that this attraction depended on the relationship between the algal host and the herbivore. Preliminary experiments showed that the generalist grazers *T. atra* were attracted to the kelp *L. spicata* when it was previously grazed by their conspecifics, while they could not discriminate control algae from kelps previously grazed by the specialist herbivore *S. scurra*. This may reflect a kind of recognition of the interaction between the kelp and the herbivore to following grazers, like the food source could be recognized as edible only after grazing by the same species. We can suggest that this could be a strategy for herbivores' fitness, which could identify a remote food source, thus avoiding poisoning or unnecessary movement towards non-edible diet.

3. Kelps perceive and integrate water-borne signals emitted by their grazed neighbors

When a kelp was co-incubated with a grazed one, metabolic regulations occurred in both algal tissues but these changes were clearly different between the two treatments. However, the co-incubation with a grazed kelp activated different metabolic regulations than co-incubation with an unchallenged alga. Thus, the grazed kelp released chemical signals in the surrounding seawater that could induce specific modifications in the receiving kelp. The different metabolic pathways induced in response to the perception of chemical compounds released in the seawater upon a grazing stress could lead to adaptive responses to a subsequent herbivore attack. Interestingly, in both *L. digitata* and *L. spicata*, field observations showed high concentrations of *P. pellucida* and *S. scurra*, respectively, on a single kelp, while its neighboring conspecifics contained limited numbers of grazers on their thalli. Given the low dispersion rate of kelp spores in the marine environment, it is admitted that close neighbors have family relationship. From the evolutionary theory of the kin selection, we suppose that the over-grazed kelp should favor the development of its offspring around, possibly through the emission of warning signals and/or the attraction of grazers, thus increasing the global fitness of the algal species. Even if chemical signaling could require metabolic costs for its production and release, it could therefore give advantages to the species, by providing benefits to the descendants.

4. Aldehydes are inducers of metabolic responses and modify algal resistance to herbivores

At the wounding site, some released compounds following grazing by herbivores and the endo-metabolome of a co-incubated kelp with a grazed alga were shown to be fatty acids and oxidized derivatives oxylipins. Elicitation by oligoguluronates and copper stress were already shown to induce the release of a wide range of aldehydes in both the air and the seawater (Ritter et al., 2008; Goulitquer et al., 2009). This previous study demonstrated that the aldehyde 4-HHE induced the synthesis of an oxylipin, and proposed that some aldehydes could act as inducers of metabolic responses. Our results suggested that the metabolic oxylipin pathway could also be involved in kelp responses to grazing stresses. Aldehydes seem to induce the production of their own precursors (oxylipins), forming an induction loop which could then be involved in switching on warning mechanisms towards the neighboring kelps for an optimized defense against grazers.

The direct effects of aldehydes on algal defense against herbivores, evaluated through algal consumption bioassays, seem to strongly depend on the chemical nature of the aldehyde, as well as on the concentration applied on the kelp. For instance, 4-HHE and 4-HNE seemed to decrease the grazing pressure in both kelp species treated by 100 $ng.mL^{-1}$, whereas dodecadienal appeared to stimulate algal consumption at this concentration. In parallel, metabolic profiling showed that the algal endometabolome was rapidly modified after application of these aldehydes, in particular regarding fatty acids and oxylipin amounts. These modifications were observed early in time after algal incubation in 4-HHE and 4-HNE (4 to 8 hours), while occurring later after incubation in dodecadienal (around 24 hours). All these results strongly suggest that the three aldehydes induced different metabolic pathways in kelp tissues, some being involved in algal defense responses.

Some aldehydes, such as 4-HHE, could be perceived by kelps in the closely neighborhood of the grazed kelp, then inducing a release of free fatty acids, their oxidation, thus forming *de novo* aldehyde production. This should result in an amplification of metabolic responses to grazing, to finally inhibit kelp grazing by either specialist or generalist herbivores. However, numerous compounds should be involved in this chemical signaling, acting altogether synergistically to activate an optimized defense against the herbivore. In contrast to 4-HHE, the aldehyde dodecadienal increased the consumption by grazers, suggesting that this compound could be perceived differently from 4-HHE and could induce pathways that do not trigger defenses of the kelp against grazers.

Moreover, the fact that these aldehydes are emitted in much lower amounts in natural conditions than the efficient concentrations in laboratry experiments could suggest that a single chemical compound would not be sufficient for triggering of defenses. Indeed, the signal could contain a complex blend of chemical compounds, acting together on algal defense induction and later protection against herbivores. Rather than being composed of specific compounds which could require high costs for their production, this bouquet could contain relative ratios of compounds constitutively present in kelps which could

induce algal defense. Moreover, it can be supposed that, in the natural environment, several kelps could respond to grazing stress, thus increasing the amounts of the chemical compounds into the seawater and allowing their perception by neighboring kelps, leading to defenses. The fact that 4-HHE was perceived by kelps, without inducing an oxidative burst, but triggering fatty acid metabolism regulation and defense responses against *P. pellucida* could suggest that this aldehyde could induce defenses in neighboring kelps during kelp/herbivore interactions.

This study should be completed by further bio-assays testing the effects of other aldehydes and oxylipins which were found in kelps in response to diverse stresses in natural conditions or in the laboratory, on algal consumption by grazers. These compounds could be tested alone and in mixture as well, using different ratios between the chemical compounds. It could also be interesting to evaluate their effects on the interaction between kelps and generalist herbivores, as well as their direct effect on grazers at different concentrations, through artificial food experiments.

In conclusion, this work allow to draw new general schemes regarding chemical signaling in kelps during interactions with herbivores (Fig 23 and 24). We highlighted the existence of chemical compounds linked to fatty acid metabolism released during grazing, such as aldehydes, and their biological role on metabolism of neighboring algae and on their defense against grazers. Like in plants and marine diatoms, aldehyde-based signals seem to play a crucial role in regulation of kelp defense mechanisms. These are the first components to continue the deciphering of fine chemical interactions between kelps and their associated herbivores, and of underlying mechanisms.

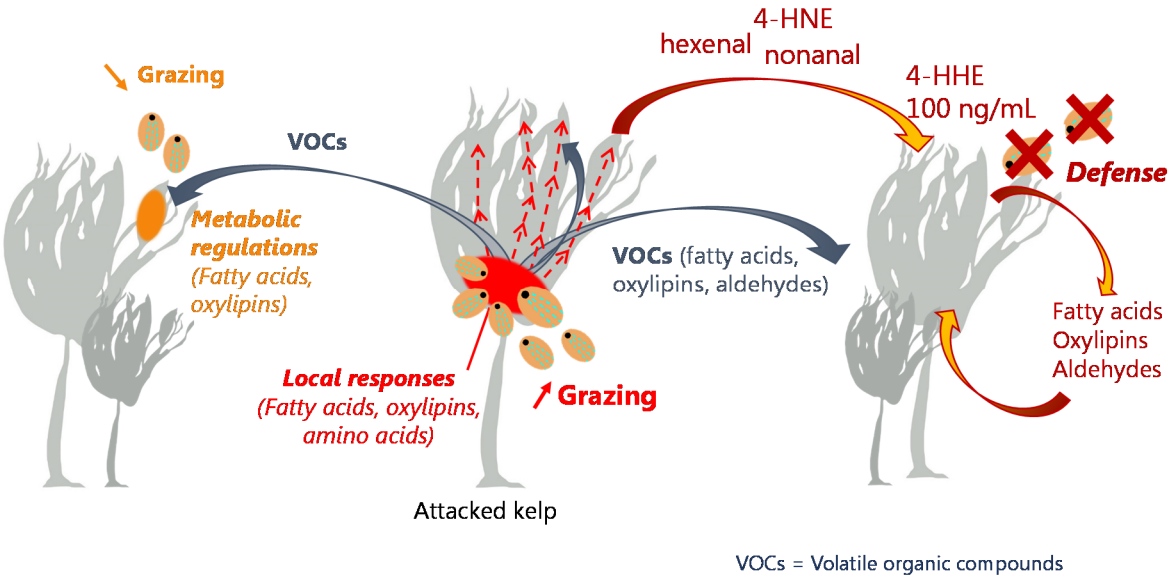

Figure 23. Schematic representation of distance signaling in kelp chemical defense during interactions with herbivores. In response to grazing by herbivores, local responses are activated such as the accumulation of fatty acids, oxylipins and amino acids, and grazers are firstly attracted to the wounding site. Simultaneously, the kelp releases diverse Volatile Organic Compounds (VOCs) into the surrounding environment, like fatty acids, oxylipins and aldehydes. Some of these emitted compounds have impacts on metabolic regulations in neighboring kelps, activating the fatty acid metabolism in algal tissues. Amoung the emitted compounds, the 4-HHE is perceived by the neighboring kelp, changing its metabolism and then inhibiting further grazing by herbivores.

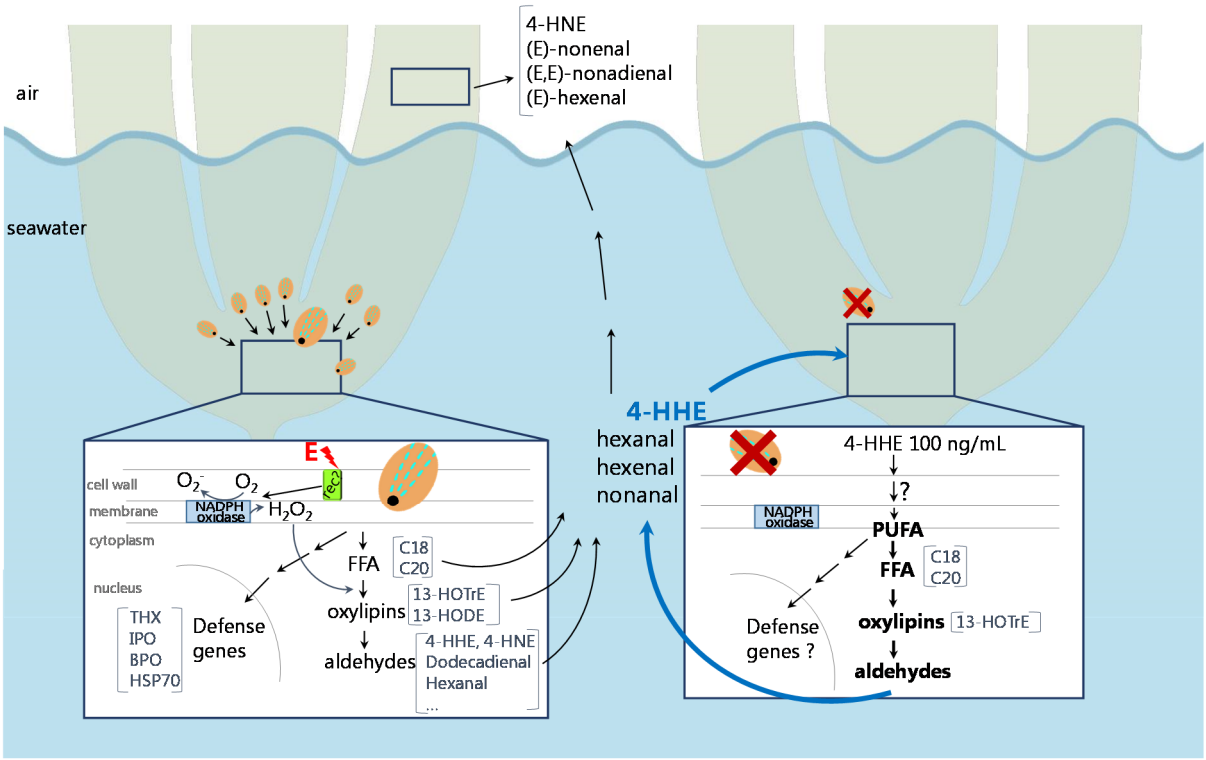

Figure 24. Schematic representation of biochemical processes in kelp chemical defense in response to grazing. Grazing by herbivores induces a release of free fatty acids which are oxidized by ROS, producing oxylipins and aldehydes, as well as activation and repression of defense genes. Some chemical compounds are emitted into the seawater and a part of them reach the air environment. Among these compounds, the aldehyde 4-HHE is perceived by the neighboring kelp and induces modification of metabolism, leading to protection against herbivores.

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Appendices

Appendix 1.

Daily Rhythm of Mutualistic Pollinator Activity and Scent Emission in *Ficus septica*: Ecological Differentiation between Co-Occurring Pollinators and Potential Consequences for Chemical Communication and Facilitation of Host Speciation

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Daily Rhythm of Mutualistic Pollinator Activity and Scent Emission in *Ficus septica*: Ecological Differentiation between Co-Occurring Pollinators and Potential Consequences for Chemical Communication and Facilitation of Host Speciation

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Abstract

The mutualistic interaction between *Ficus* and their pollinating agaonid wasps constitutes an extreme example of plant-insect co-diversification. Most *Ficus* species are locally associated with a single specific agaonid wasp species. Specificity is ensured by each fig species emitting a distinctive attractive scent. However, cases of widespread coexistence of two agaonid wasp species on the same *Ficus* species are documented. Here we document the coexistence of two agaonid wasp species in *Ficus septica*: one yellow-colored and one black-colored. Our results suggest that their coexistence is facilitated by divergent ecological traits. The black species is longer-lived (a few more hours) and is hence active until later in the afternoon. Some traits of the yellow species must compensate for this advantage for their coexistence to be stable. In addition, we show that the composition of the scent emitted by receptive figs changes between sunrise and noon. The two species may therefore be exposed to somewhat different ranges of receptive fig scent composition and may consequently diverge in the way they perceive and/or respond to scents. Whether such situations may lead to host plant speciation is an open question.

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Introduction

Plant-insect interactions are at the origin of huge diversification within the living world [1]. Plants need to attract pollinators and defend themselves against phytophagous insects, resulting in chemical diversification, and this diversity has led to radiations of whole insect groups. Despite the biological importance of this diversity, how plants and their associated insect communities diversify is largely unknown. For instance, what levels of ecological specialization will accelerate or impede the macroevolutionary diversification process is still largely unknown [2].

The mutualistic interaction between *Ficus* and agaonid wasps constitutes an extreme example of plant-insect co-diversification [3]. Agaonid wasps only develop in figs (the urn-shaped inflorescence of *Ficus*) of their host species (generally one host species, sometimes more [4]), and they are almost the sole pollinators of *Ficus* [5]. While many of the 800+ currently recognized *Ficus* species seem to be associated with a single pollinator species, some have two or more [6]. In some exceptional cases, co-pollinators of a *Ficus* species belong to different genera, a feature which should facilitate co-existence through strong ecological differentiation, as when diurnal *Elisabethiella* species coexist with nocturnal *Alfonsiella* species [7]. Much more often, the pollinators associated with a given *Ficus* species are sister species. The presence in different parts of the range of a *Ficus* species of different but closely related pollinator species could be an intermediate step in a process of allopatric speciation in which pollinator speciation could precede and maybe facilitate host speciation. In such situations, two or more species of pollinators may be observed to co-occur locally, but only in contact zones between their respective ranges. Examples include the three species of the *Wiebesia pumilae* complex associated with *Ficus pumila*, [8], and the two species associated to *Ficus sur* in West Africa. In the latter case, *Ceratosolen flabellatus* is a forest specialist, *C. silvestrianus* is a savanna specialist and both species coexist in forest-savanna mosaics [9].

In some situations, however, two or more sister species of pollinating wasps colonize the same trees and often even the same figs, over larger parts of the range of a *Ficus* species. In such

situations, we may expect strong interspecific competition between the wasp species that should select for niche differentiation. For instance, co-occurring sister species could evolve different compromises between dispersal capacity (which in fig wasps is tightly linked to lifespan as these very short lived wasps disperse by drifting in the wind) and competitiveness (*e.g.* the ability to rapidly locate receptive figs, to enter them, and to oviposit faster than the other pollinator species).

There are five documented cases of relatively widespread co-occurrence of closely related pollinator species on a fig host, for which habitat differentiation has not been suggested as the mechanism allowing co-existence. The first example concerns *Ficus microcarpa* in Hainan [10] but no biological information or wasp description are provided and the evidence for widespread co-occurrence is preliminary. In Yunnan, however, one of the two sister species of agaonid wasps colonizing *F. microcarpa* does not carry pollen so that its larvae develop mainly in unfertilized fig ovules as opposed to mainly in fertilized ones in the case of pollinators [11], suggesting that, in this case, co-occurring sister species present highly divergent ecologies [12]. The second example concerns the co-occurrence of the sister species *Elisabethiella stuckenbergi* and *E. socotrensis* on *Ficus natalensis* in South Africa [4]. *E. stuckenbergi* has a shorter head than *E. socotrensis*, a characteristic that suggests different fig-entering capacities since head shape of agaonid wasps is strongly correlated with the shape of the entrance into the fig [13].

The three other examples involve differences in coloration among otherwise morphologically-similar wasp species. *Ficus tuerckheimii* is pollinated by two co-occurring species of *Pegoscapus* in both Mexico and Costa Rica [14]. *Pegoscapus carlosi* is uniformly black while *Pegoscapus mariae* is ventrally honey-colored and dorsally blackish [15]. Similarly, in Australia, *Ficus rubiginosa* is pollinated by four sister species constituting the *Pleistodontes imperialis* species complex [16]. The color varies from entirely dark testaceous (all specimens collected close to the city of Sydney, New South Wales), to dorsally testaceous and ventrally yellow (some specimens collected close to the city of Mareeba, Queensland), to nearly completely yellow (some specimens collected close to the city of Townsville, Queensland) [17]. The yellow-colored individuals correspond to one cytotype while the correspondence between color and cytotype is not yet ascertained for the other color morphs. Finally, *Ficus septica* is pollinated by a yellow and a black species that co-occur in south Taiwan [18], a situation we have also observed in populations from the Philippines. In both *F. septica* and *F. rubiginosa*, only the dark colored pollinators are present at the more temperate limits of their distributions.

In fig-pollinating wasps, light body color (qualified by authors as yellow, amber or honey) is almost always associated with large eyes and nocturnal flight, while dark color is always associated with smaller eyes and diurnal dispersal. In the three aforementioned cases however, both dark and light sister species have relatively small and similarly-sized eyes that suggest diurnal flight [15,17,19,20]. In insects, melanisation or lack of melanisation may correlate with a diversity of adaptive traits [21]. Production of melanin is physiologically costly in insects: it can only be maintained if it confers a selective advantage [22]. For instance intra-population color variation in *Drosophila melanogaster* correlates strongly with resistance to dehydration, with light morphotypes being most sensitive [23]. The three cases reported above constitute the sole examples we have detected, in a systematic survey of pollinating fig wasp descriptions, of most probably diurnal light colored fig pollinating wasps. Therefore, we predict that the light color corresponds to an unusual life history strategy in diurnal pollinating fig wasps that is made possible because of selection for ecological niche separation between closely related species using the same resources. Given classical explanations of advantages associated with dark color, we surmise that dark pollinating wasps are better protected against diurnal stresses such as oxidative stress due to exposition to UV and ozone. We therefore predict that differences in body color might correlate with trade-offs between competitiveness and lifespan. Diurnal fig-pollinating wasps usually emerge from their natal fig early in the morning and survive only a few hours [24–26]. The black species, being better protected, would survive longer and have a more extended fig colonization time window than the yellow species. As a consequence it would be sufficiently long lived to reach somewhat more isolated receptive figs. Under the competitiveness-lifespan trade-off scenario, the yellow species would be more efficient than the black one at rapidly locating and entering receptive figs but would be shorter lived. A similar trade-off was found in the dark and pale males of the non-pollinating *Walkerella* sp. associated to *Ficus benjamina*, where males fight to access females [27]. Pale males were better fighters whereas dark males were more susceptible to disperse out of their natal figs in search for alternative mates. Dark males also tended to survive longer in laboratory conditions.

In *Ficus*, scent is the major cue used by pollinators to locate receptive figs and it facilitates the species-specificity of the interaction: each *Ficus* species produces a specific volatile blend that is only attractive to its specific pollinators [28–31]. However, variation of this volatile blend during the course of a day is expected as it has been reported for flowers as well as leaves of other plant species [32–34], and potential consequences of such variation on fig wasp behavior has not been investigated. One source of this variation originates in the plants being exposed, as the wasps, to a variety of stresses during daytime, involving oxidative stresses and thermal stresses. Volatile isoprenoids, including monoterpenes, protect plant tissues against these stresses [35,36], and their emission varies during the day [37]. Monoterpenes are also perceived by insects and are involved in their attraction to plants (e.g. in fig wasps [38,39]). We may therefore expect daily variation of the scents produced by receptive figs to result from responses to selection stemming from both daily patterns of pollinator activity and daily variation in abiotic stress intensity. On the contrary, leaves do not contribute to pollinator attraction [40] but they are exposed to the same abiotic stresses as figs. Daily leaf scent variation can therefore be used as a control to disentangle both functions in figs, and determine whether daily fig scent variation is the result of an adaption to increase pollinator attraction. Moreover, the presence of two pollinators instead of one is an exceptional situation for a fig species, and this may have selected for particular adaptations. It is therefore necessary to include a second type of control: a closely related *Ficus* species pollinated by a single wasp species.

Whatever the adaptive reasons are behind daily fig scent variation, we may expect adaptation of the pollinators to recognize the range of receptive fig scents to which they are regularly exposed. Two pollinator species presenting different daily activity patterns could be exposed to somewhat different ranges of attractive scent composition. If the hypothesis of more extended daily activity period of the black pollinators is upheld, and if scents emitted by receptive figs vary during the day, then we may predict some divergence over evolutionary times in the way the black and the yellow wasp species perceive and respond to volatile cues, a trait that could ultimately facilitate host plant speciation via assortative mating among plant genotypes if there is some heritable variation in receptive fig scent composition.

In this contribution, we document pollinator coexistence in *Ficus septica*. 1) We show that the yellow and black pollinator species associated with *Ficus septica* emerge from figs in the morning but differ in their lifespan, 2) we compare the daily rhythm of scent production by receptive figs with the rhythm of scent production by leaves and by figs and leaves of the closely-related *Ficus nota* and 3) we document that the composition of *Ficus septica* receptive fig scent varies during the course of a day. We discuss the potential consequences of these findings for the future evolution of this system, especially in terms of diversification processes.

Materials and Methods

Biology of the model system, study site and species

The fig is an urn-shaped inflorescence. Its inside is lined by uniovulate female flowers and male flowers. When the fig is receptive it emits a scent that attracts pollinating wasps. The wasps enter the fig, oviposit in some of the female flowers and pollinate. Female wasps that have colonized a fig and deposited their offspring in it are called foundresses. In monoecious *Ficus* species, seeds and galled flowers that each contains a single wasp larva develop side by side. Some weeks later, male wasp offspring emerge into the fig cavity and mate with the females still enclosed in their galls. The female wasps then emerge into the fig cavity, become loaded with pollen, and leave in search of a new, receptive fig. Finally, the fig ripens, becoming attractive to a large set of frugivorous animals. In dioecious *Ficus* species, functionally-male trees bear figs that produce wasps, pollen, but no seeds. Female trees bear figs that do not allow wasp oviposition and do not produce pollen, but do produce seeds. The adult lifespan of pollinating fig wasps is usually a few hours and is entirely devoted to searching for a receptive fig and subsequently ovipositing inside that fig. In *Ficus* in general, and in *Ficus septica* in particular, flowering is relatively synchronous within a given tree but asynchronous among trees resulting at the population level in the production of receptive figs and adult wasps throughout the year. The set of figs developing synchronously on a fig tree is called a crop.

The experimental work was carried out in the Diliman Campus of the University of the Philippines, in Quezon City, on the island of Luzon, Philippines (N 14°38′E 121°03′). Because the work was carried out in the campus of the University, no specific permit was required to conduct this study. Further, none of the studied species is protected or endangered. The campus is located in an urban zone and a large part of its area is made of more-or-less natural patches of vegetation. In the campus, native *Ficus* species grow wild in gardens, along roads and streams and in less intensively-tended places. Experimental work was carried out during the dry season, from January 14th 2013 to April 12th 2013. During this period, sunrise shifted from 6:30 a.m. to 5:45 a.m. and sunset from 5:45 p.m. to 6:15 p.m.

In Quezon City and more generally in the island of Luzon, *F. septica* is associated with two closely-related *Ceratosolen* species belonging to the *Ceratosolen bisulcatus* species complex (F. Kjellberg, L.J.V. Rodriguez unpublished observations, J.Y. Rasplus pers. com.): *Ceratosolen jucundus*, yellow-colored [19], and *Ceratosolen* sp., black-colored. Hereafter, they will be called "yellow pollinator" and "black pollinator" respectively.

In order to determine if the presence of two pollinators could have induced a shift in the period of the day when *Ficus septica* is pollinated and in the daily rhythm of fig scent production, some parallel observations were also done on a *Ficus* species associated to a single pollinator species. *Ficus nota* is taxonomically close to *F. septica* (both belong to subgenus *Sycomorus*, section *Sycocarpus*, subsection *Sycocarpus*) and is pollinated by the black-colored *Ceratosolen notus*, a close relative of the pollinators of *F. septica* [3]. Both species are found throughout the Philippines. A set of male trees of both species was surveyed every 2–3 days for the presence of figs and their developmental stage. Trees bearing figs close to receptivity or close to wasp emergence were visited daily.

Traits associated with wasp lifespan and competitiveness

The aims of the following experiments were 1) to examine differences between black and yellow pollinators in traits associated with their daily activity patterns and 2) to test whether the daily patterns of yellow and black pollinator emergence and presence around receptive *Ficus septica* trees differ from those of the pollinator species associated to *Ficus nota*.

Wasp emergence patterns and lifespan of the black and yellow pollinators of *F. septica*. At 4–6 p.m., we collected male *F. septica* and *F. nota* figs from which wasps were to emerge on the following day. These figs were recognizable due to their swelling and their softness. Each fig was put into a separate plastic pot closed with plankton net. The figs were then stored outdoors under the shade of a tree so that physicochemical conditions were as close as possible to *in natura*. Wasp emergences from figs were recorded every hour on the following day, from 5 a.m. to sunset.

During the survey of wasp emergences, pots containing *F. septica* figs from which both black and yellow pollinators were emerging were put aside. The fig was removed from the pot in order to keep only the insects that had emerged during the preceding single hour. Dead pollinators were counted after 6 hours and every 3 hours thereafter until all the insects were dead. The survey was replicated on eight figs taken from five different mother trees on six different dates. Therefore, yellow and black species survival rates were first compared for each fig separately.

For every fig, all the insects were dead within 12 hours after their emergence. We therefore calculated for each fig the survival rate of emerging black and yellow pollinators at two time points: six and nine hours after emergence. To determine if one species had a longer lifespan than the other, we performed a binomial test for each time point: we ranked yellow and black species survival rate and transformed this ranking into a binomial variable B ($B = 0$ if yellow survival>black, $B = 1$ if black survival>yellow). Under the null hypothesis (yellow and black lifespan identical) we expected to observe $B = 1$ with a probability of 0.5 i.e. black survival rate to exceed yellow survival rate for just half of the figs. Our prediction was that black wasps would survive longer than yellow wasps ($p(B = 1) > 0.5$).

Day round pattern of pollinating wasp presence on trees during their period of fig receptivity. In order to determine the day round presence on trees bearing receptive figs of the two pollinator species associated with *F. septica* and of the pollinator associated to *F. nota*, passive insect traps were hung in male trees bearing receptive figs. The insect traps were made of transparent A4 plastic sheets, rolled into a cylinder and coated with transparent odorless glue. Four such traps were suspended in branches close to receptive figs. The first insect traps were installed at 6 a.m., and were replaced every three hours until 6 a.m. on the following day. The experimental day was thus partitioned into eight time intervals. The exact timing of first trap setup, transition between fourth and fifth trap as well as last trap removal were adapted to match sunrise and sunset as close as possible. The experiment was repeated on six *F. septica* and three *F. nota* trees. After trap collection, the number of pollinators of each species captured during each three-hour time interval was counted.

Our prediction was that numbers of wasp trapped would decrease as the day progressed 1) because of limited lifespan of the wasps emerging in the morning and/or 2) because all the day's supply of receptive figs would no longer be attractive as they would have been pollinated in the morning. The following experiment was set up to discriminate between these two hypotheses.

Consequences of manipulating the period of accessibility of receptive *F. septica* figs on the abundance of black and yellow foundresses. Two branches bearing figs close to receptivity on each of three *F. septica* male trees were enclosed in plankton net bags for four to five days in order to let the figs become receptive without being pollinated. On one of the two branches per tree, the bag was removed at sunset the day before the experiment, at a time when pollinators were no longer active. Figs on this branch were thus accessible to pollinators for the whole experimental day. On the second branch the bag was removed at 0:30–1 p.m. on the day of the experiment, so that figs were accessible to pollinators only during the afternoon. All the figs were collected at sunset on the day of the experiment, and the number of black and yellow wasps that had penetrated each fig (number of foundresses) was determined. For each pollinator species, the mean number of foundresses per fig was compared between figs exposed the whole day and those exposed only in the afternoon, using Student's t-tests.

Our prediction was that because a lower proportion of the yellow pollinators would be alive and visiting figs in the afternoon than in the morning, figs exposed only during the afternoon would contain a higher proportion of black foundresses than fig already exposed to pollinators in the morning.

Daily pattern of scent production by *F. septica* and *F. nota* at the time of fig receptivity

The aims of the following experiment were to establish 1) whether volatile organic compound (VOC) release by figs varied in quantity and/or in composition during the course of a day, 2) whether the pattern observed in figs is similar to the pattern observed in leaves or whether it is adjusted to the daily pattern of pollinator activity, and 3) whether *Ficus septica* figs display an unusual pattern (potential adaptive response to the presence of two pollinators using *Ficus nota* figs as a control).

Scent sampling design. We compared daytime variation in volatile emissions (sunrise and noon) between receptive male figs of *Ficus septica* (two pollinators) and *Ficus nota* (one pollinator) and between figs and leaves.

Pre-receptive figs were enclosed in plankton net bags in order to prevent pollination. Large numbers of pollinators flying around the tree was taken as a signal that many figs had become receptive. We then performed receptive fig scent and leaf scent extractions simultaneously, once at sunrise and then once at noon. This sampling protocol was repeated in five male individuals of each *Ficus* species.

Flowering phenology was somewhat asynchronous in both *Ficus* species, so that it was necessary to select the figs from which VOC emissions were collected in order to obtain a sample as homogenous as possible in developmental stage. To avoid any bias due to haphazard allocation of figs to sunrise and noon extractions, we randomized the selection process. Prior to each sunrise scent collection, 40 figs were chosen from the tree to be sampled and 20 of them were randomly assigned to the sunrise scent collection. The remaining 20 were used for the noon scent collection.

Scent extraction methods. Scent extraction was performed using the headspace technique [31]. The filters were designed to fit inside a chromatoprobe thermodesorption kit (see next section) and filled with 1.5 mg of Carbotrap 20–40 and 1.5 mg of Tenax 60–80. One microliter of a solution of internal standards (nonane and dodecane) in known concentrations was injected in each filter prior to extraction to allow later estimation of emission rates. Each sample was taken from either 20 receptive figs (selected as indicated above) or five leaves, cut off from branches and enclosed in polyethylene terephtalate bags. We standardized bag size to limit the variability in headspace volume and improve the repeatability of emission rate estimation. Scent was left to accumulate inside the bags for 30 minutes, and then the air was pulled out of the bag through the filter for five minutes with a flow rate of 160 mL/min. For each paired sample (one fig sample and one leaf sample extracted simultaneously), a control was made using an empty bag.

Identification and quantification of the volatile compounds. GC-MS analyses were carried out using a gas chromatograph CP-3800 (Varian Inc., Palo Alto, CA) equipped with an FID detector and coupled with a Saturn 2000 mass spectrometer (Varian). The samples were injected using a 1079 programmed temperature injector with a chromatoprobe kit (Varian), and was programmed as follow: 40°C hold for 0.5 min, and increased to 250°C at 200°C/min, hold for 3 min, and finally cooled down to 40°C with a fan. Chromatographic separation was performed using a fused silica capillary column (30 m×0.25 mm×0.25 µm Optima 5 Accent, Macherey-Nagel, Düren, Germany) with the following oven program: 40°C hold for 3 min, from 40°C to 100°C at 3.3°C/min, from 100 to 140°C at 2.9°C/min, from 140 to 180°C at 2.7°C/min, and finally upped to 250°C at 10°C/min and hold 8 min. The carrier gas was helium with a constant flow rate set close to 1.0 mL/min. The samples were injected in splitless mode. The energy for ionization by electron impact was 70 eV. The temperature of the transfer line, manifold and trap were respectively 250°C, 80°C and 170°C. The spectrometer was used in scan mode, from 38 to 300 m/z ratio.

All the volatile organic compounds (VOC) were tentatively identified by comparison with mass spectral libraries NIST98 MS and Adams 2007 [41], and retention indices found in Adams 2007 [41], online libraries (pherobase [42], NIST webbook [43]) and published data for (Z)- and (E)-DMNT [44]. Internal standards injected into each filter prior to scent extraction (0.08 µg nonane, 0.1 µg dodecane) allowed estimating the quantity (µg) of each identified compound contained in each sample.

Statistical analysis of scent profiles. Only VOCs that appeared in at least two different scent samples were retained to determine scent profiles. We checked that the presence/absence of the major VOCs was not affected by cutting off figs and leaves from branches. The only major VOC whose presence was due to cutting was (Z)-3-hexen-1-ol. Therefore, all the statistical analyses presented below were done after removing (Z)-3-hexen-1-ol from scent profiles. This had no qualitative effect on the results unless mentioned. From this VOC set, we calculated total emission rate and the relative composition of each scent profile. Total emission rates were the sum of emission rates of all VOCs detected in a given sample, calculated as µg/fig*hour for figs and as µg/cm^2*hour for leaves. Relative scent composition was the relative contribution of each VOC to the scent profile, expressed as a percentage.

Emission rate variation among species and extraction hours were analyzed separately on figs and leaves, using Wilcoxon signed rank tests. Relative scent composition variation among species, organs, and extraction hours were analyzed with methods based on Bray-Curtis distances, implemented in the R package Vegan [45,46]: Patterns of variation were visualized using non-metric multidimensional scaling (NMDS, [31]) and their significance

tested with PERMANOVA [47]. NMDS is an ordination method which computes a locus for each sample within a space of given dimensionality so that the distances between samples on the final ordination are as close as possible to the original distances. The discrepancy between distances on the graph and actual distances is measured by the stress value, which varies from 0 (perfect correspondence) to 100% (no correspondence). According to the subset of samples to be included, we set the dimensionality to either 2 or 3 in order to always obtain stress values below 15%.

For the factors whose effect was detected to be significant by the PERMANOVA, we identified individual VOCs that contributed most to the overall difference by performing univariate Mann-Whitney tests between couples of sample categories. We tagged individual VOCs for which the p-value was lower than 0.05. We preferred this method to the dedicated test in the vegan package (simper function) because the latter is known to highlight variables presenting the highest intragroup variance rather than those that differ among groups [48]. Indeed, the simper function produced biologically meaningless results on our dataset.

Results

Traits associated with wasp lifespan and competitiveness

Wasp emergence patterns and lifespan of the black and yellow pollinators of *F. septica*. Most emergences from figs occurred in the morning in both *F. nota* and *F. septica*, independently of the pollinating wasp species. Indeed for 61% of 117 *F. septica* figs and for 57% of 89 *F. nota* figs, peak pollinator emergences occurred before 7 a.m. We did not detect any difference in timing of peak wasp emergence between *F. septica* figs hosting yellow, black or both pollinator species, and when both species emerged from the same fig they did so simultaneously. Since only 15 *F. septica* figs contained black pollinators (alone or together with yellow wasps), however, we could not exclude some slight difference in timing.

For all eight *Ficus septica* figs for which emerging pollinator lifespan was followed, all insects were dead within 12 hours after their emergence, so that survival was counted for each fig 6 hours and 9 hours after emergence. There was no single case of higher survival rate of yellow pollinators comparatively to black ones, resulting in globally significantly higher survival of black wasps 6 and 9 hours after emergence (Table 1).

Day round pattern of pollinating wasp presence on trees during their period of fig receptivity. Black *F. septica* pollinators were much less abundant than yellow ones during the field session (1043 yellow wasps caught versus 71 black ones), a pattern also observed when we were monitoring emergences. Both *F. septica* pollinators and *F. nota* pollinators were virtually always caught during daytime on sticky traps (Figure 1, Table S1). In *F. septica*, the presence patterns of black and yellow wasps were very similar (Figure 1): most individuals were caught in the morning (87% of yellow and 85% of black pollinators were caught between sunrise and 12 a.m., see Table S1 for detailed results), with detections decreasing during the afternoon and approaching zero during the night. Given the difference in lifespan between both species, we would have expected the relative frequency of black wasps around receptive trees to peak in the afternoon. However, in addition to wasp lifespan, the actual presence of attractive figs must also influence the daily patterns of wasp presence around receptive trees. Morning-pollinated figs could rapidly lose attractiveness, a feature that could explain the low numbers of black wasps trapped on the trees in the afternoon. The following experiment was set up to test that hypothesis.

Consequences of manipulating the timing of accessibility of *F. septica* figs on the abundance of black and yellow foundresses. The figs that had been left accessible to wasps all day long contained many more yellow foundresses at the end of the experimental day than those that had been left accessible only during the afternoon (Wilcoxon rank sum test: $W = 815$, $p < 0.001$, Figure 2). On the contrary, the number of black foundresses that had entered the figs did not differ according to their period of accessibility ($W = 357.5$, $p = 0.35$, Figure 2). The mean proportion of black wasps was significantly higher in figs left accessible only during the afternoon (whole day: 5+/−9% black wasps; afternoon: 34+/−28% black wasps; generalized linear model with quasibinomial distribution: $t = -3.43$, $p = 0.0011$). An interpretation of these results is that decreased pollinator densities in the afternoon in natural conditions could be due to a rapid loss of fig attractiveness once pollinated. We propose that when some figs remained attractive in the afternoon, black pollinators were more efficient at colonizing them probably because of their longer

Table 1. *Ficus septica* pollinating wasp lifespan survey: compared survival rates of yellow and black pollinators emerged from the same figs.

			survival rate (%)			
	number of wasps		6 hours after emergence		9 hours after emergence	
fig N°	yellow	black	yellow	black	yellow	black
1	28	150	28.57	78.66	0	5.33
2	106	24	56.60	75.00	0	4.16
3	136	39	84.55	100.00	0	30.76
4	29	28	48.27	89.28	0	0
5	31	125	77.41	81.60	0	0
6	77	36	58.44	88.88	14.28	38.88
7	214	57	77.10	98.24	5.14	75.43
8	203	25	46.79	60.00	0	12.00
binomial test p-value[1]			0.0039		0.016	

[1]null hypothesis: if yellow and black pollinators have the same lifespan we expect that at any point in time the survival rate of the black species exceeds that of the yellow species in half of the replicates (p-values are for one-tailed tests, excluding ex-aequo).

doi:10.1371/journal.pone.0103581.t001

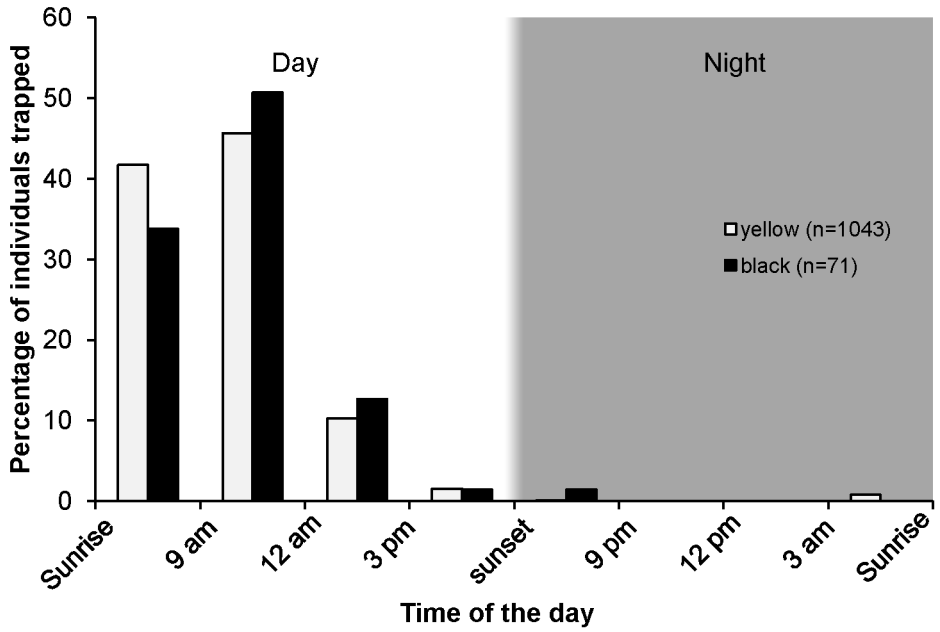

Figure 1. The daily pattern of *Ficus septica* pollinator activity around trees bearing receptive figs. Number of yellow and black pollinators trapped at different times of the day, expressed as percentage of the total number of individuals trapped over 24 hours on 6 different trees. Raw data provided as Table S1.
doi:10.1371/journal.pone.0103581.g001

lifespan. Because wasp lifespan should be counted in hours, the longer longevity of black pollinators should enable them to colonize more distant host trees.

Daily patterns of scent production by *F. septica* and *F. nota* at the time of fig receptivity

Variation in scent emission rates. Figs and leaves of the two species displayed the same pattern of variation: emission rates were significantly higher at noon than at sunrise (Wilcoxon signed rank test on both species pooled; figs: $V = 8$, $p = 4.8*10^{-5}$, Figure 3.A.; leaves : Figure 3.B., $V = 8$, $p = 0.049$). Hence the general physiology of the trees and protection against abiotic stress is sufficient to explain the rhythm of fig VOC production. We have no evidence in favor of an emission rhythm that would reflect adaptation to pollinator activity rhythm or number of pollinator species.

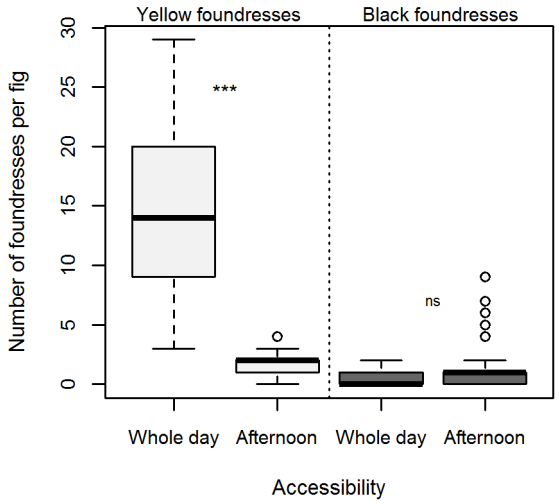

Figure 2. Colonization by pollinators of *Ficus septica* receptive figs whose accessibility has been manipulated. Number of yellow (light grey) and black (dark grey) foundresses found inside figs that have been left accessible to pollination for the whole day or in the afternoon only. Raw data provided as Table S2.
doi:10.1371/journal.pone.0103581.g002

Variation in relative scent composition. Seventy VOCs were detected in at least two samples and retained in the computation of scent profiles (Table S3, all statistical analyses exclude (Z)-3-hexen-1-ol, see material and methods). Forty-eight of them were tentatively identified. A further 16 of the non-identified ones were assigned to a biosynthetic category. Both *F. septica* and *F. nota* emitted mainly terpenoids (mean relative contribution varying from 48 to 94% depending on the species, organ and hour of extraction). Overall, figs emitted a larger number of different VOCs than leaves, and noon scents were comprised of a larger number of VOCs than morning scents (Table S3).

Relative scent composition varied significantly according to species, organ type and time of extraction (global PERMANOVA, Table 2). The species*organ type and organ type*time of extraction interactions were also significant. On the 3 dimensional NMDS ordination, fig and leaf scents were separated along axis 1 (Figure 4). Morning and noon scents were separated on axes 2 (Figure 4.A) and 3 (Figure 4.B, Wilcoxon rank sum tests comparing position along the axes: $W = 326$ and $p = 0.0004$ for axis 2, $W = 331$ and $p = 0.0002$ for axis 3).

In order to get better insights into sources of variation, we performed some further analyses separately on fig and leaf scents.

Separate analysis of daily variation in fig and leaf relative scent composition. Sixty-seven VOCs were present in at least two fig samples. Receptive fig scent composition differed significantly between the two species and according to time of extraction (PERMANOVA, Table 3). The interaction term had no significant effect, suggesting that the two effects were orthogonal. Indeed, on the 2 dimensional NMDS ordination (Figure 5.A), the two species are separated along axis 1, and

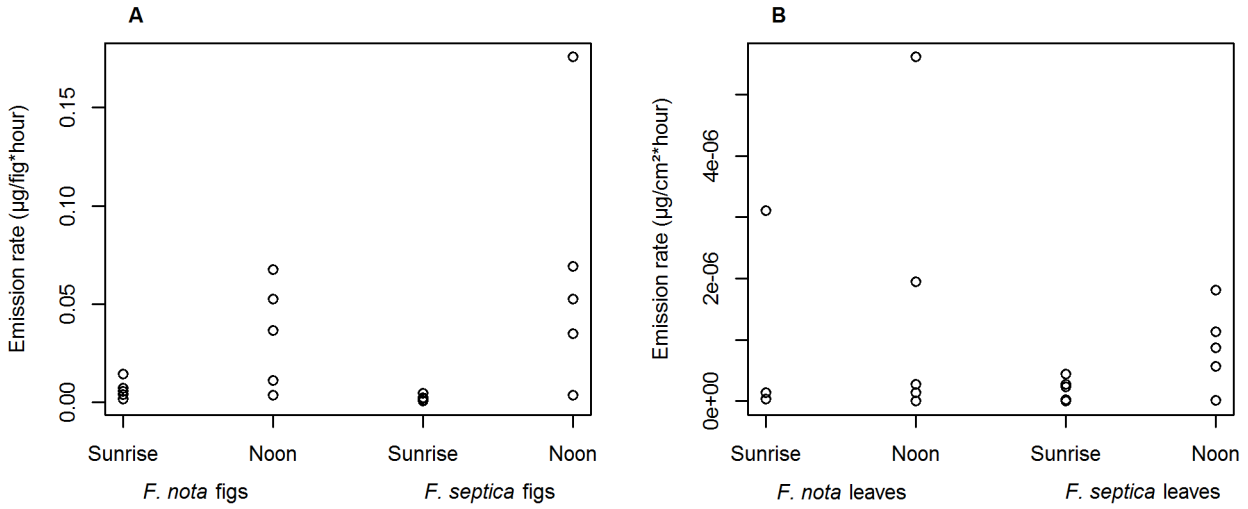

Figure 3. Daily variation of scent emission rates in *Ficus septica* and *Ficus nota*. Total scent emission rates from (A) figs (µg/fig*hour) and (B) leaves (µg/cm^2*hour) of both species at sunrise and at noon.
doi:10.1371/journal.pone.0103581.g003

sunrise and noon samples along axis 2. Again, this suggests the absence of special features in *Ficus septica* scent production rhythm.

Twenty VOCs were present in at least two leaf samples. Leaf scent composition varied significantly according to the hour of extraction, but the species effect and the interaction term were not significant (PERMANOVA, Table 4). Although we observed no segregation on the NMDS ordination (Figure 5.B.), sunrise samples were more dispersed than noon samples. Sunrise leaf scent composition was actually poorly consistent across samples, as only one VOC ((E)-caryophyllene) was detected in at least 4 out of 5 sunrise leaf samples in *F. nota*, and none in *F. septica*. In other words, we cannot define a clear mean profile for leaf scents in the morning. (Z)-3-hexen-1-ol was the only other compound consistently present in most morning leaf samples. When it was included in the analysis, sunrise and noon leaf scents were segregated along axis 1of the NMDS ordination (not shown). The lack of consistency in the composition of morning leaf scents may be due to low emission rates, as most compounds detected in these samples were at or near to the detection limits.

Identification of the VOCs responsible for the difference between sunrise and noon scents. Regardless of species and organ type, the difference between sunrise and noon scent composition was mainly explained by a set of monoterpenes and both DMNT enantiomers (Table 5), whose relative proportion was higher at noon. The exact identity of the VOCs involved was different in figs of both species and in leaves, but (Z)- and (E)-β-ocimene were common to all. Consistent with this, there was a

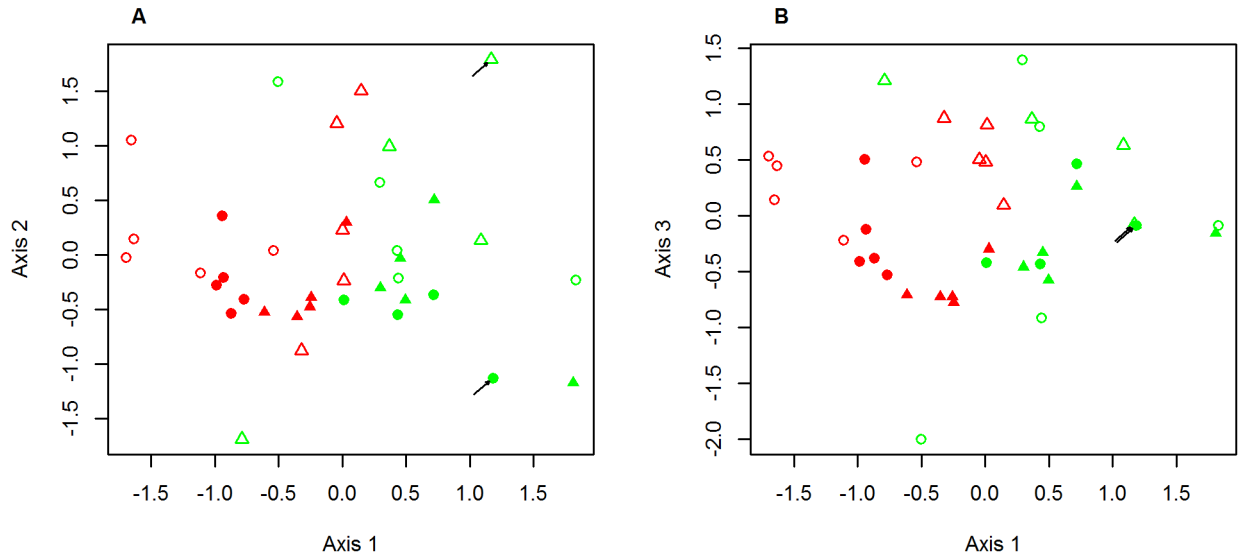

Figure 4. Patterns of variation of the composition of scents emitted by *Ficus septica* and *Ficus nota*. Three-dimensional NMDS ordination on the relative composition (% each VOC) of scents emitted by figs (red symbols) and leaves (green symbols) of *Ficus septica* (triangles) and *Ficus nota* (circles) at sunrise (open symbols) and at noon (closed symbols). (A) Axes 1 and 2, (B) Axes 1 and 3. Stress-value = 10%. Black arrows indicate places where two samples of the same category share the same locus.
doi:10.1371/journal.pone.0103581.g004

Table 2. PERMANOVA analysis on the relative composition of scents emitted by *Ficus septica* and *Ficus nota* figs and leaves at sunrise and noon.

Factor	Df	sum of squares	F-value	p-value
species	1	0.86	3.55	0.001
organ	1	1.56	6.45	$1*10^{-4}$
hour	1	1.82	7.57	$1*10^{-4}$
species*organ	1	0.67	2.78	0.007
species*hour	1	0.34	1.42	0.16
organ*hour	1	0.53	2.21	0.02
triple interaction	1	0.33	1.38	0.18
residuals	32	7.72		

doi:10.1371/journal.pone.0103581.t002

generalized increase in total monoterpenoid proportion at noon compared to sunrise scents, in figs of both species as well as in leaves (Table S3). In leaves, the relative proportion of (E)-DMNT was also higher at noon. This difference is due to a much stronger increase in the absolute quantity of several monoterpenes (and of (E)-DMNT in leaves) between sunrise and noon samples relative to other VOC categories (not shown). Consistent with the orthogonality of species and hour of extraction effects on fig scent composition, a set of sesquiterpenes were responsible for a large part of the difference in scent composition between figs of both species (Table S4).

Discussion

While it is rarely mentioned in the literature, field evidence shows that light colored fig pollinating wasps are generally nocturnal and dark colored fig pollinating wasps are diurnal [49]. To our knowledge, our results provide the first demonstration of the occurrence of a diurnal light colored fig pollinating wasp. This light colored fig pollinating wasp co-occurs on its host tree with a very closely related species that is dark colored. Studies using molecular markers have allowed detecting numerous cases in which several fig wasp species, generally qualified as cryptic, pollinate the same host [6]. We provide here one more example in which it is demonstrated that co-pollinators present divergent ecological traits, a feature which should facilitate co-existence. We suggest that most cases of several species of fig pollinating wasps co-occurring locally on a host will turn out to correspond either to contact zones between different species or to the co-occurrence of species presenting strongly divergent ecological traits. We show that the black pollinator species associated with *Ficus septic*a is longer lived than the yellow species a feature which should enable it to drift further in the wind in search of receptive figs, and hence reach receptive figs located further away from the one they were born in. This should give some advantage to the black species comparatively to the yellow one. The two species coexist throughout the island of Luzon, and a similar situation is found in Taiwan with a yellow and a black species associated with *F. septica* [18]. This widespread coexistence can only be explained if some trait of the yellow species compensates for its shorter survival.

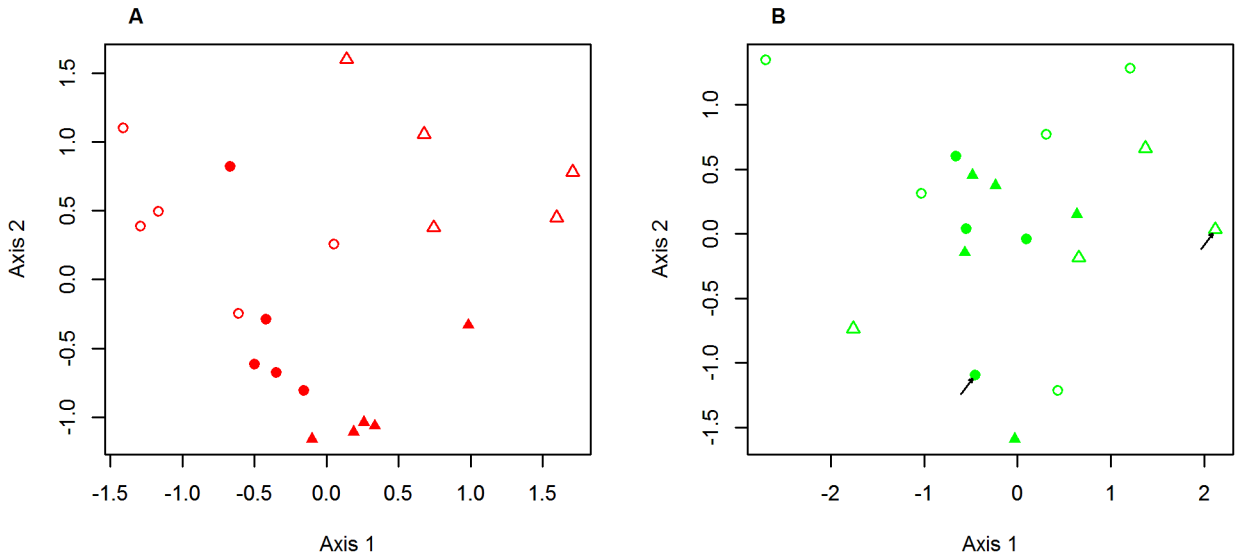

Figure 5. Variation in the composition of the scents emitted by figs (A) and leaves (B). Two-dimensional NMDS ordinations on relative scent composition (% each VOC) computed separately on (A) fig scents (stress-value = 14%) and (B) leaf scents (stress-value = 11%). Circles represent *Ficus nota* and triangles *Ficus septica* samples, open symbols are sunrise samples and closed symbols are noon samples. Black arrows indicate places where two samples of the same category share the same locus.
doi:10.1371/journal.pone.0103581.g005

Table 3. Refined PERMANOVA analysis on the relative composition of scents emitted by figs of *Ficus septica* and of *Ficus nota* at sunrise and at noon.

Factor	Df	sum of squares	F-value	p-value
species	1	0.97	4.79	0.0005
hour	1	1.16	5.72	0.0002
species*hour	1	0.39	1.93	0.072
residuals	16	3.26	0.56	

doi:10.1371/journal.pone.0103581.t003

We therefore predict that the yellow species is more efficient than the black species at rapidly locating, entering and/or ovipositing in receptive figs. If this competitiveness-lifespan trade-off hypothesis holds true, we would expect the black species to be more abundant in places where *Ficus septica* population density is low and the yellow species to be more abundant where population density is high. Hence, their coexistence would be facilitated by spatial heterogeneity of density of the resource they compete for. While *Ficus septica* fruits throughout the year, its fruiting frequency varies across seasons [50]. Therefore, we predict that the identity of the favoured species at a given location may also vary throughout the year.

We found published data and species descriptions suggesting the presence of the same pattern for the wasps associated with two other *Ficus* species: *F. rubiginosa* (subgenus *Urostigma* section *Malvanthera*) and wasp genus *Pleistodontes* in Australia, and *F. tuerkheimii* (subgenus *Urostigma* section *Americana*) and wasp genus *Pegoscapus* in America [14–17]. There are therefore potentially three independently evolved cases of diurnal light colored fig pollinating wasps pollinating a fig tree in competition with a very closely related black species. If diurnal behavior is confirmed, then it will be possible to test the prediction that in all three cases, the yellow species is shorter lived than the black one. Further investigations would then be needed to establish precisely what are the traits enabling yellow species persistence despite their shorter lifespan.

Other cases of *Ficus* species colonized by light colored and dark colored fig wasps are strikingly different. They include some species of section *Galoglychi*a pollinated by genera *Alfonsiella* and *Elisabethiella* [7]. But in those cases the *Alfonsiella* species are light colored and nocturnal and the *Elisabethiella* species are dark and diurnal. Similarly, *Ficus sycomorus* is visited by two agaonid wasps of genus *Ceratosolen*. *C. arabicus*, the pollinator, is light colored and nocturnal while the distantly related *C. galili* does not carry pollen and is dark colored and diurnal [26].

In order to establish whether the two wasp species pollinating *Ficus septica* encountered the same receptive fig scents, we analyzed daily variation of receptive fig scent composition in *F. septica* and in another fig species, *F. nota*, as a control. In both species the quantity of volatile compounds produced increased between morning and noon. Further the composition of the scent varied, notably the monoterpene content increased, i.e. compounds that are known to be detected by insects and among them fig wasps [38–39] and that are known to be used by insects to locate flowers. Hence, despite the central role of receptive fig scent in attracting fig wasps [28–31], this has not led to highly stereotyped receptive fig scents throughout the day. This new result is in agreement with previous studies which have evidenced strong variation within population among trees in receptive fig scent composition (one exception, *F. semicordata* uses a private channel to attract its pollinator [51]). Because of 1) the similar patterns of scent emission by the two *Ficus* species and 2) the similitude between figs and leaves in the daily pattern of scent variation, and 3) because monoterpenes are known to protect plant tissues against temperature and oxidative stresses [35,36], we suggest that this variation is, at least in part, due to the production of volatile compounds protecting the organs against temperature and oxidative stresses. The biology of *Ficus sycomorus* suggests that the tree has limited control on the production of volatiles used by wasps to detect receptive figs. Indeed, *F. sycomorus* is pollinated at night by *Ceratosolen arabicus*, and in the daytime it is colonized by *C. galili*, a species that does not provide any pollination service. Despite this pattern of visitation, *F. sycomorus* receptive figs produced at noon the same main volatile compound and similar total quantities of volatile compounds as the closely related diurnally pollinated *F. sur* [31].

In this study, f we demonstrated that black and yellow pollinating wasps of *Ficus septica* have different lifespan and that receptive fig scent composition varies during the day. The two wasp species are therefore submitted to somewhat different ranges of receptive fig scents. We may therefore expect that they use a somewhat different range of chemical cues to locate receptive figs. If this is the case, then we may speculate on whether they respond differently to the within population variation of receptive fig scent among individual trees. Any such variation could lead to some assortative mating of the fig trees, thus structuring the gene flow

Table 4. Refined PERMANOVA analysis on the relative composition of scents emitted by leaves of *Ficus septica* and of *Ficus nota* at sunrise and at noon.

Factor	Df	sum of squares	F-value	p-value
species	1	0.47	1.72	0.10
hour	1	1.04	3.78	0.0015
species*hour	1	0.3	1.09	0.37
residuals	16	4.4	0.71	

doi:10.1371/journal.pone.0103581.t004

Table 5. Main VOC responsible for the difference between sunrise and noon scent composition in figs of each species and in leaves.

	F. nota figs	*F. septica* figs	Leaves
monoterpenes			
sabinene	*	ns	ns
myrcene	*	*	ns
α-terpinene	*	ns	ns
1,8-cineole	*	ns	ns
(Z)-β-ocimene	*	**	*
(E)- β -ocimene	*	*	*
γ-terpinene	*	ns	ns
menthatriene	ns	*	ns
allo-ocimene	ns	**	*
irregular terpenes			
(Z)-DMNT	ns	ns	**
(E)-DMNT	ns	**	**
phenylpropanoids/benzenoids			
Aromatic 1	*	*	*
p-cymene	*	ns	ns
aromatic 2	*	ns	ns
p-cymenene	ns	*	ns
aromatic 5	ns	**	*
unidentified			
unidentified alcohol	ns	*	ns

P-values for the Mann-Whitney tests comparing the mean relative contribution of individual VOC to sunrise and to noon scent samples. Significance codes (values non corrected for multiple testing): ns = non-significant,
*<0.05,
**<0.01.
doi:10.1371/journal.pone.0103581.t005

within the *Ficus* species. Whether such processes are at work and whether they could ultimately lead to host speciation is an open question.

Supporting Information

Table S1 The daily rhythm of pollinator activity around trees bearing receptive figs: detailed results.
(DOCX)

Table S2 Tree by tree results of the experiment where the accessibility of receptive *Ficus septica* figs was manipulated. Number of yellow and black foundresses found inside receptive figs that have been accessible to pollination either for the whole day or in the afternoon only.
(DOCX)

Table S3 Mean relative composition of the scents emitted by figs and leaves of *Ficus septica* and *Ficus nota* at sunrise and at noon. Mean relative contribution of each VOC to the scent of each sample category, expressed as a mean percentage +/− standard deviation.
(DOCX)

Table S4 Main VOC responsible for additional differences of interest. P-values for the Mann-Whitney tests comparing the mean relative contribution of individual VOC between categories indicated by column titles. Each comparison is between groups of 10 samples. Significance codes (values non corrected for multiple testing): ns = non significant, *<0.05, **< 0.01, ***<0.001.
(DOCX)

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Author Contributions

Conceived and designed the experiments: L. Conchou LR FK. Performed the experiments: L. Conchou L. Cabioch LR. Analyzed the data: L. Conchou L. Cabioch. Contributed reagents/materials/analysis tools: L. Conchou LR FK. Contributed to the writing of the manuscript: L. Conchou L. Cabioch LR FK.

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Appendix 2. List of figures

Appendix 3. List of tables

Appendix 4. Poster presented at the 6th European Phycological Congress

Chemical signaling and defense in brown algal kelps during interactions with herbivores

Cabioch Léa[1,2], Ritter Andrés[1], Goulitquer Sophie[1], Potin Philippe[1], Faugeron Sylvain[2], Leblanc Catherine[1]

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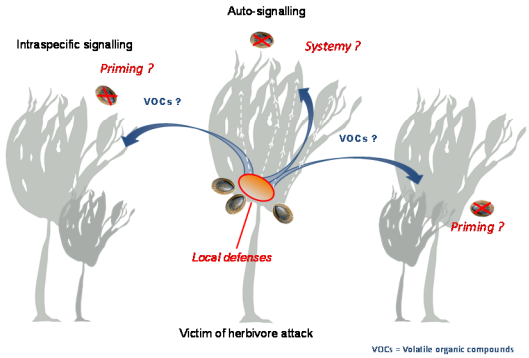
2- Departamento de Ecologia, Pontificia Universidad Catolica de Chile, Santiago de Chile, Chile

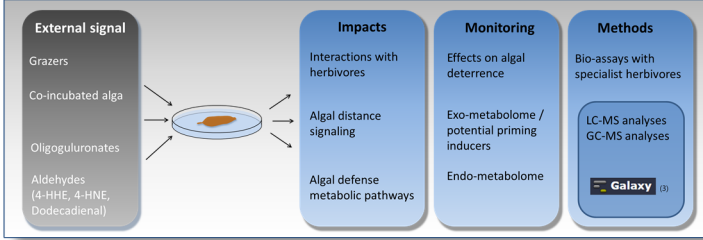
Introduction

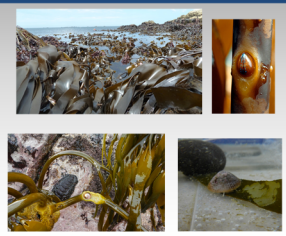
In the terrestrial environment, **volatile organic compounds**, such as aldehydes, are emitted by plants following herbivore's attack, and perceived by neighboring plants as **warning distance signals**, leading to priming and systemic defense. Marine algae actively respond to biotic stress, such as grazer's attack, by regulating transcription and metabolic pathways. We have recently shown systemic responses in the brown alga *Laminaria digitata* upon defense elicitation by oligoguluronates (1) and demonstrated a priming effect, induced by **chemical cues** present in seawater surrounding a kelp bed and/or released from neighboring brown algae (2). In the context of **kelp/herbivore interactions**, we are developing metabolomic approaches to identify the chemical signals involved in the defense upon herbivory in two emblematic kelp species, *L. digitata* from North-Atlantic Brittany and *Lessonia spicata* from Chile.



Hypothetical defense signaling during kelp/herbivore interactions

Methodology



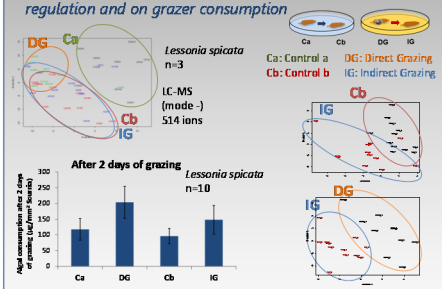


Laminaria digitata
Patella pellucida

Lessonia spicata
Scurria scurra

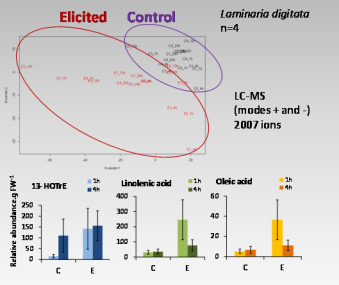
Results

Effects of direct and indirect grazing on algal metabolism regulation and on grazer consumption



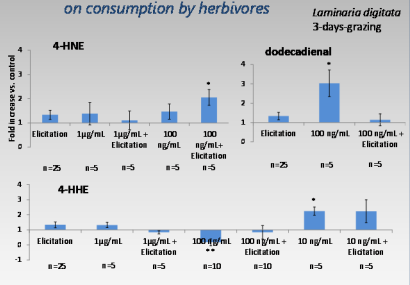
- ✓ Metabolomic analyses show that grazing by herbivores (DG) induces modifications of the endo-metabolome, as well as co-incubation with a grazed alga (IG ≠ Cb, IG ≠ DG).
- ✓ Moreover, the bio-assay shows that previous grazing or co-incubation with a grazed alga seem to increase subsequent algal consumption by herbivores.

Kinetics of the exo metabolome of an elicited alga



- ✓ Statistical analysis (PLS-DA) shows that defense elicitation by oligoguluronates significantly modifies the exo-metabolome released by kelps in seawater.
- ✓ Upon defense elicitation, fatty acids and oxylipins are rapidly over-produced and released in seawater after 1h.

Effects of algal incubation with an aldehyde on consumption by herbivores



- ✓ Chemical compounds, like the aldehydes 4-HNE or dodecadienal, tend to increase grazing impacts by herbivores.
- ✓ However, incubation of algae in the aldehyde 4-HHE at 100 ng/mL significantly decreases algal consumption by herbivores, and seems to confer a better protection against grazing.

Conclusions and perspectives

Upon grazing by herbivores and perception of waterborne chemical cues emitted by a grazed alga, **specific metabolic pathways** seem to be induced in a neighboring kelp. This appears to increase subsequent grazing after a previous grazing or perception of chemical compounds. Moreover, defense elicitation induces the release of numerous **waterborne compounds** in seawater, such as **fatty acids and oxylipins**, or aldehydes. The aldehyde **4-HHE**, at the concentration of 100 ng/mL, seems to trigger defense responses or **priming** state, leading to a **better protection against grazers.** The chemical identification of waterborne cues in seawater and differentially-expressed compounds in the algae should lead to a better understanding of the metabolic regulations occurring during defense and distance signaling in kelps.

References: (1) Thomas *et al.* 2011. Waterborne signaling primes the expression of elicitor-induced genes and buffers the oxidative responses in the brown alga Laminaria digitata. PlosOne 6(6): e21475. (2) Thomas *et al.* 2014. Kelps feature systemic defense responses: insights into the evolution of innate immunity in multicellular eukaryotes. New Phytologist. 204: 567–576. (3) Giacomoni *et al.* 2014. Workflow4Metabolomics: A collaborative research infrastructure for computational metabolomics. Bioinformatics doi:10.1093/bioinformatics/btu813.

Appendix 5. Training

- Expertise Flore, 10-31 juillet 2013, Roscoff
- Anglais « Scientific Writing », 23-24 janvier 2014, Paris (2 jours)
- Traitement de données métabolomiques sous Galaxy, 28 janvier 2014, Roscoff (1 jour)
- Linux, 12 mai 2014, Roscoff (1 jour)
- Cluster, 15 mai 2014, Roscoff (1 jour)
- Cycle ”Projet Professionnel et Gestion de Carrière”, 20-24 avril 2015, Roscoff (4 jours)
- Ecole Workflow4metabolomics, 21-25 septembre 2015 , Roscoff (5 jours)
- Stage ”Vulgariser les sciences”, 7-9 mars 2016, Orsay (3 jours)
- Journée Entreprise ”les métiers de la culture”, 13 juin 2016, Paris (1 jour)

Appendix 6. Conferences

Journées Idealg	Roscoff	October 21st-23rd, 2013	Poster **2nd poster price**
AG GdR BioChimar	Lorient	November 7th, 2013	
Journée de la plateforme Corsaire	Rennes	November 8th, 2013	
Journée des Jeunes Chercheurs de la Station Biologique de Roscoff	Roscoff	December 5th, 2013	Poster **1st poster price**
Journées de la Société Phycologique de France	Roscoff	December 16-18th, 2013	
Ecole Thématique en Ecologie Chimique (ETEC)	Marseille	June 16-20th, 2014	
Journées du Réseau Français de Metabolomique et de Fluxomique (RFMF)	Lille	June 9-11th, 2015	Poster **1st student poster price**
6th European Phycological Congress	London	August 23-28th, 2015	Poster **1st poster price**
Journées de la Société Phycologique de France	Vannes	September 23-25th, 2015	Poster
2è journées scientifiques du GdR MediatEC	Banyuls-sur-Mer	October 28-30th, 2015	Oral communication
Journées Idealg	Roscoff	November 5-6th, 2015	Oral communication
Workshop CeBiB (Centro de Biotecnología y Bioingeniería)	Santa Cruz, Chile	December 2nd-4th, 2015	Poster
International Conference on Ecological Sciences – Sfécologie 2016	Marseille	October 24-27th, 2016	Oral communication
3è journées scientifiques du GdR MediatEC	Marseille	October 28-29th, 2016	

Appendix 7. Other participations

- Membre élue du collège étudiant au Conseil d'Administration de la Station Biologique de Roscoff (2014 – 2016)
- Suppléante du collège étudiant au Conseil de Laboratoire de l'UMR 8227 (2014 – 2016)
- Présidente de l'Association Française des Jeunes Chercheurs en Ecologie Chimique (AFJCEC), oct 2014 – oct 2015.
- Organisation de la Journée des Jeunes Chercheurs de la Station Biologique de Roscoff, décembre 2014.
- Participation au Dance Your PhD contest (2015) "Chemical signaling in brown macroalgae during herbivory"
- Participation à l'organisation des 2èmes journées scientifiques du GdR MediatEC (2015).
- Participation à la Fête de la Science, Station Biologique de Roscoff (2014, 2015)
- Nuit des Chercheurs, Océanopolis, Brest (2014, 2015)
- Participation au stand Macroalgues sur le Quai des Sciences, Fêtes Maritimes de Brest 2016 (juillet 2016)