DEVELOPMENT AND MORPHOLOGICAL EVOLUTION IN FLOWERS: ONTOGENETIC INTEGRATION PATTERNS AND STRUCTURAL FACTORS IN THE FLORAL MERISTEM.

DESARROLLO Y EVOLUCIÓN MORFOLÓGICA EN FLORES: PATRONES DE INTEGRACIÓN ONTOGENÉTICA Y FACTORES ESTRUCTURALES EN EL MERISTEMA FLORAL

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"La naturaleza es un templo donde pilares vivientes

a veces dejan salir confusas palabras;

allí se adentra el hombre entre bosques de símbolos

que lo observan con miradas familiares."

Correspondencias. Las Flores del Mal (1861)

Charles Baudelaire.

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INDEX

RESUMEN

Los patrones de diversificación floral están determinados por cambios tanto en la presión selectiva mediada por polinizador como en las trayectorias ontogenéticas. Particularmente, cambios en las proporciones entre órganos florales se asocian con cambios rápidos en el síndrome de polinización. Estos cambios han sido estudiados principalmente por tres aproximaciones: la co-variación entre órganos florales (integración morfológica), las tasas de crecimiento de cada órgano floral (alometría morfológica) y el cambio evolutivo en la temporalidad del desarrollo (heterocronía). En esta tesis, se estudiaron los patrones ontogenéticos de integración, alometría y heterocronía y su relación con la diversificación mediada por polinizador en el género *Schizanthus*. Tanto la integración como la alometría fueron medidos usando una división de dos estadios ontogenéticos o realizando un rastreo por rangos solapados de tamaños de yema creciente, para contestar las siguientes preguntas: 1) cómo la integración y las pendientes alométricas varían a lo largo de la ontogenia; 2) cómo estas variaciones se relacionan con los patrones de diversificación mediada por polinizador y 3) su relación a lo largo de la ontogenia y entre especies. Respecto a la heterocronía, análisis cuantitativos de tamaño y forma en un amplio rango de estadios ontogenéticos fueron usados para construir las trayectorias ontogenéticas y describir su relación con los patrones de diversificación mediada por polinizador en el género. Este estudio encontró que tanto la integración como la alometría variaron a lo largo de la ontogenia y entre las especies. Estos

parámetros tendieron a co-variar a lo largo de la ontogenia, dado que eventos de baja integración tendieron a correlacionarse con una mayor diferencia de pendientes alométricas entre rasgos. Sin embargo, esta relación varió entre especies, sugiriendo que existe una relación biológica mucho más compleja que la sugerida la relación matemática entre integración y alometría a lo largo del desarrollo. Los patrones de integración y alometría durante el desarrollo fueron congruentes con los patrones de diversificación mediada por polinizador en el género: estructuras reducidas que perdieron su función en el proceso de polinización tienden a estar desacopladas del resto de la corola y tener menores pendientes alométricas durante el desarrollo. Mientras que estructuras más reducidas presentaron menores pendientes alométricas, estas no estuvieron más desacopladas, mostrando nuevamente que la relación entre integración y alometría durante el desarrollo es más compleja de lo esperado. Respecto a la heterocronía, los patrones de diversificación floral en el género se produjeron por transiciones a morfologías pedomorficas y peramorficas. Estas transiciones ocurrieron mediante diferentes combinaciones de procesos ontogenéticos (cambios en la aparición del rasgo o en sus tasas de crecimiento), los cuales pueden presentar efectos compensatorios a lo largo del desarrollo. De forma interesante, estructuras reducidas que no presentaron marcados patrones de desacople durante el desarrollo resultaron encontrarse más afectadas por cambios en su aparición, evidenciando que los patrones de integración se encuentran más afectados por cambios en las tasas de crecimiento. En conclusión, la integración y la alometría ontogenética y los cambios heterocronicos son procesos interrelacionados que varían a lo largo de la ontogenia, presentando efectos compensatorios y donde los cambios entre especies se relacionan con la diversificación floral mediada por polinizador.

ABSTRACT

Patterns of floral diversification are shaped by changes in both pollinator-selective pressure and ontogenetic trajectories. Particularly, changes in the proportions among floral organs are associated with rapid evolutionary shifts in the pollination syndrome. These changes have been studied mainly by three approaches: the co-variation among floral organs (morphological integration), the growth rate of each floral organ (morphological allometry) and the phyletic change in the timing of ontogeny (heterochrony). In this thesis, ontogenetic patterns of integration, allometry, and heterochrony and their relation with pollinator-driven diversification in the genus Schizanthus is studied. Integration and allometry were studied using either a two stages division of ontogeny or overlapping ranges of bud sized along ontogeny to answer the following questions: 1) how integration and allometry vary along ontogeny; 2) how these variations relate to pollination-driven diversification and 3) their relationship along ontogeny and among species. Regarding heterochrony, quantitative analysis of size and shape in a broad range of ontogenetic stages were used to build ontogenetic trajectories and describe their relation to pollinator driven evolution. This study found that ontogenetic integration and allometry vary along ontogeny and among species. These parameters tend to co-varied along ontogeny as low integration events tend to correlate with a greater difference among allometric slopes of traits. However, this relationship varied among species, suggesting a more complex biological relationship than expected by the mathematic relationship between integration and allometry along ontogeny. Ontogenetic integration and

allometry were related with pollinator-driven diversification: reduced structures that lost their function in the pollination process tended to be decoupled from the rest of the corolla and have lower allometric slopes along ontogeny. While more reduced structures had lower allometric slopes, they were not more decoupled, showing again that the relationship between ontogenetic integration and allometry is more complex as previously thought. Regarding heterochrony, pollinator-driven diversification in the genus is produced by a transition to paedomorphic and peramorphic morphologies. These transitions occurred by different combinations of ontogenetic processes (changes in the onset of the trait or its growth dynamics), which can present compensatory effects. Interestingly, reduced structures that did not present strong decoupling patterns along ontogeny resulted in being more affected by changes in their onset, suggesting that integration patterns are more affected by the growth dynamics of each organ. Overall, ontogenetic integration and allometry, as well as heterochrony, are interrelated processes that varied along ontogeny presenting compensatory effects and which changes among species related to pollinator-driven diversification in Schizanthus.

INTRODUCTION

Flowers are modular structures composed by repeated units of the same type such as perianth (sepal, petals or tepals), androecium (stamens) and gynoecium (ovules and pistil). However, the arrangement, number, position, size, and shape of these organs may change, producing an astonishing diversity of flower morphologies. In basal Angiosperms, characterized mostly by spiral phyllotaxis and unfused floral organs, floral diversification occurred mainly by changes in arrangement, number, and position of organs (Endress 1990; Endress and Doyle 2015). In contrast, floral diversification in higher groups occurs mostly by flexibility in shape and size together with key innovations (Endress 2001; Ronse De Craene 2018). This increase in the stability of flower structure is related to evolutionary tendencies such as the appearance of petals stablishing the four-organ category, whorled phyllotaxis, syncarpy, and sympetaly (Endress 1990, 2010a).

Changes in the proportions of floral organs are associated with rapid evolutionary changes and diversification in higher Angiosperms. These changes have been closely related to pollinator-mediated selection enhancing cross-pollination and evolutionary shifts in the pollination system (Darwin 1859; Stebbins 1970; Armbruster *et al.* 2009; van der Niet and Johnson 2012; Van Der Niet *et al.* 2014). Particularly, specialization for particular functional groups of pollinators has triggered both floral divergence among related taxa and floral convergence in

unrelated plan lineages (Faegri and van der Pijl 1979; Fenster *et al.* 2004). In this context, changes in floral size and shape are associated with changes in the function of floral parts during pollination (Castellanos *et al.* 2003; Armbruster *et al.* 2009). For example, the transition to bee pollination needs of a landing platform, which is usually reached by an expansion of the lower lip in bilabiate flowers (Castellanos *et al.* 2004; Wilson *et al.* 2007). In the other hand, transitions to hummingbird or moth pollination are more associated with an elongation of the corolla tube in order to facilitate the fit with the mouthpart of these pollinators (Nilsson 1988; Alexandersson and Johnson 2002; Muchhala and Thomson 2009; van der Niet and Johnson 2012; Johnson *et al.* 2016).

It is widely recognized that changes in developmental processes produce the pre-existing diversity in which pollinator-selective pressure acts (Endress 1990; Ronse De Craene 2003, 2018; Specht and Howarth 2015; Benítez *et al.* 2015). However, in comparison with evolutionary and ecological approaches, changes in flower proportions have been only recently studied under a developmental approach. Several studies have focused on the role of development in evolutionary novelties, such as extra-floral organ whorls (e.g., staminodes), highly derived floral organs (e.g., Pappus) and floral outgrowths (e.g., Spurs)(reviewed in Becker *et al.* 2011; Glover *et al.* 2015). Equally important has been their role on diversification of size and shape: changes in reproductive meristem size may affect organ size and shape proportionally, leading to smaller or larger flowers, or not proportionally, being restricted to a single whorl or affecting particular organs within a whorl (reviewed in Moyroud and Glover 2017; De Craene 2018). Changes in organ shape and size, and their required a change in symmetry, has been studied at the molecular level regarding the identity and development of all organs within a whorl (Moyroud and Glover 2017), but also at the organ

level with their correspondent changes of timing, size and/or pressure along ontogeny (Ronse De Craene 2018).

Fixation of the number and relative position of organs favored not only the variation in shape and size of each fixed organ but also the structural coordination among them into intra-floral functional units or synorganization (Remane 1956, for animals; Vogel 1959, 1969, for plants). Variation in shape and size of synorganized elements opened a new dimension of morphological change. In higher groups, sympetaly and syncarpy increase the potential for synorganization (Endress 1990, 2011), imposing an evolutionary trend in angiosperms and being in some cases considered a key innovation (Endress 1987, 1990, 2001). Synorganization commonly occurs by means of developmental fusion of floral parts: either by congenital or postgenital fusion (Classen-Bockhoff and Heller 2008; Specht and Bartlett 2009; Endress 2016). However, the coordination of organs without fusion is also possible but has been much less considered in developmental studies (Endress 2006, 2010b).

In this thesis, flower morphological diversification will be approached considering how changes in size and shape of floral organs and their coordination among species appeared during development. These two factors should be considered together, as changes in proportions in size and shape of an organ affect directly to its coordination with other organs, and therefore, the functional unit of which it is part. Changes in the coordination among organs without fusion will be studied under the conceptual framework of morphological integration (Armbruster *et al.* 2014; Klingenberg 2014), while the change of size and shape of floral organs will be studied under the conceptual framework of allometry and heterochrony (Gould 1965, 1977; Klingenberg 1998, 2016).

Changes in the coordination among organs: morphologic integration.

Synorganized units not assured by fusion process relies only in the coordinated growth among floral organs for their maintenance. The coordination among floral organs or floral integration is usually measured as the pattern and strength of correlations among traits in a population (Hallgrímsson *et al.* 2009; Haber 2011; Armbruster *et al.* 2014). Morphological integration is an important evolutionary trend in angiosperms, being considered a potential evolutionary route to modularity (Armbruster *et al.* 2014) and an important factor constraining and canalizing morphological evolution (Gould 1977, 2002; Lewontin 1978). Levels and patterns of integration can be shaped by different processes, such as pollinator driven selection, pleiotropy, coordinated gene expression or developmental factors (Cheverud 1982; Murren 2002; Pigliucci 2003; Pigliucci, Massimo; Preston 2004; Armbruster *et al.* 2014; Klingenberg 2014; Smith 2016).

The action of natural selection on traits functionally linked during pollination result in higher correlations among those traits than with others (Diggle 2002; Ordano *et al.* 2008; Armbruster *et al.* 2009). Most studies focus on trait correlations in mature flowers (static integration sensu Klinberger, 2014) showing that integration can vary within and among species. Integration can vary within a whorl and among different functionally related whorls (Conner and Sterling 1996; Delph 1996; Herrera 2001; Diggle 2002; Herrera *et al.* 2002), and among species or populations in accordance with pollinators (Pérez *et al.* 2007; Rosas-Guerrero *et al.* 2011; González *et al.* 2015). In any case, floral traits having different functions in the pollination process, such as traits that enhance pollinator attraction or those that favor a precise fit with the pollinator, can present different patterns of integration (Armbruster *et al.* 2009, 2014; Diggle 2014).

Floral integration can also result from developmental factors imposing evolutionary tendencies that can evolve to match functional relationships or constraint them (Armbruster *et al.* 1999; Herrera 2001; Herrera *et al.* 2002; Armbruster 2004). Accordantly, a key issue to understand the developmental basis of floral diversification is to examine how floral traits become divided into functional units during ontogeny. To our knowledge, no studies have yet examined flower integration across ontogenetic stages (ontogenetic integration sensu Klingenberg, 2014), being usually studied at a particular developmental stage (developmental integration, Armbruster et al., 2014). Therefore, it is not known how ontogenetic integration varies along ontogeny and how this variation is related to static integration of mature flowers and differences among species. Changes in floral integration are expected to be closely related with changes in size and shape of each floral organ. However, of our knowledge there are no studies dealing with the relationship between integration and growth dynamics of particular organs through ontogeny.

Changes of size and shape of organs: allometry and heterochrony studies

Changes in size are usually studied under the conceptual framework of allometry, where growth curves are built to study how a traits varies in relation to either another traits or the overall size of the organism (Huxley *et al.* 1932; Mosimann 1970). Allometry can be measured at different levels: in adults populations within and among species, known as static allometry, and along ontogeny, known as ontogenetic allometry (Klingenberg and Zimmermann 1992; Klingenberg 1998). Changes of static allometry have been related to morphological diversification in flowers (Humeau and Thompson 2001; Hornung-Leoni and Sosa 2005; Feng *et al.* 2009) and, particularly, the transition among pollination systems (Ushimaru and Nakata 2002; Temeles *et al.* 2002; Summers *et al.* 2015). In the other hand,

ontogenetic allometry is usually studied by allometric trajectories, which are built as the bi-variated plots between traits or between traits and overall size through ontogeny (Klingenberg 1998). In plants, allometric trajectories can be conserved between ancestors and descendants (ontogenetic scaling) imposing evolutionary tendencies (Olson et al, 2009; Fen et al, 2009), or can evolve in coordination with pollinator-selective pressure (Hazle and Canne-Hilliker, 2005; Summers et al, 2015, Streling et al, 2017).

Ontogenetic allometry is closely related to heterochrony, which is defined as a phyletic change in the timing of development (Gould 1977, 2002, Alberch et al, 1979). Heterochrony relates only to developmental process such as changes in onset of organs, in the cessation of growth, and/or in their growth rates, where the last directly relates with ontogenetic allometry (Gould 1966, 1977; Alberch *et al.* 1979). Due to this, is common that heterochronic process related to changes in the growth rate are assessed through allometric studies (e.g. Minter and Lord 1983; Altesor *et al.* 1994). However, to evaluate heterochronic changes in general, growth curves need to focus on the variation of shape, instead on the variation of size of a trait, in relation to another trait or overall size of the organism (Gould 1966).

Heterochrony in plants has been usually studied in flowers, as they represent closed developmental systems similar to animals. However, as in flowers there is no clear differentiation among juvenile and adult phases, heterochrony have been defined differently than in animals. In flowers, Paedomorphosis and peramorphosis are defined as an extension and a contraction of trait development, respectively, and can occur by changes in the onset of the traits, in the rate of growth and/or in cessation of growth, but no longer related to the reach of maturity (Bateman and DiMichele 1994; Box and Glover 2010). Paedomorphosis is broadly extended in flowers and usually underlie transitions in the pollination system from cross to

self-pollinated species (Lord 1982; Minter and Lord 1983; Gallardo *et al.* 1993; Stewart and Canne-Hilliker 1998; Pedersen and Ehlers 2000; Porras and Muñoz 2000; Li and Johnston 2001, 2010; Georgiady and Lord 2002; Box and Glover 2010). But also changes among in the pollination system among cross pollinated species: between specialized systems (Guerrant Jr 1982), from specialized to generalized system (Laurent *et al.* 1998; Box *et al.* 2008) and from generalized to specialized cross-pollinated species (Armbruster *et al.* 2013).

How ontogenetic allometry and heterochrony are measure varies: while ontogenetic allometry is usually measured as a tendency during ontogeny (Pélabon *et al.* 2013), heterochrony considered change at discrete stages of ontogeny (Alberch *et al.* 1979). In contrast, only few studies on heterochrony consider comparative quantitative measurements among species (Bull-Hereñu *et al.* 2016; Naghiloo and Claßen-Bockhoff 2017). In plants, growth rates may vary during ontogeny (Mikhalevskaya, 2008) producing non-linear allometry during development (Kampny et al., 1993; McLellan, 1993), suggesting that scaling relationships among floral traits can vary along ontogeny. Similarly, different heterochronic processes can occur at discrete stages of ontogeny (Li and Johnston 2000) and the effect of this changes in floral morphology can be enhanced, reduced, or eliminated by later changes during development (Naghiloo and Claßen-Bockhoff 2017). Therefore, Quantitative analysis of allometry and heterochrony trough a broad range of ontogenetic stages are of key importance to determine the role of growth dynamics in floral morphological evolution.

Model system: *Schizanthus* genus

The genus *Schizanthus* Ruiz and Pavón (Solanaceae) comprises 13 species of annual and biannual herbs endemic to the southern South American Andean region (Grau and Gronbach

1984; Rodríguez *et al.* 2018). The genus diverged early from the rest of the Solanaceae family and represents a monogeneric tribe (Olmstead and Palmer 1992; Wikström *et al.* 2001; Knapp 2002; Olmstead *et al.* 2008). *Schizathus* flowers are morphologically unusual in the family with oblique zygomorphy and resupinated open flowers (Walters 1969; Grau and Gronbach 1984; Cocucci 1989a). The flower is sympetalous and pentamerous with highly zygomorphic corolla and androecium. The corolla presents a corolla tube and is bi-labiate: three petals, the banner, and the two lateral sections, compose the upper lip and two petals fused in their inner part (the keel) and their outer outgrowing (the wings) compose the lower lip. The androecium is composed of five stamens fused to the corolla tube, where only the two transverse stamens will develop into fertile bithecate anthers; the rest will remain as staminodes.

Evolutionary transitions from bee- to hummingbird- and moth- pollination have occurred among the few species of the genus in association with marked changes of form and function of the lower lip (Pérez *et al.* 2006). Bee pollination is considered the ancestral morph in the genus and is characterized by extended lower lip (Pérez *et al.* 2006), where the keel retain the exerted stamens forming an explosive pollen discharge mechanism (Cocucci 1989b) and the wings act as a lading platform for insects. Transitions to hummingbird and moth pollination are accompanied by a complete lower lip reduction, in which the pollen-discharge mechanism and the landing platform is completely lost (Pérez *et al.* 2006). There is only one case of incomplete lower lip reduction, in which only the wings are reduced and the keel function as both the pollen discharge mechanism and as a landing platform (Pérez *et al.* 2006). Finally, the evolution of autonomous selfing accompanies increased specialization in pollination systems in some species (Pérez *et al.* 2009).

The huge morphological diversity closely associates with shifts in the pollination described above can be summarized by comparing only five species in the genus. These characteristics make the genus particularly interesting and amenable to study the role of ontogenetic changes related to growth dynamics in pollinator-driven diversification.

HYPOTHESIS

In this thesis, a quantitative analysis of growth dynamics is used to answer the following questions:

1. *Does ontogenetic integration and allometry vary through the development and among species?*

Hypothesis: It has been described in the literature that growth dynamics of each organ can vary independently through ontogeny. Therefore, both integration and allometry should also vary along ontogeny and among species.

2. *Are changes in ontogenetic integration congruent with changes of ontogenetic allometry in all species?*

Hypothesis: Strong variation of allometric slopes among floral traits are expected to reduce floral integration. Therefore, ontogenetic allometry and integration are expected to be strongly correlated through ontogeny in all species.

3. *How does changes in ontogenetic integration and allometry among species relate to pollinator-driven morphological diversification in the genus?*

Hypothesis: intra-floral functional units tend to be maintained by natural selection and in consequence, being highly integrated. Thus, reduced traits that lost their function during pollination should present lower allometric slopes and being less integrated with the rest of the corolla.

4. *How heterochrony does contributes to the diversification of Schizanthus species?*

Hypothesis: the relatively recent flower diversification of *Schizanthus* is expected to be produced by changes in the timing of development. Particularly, as the most common cases described in the literature, it is expected that morphological reductions represent paedomorphic morphologies produced by a neotenic process.

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Ontogenetic integration in two species of *Schizanthus* (Solanaceae): a comparison with static integration patterns

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Abstract

Floral integration refers to the patterns of co-variation among floral traits and can result from genetic correlations, developmental constraints and the function of traits in pollination and reproduction. Most studies have examined variation of mature flowers within populations or species (static integration) with contrasting pollination systems in order to test the role of pollinator-mediated selection in floral integration. But little is known about the ontogenetic pattern of floral integration and how it is related to floral morphology and floral integration patterns. Here we examined floral variation across ontogeny (ontogenetic integration) in two related *Schizanthus* species with contrasting floral morphology, pollination syndromes and static integration patterns. We also estimated ontogenetic integration in early and late phases of ontogeny. We found that ontogenetic integration varies among species and during ontogeny in accordance with the function of traits in pollination and static integration patterns of mature flowers. Our results suggest that both ontogenetic and static integration patterns are showing functional relationship related to the pollination process.

1. **Introduction**

The tendency to co-variation in morphological traits is known as morphological integration (Olson and Miller, 1958); it can result from different processes including pleiotropy, coordinated gene expression, functional relationships among traits and developmental factors (Cheverud, 1982a; Murren, 2002; Pigliucci, 2003; Pigliucci and Preston, 2004; Armbruster et al., 2014; Klingenberg, 2014). Trait correlations among individuals within a population or species are used to characterize integration patterns (Herrera, 2001;Herrera et al., 2002; Armbruster et al., 2009; Ellis et al., 2014; Gómez et al., 2014), but variation across ontogenetic stages of a single species has also been considered. In order to differentiate between these two levels of variation, Klingenberg (2014) proposed the terms "static integration" and "ontogenetic integration", respectively, following the well-established nomenclature used in literature on allometry. The term allometry originally referred to the scaling relationship between the size of body parts of growing organisms (ontogenetic allometry), but the term was then extended to include variation among conspecific individuals at a particular ontogenetic stage (static allometry) (Cock, 1966; Gould, 1966). Little is known about how these two levels of variation are related and the few comparisons made have given contrasting results (Cheverud, 1982b; Klingenberg and Zimmerman, 1992; Klingenberg et al., 2001, 2012; Laffont et al., 2009; Gonzalez et al., 2011; Pelabón et al., 2013).

Morphological integration has been considered a potential evolutionary route to modularity (Armbruster et al., 2014), playing an important role in constraining and canalizing morphological evolution (Lewontin, 1978; Gould, 1977, 2002). Berg (1960) was the first to suggest that flowers and vegetative traits can evolve in different modules, comprising different "correlation Pleiades". She argued that a decoupling between floral and vegetative traits

should occur in plants with specialized pollination systems as result of pollinator-mediated selection. The same mechanism can operate at the organ level: floral traits having different functions in the pollination process, such as traits that enhance pollinator attraction (like petals) or those that favor a precise fit with them (like style and stamen length), can comprise separate functional modules (Diggle, 2014; Armbruster et al., 2009, 2014). However, rather than reflecting only the result of pollinator-mediated selection, floral integration can result from developmental and genetic factors. Flowers have a metameric organization; each whorl comprises a different type of metamer that can be viewed as an independent developmental unit. Developmental factors are usually considered in terms of pleiotropic effects or spatial restrictions in the bud (Armbruster et al., 1999, 2004; Herrera, 2001; Herrera et al., 2002) that constrain the response to pollinator-mediated selection. Indeed, genes can simultaneously affect multiple floral traits through direct interaction between developmental pathways or through parallel effects on separate pathways, and therefore adaptive changes in one trait can cause negative changes in other, constraining morphological evolution and the response to natural selection (Wagner & Zhang, 2011). However, it has been shown that pleiotropy can be adaptive, i.e. it can have multiple positive effects on fitness, facilitating evolution (Ostrowski et al., 2005, Lovell et al., 2013). This evidence plus the fact that pleiotropy can itself evolve points out that pleiotropy may play an important role in achieving the coordinated evolution of interacting floral traits (Smith, 2016).

To explore the role of pollinator-mediated selection and development on floral integration, several studies have measured floral trait correlations in mature flowers (static integration *sensu* Klinberger, 2014). It has been tested whether traits belonging to the same floral whorl exhibit higher or lower integration than traits from different functionally related whorls

(Herrera et al., 2002; Conner and Sterling, 1995; Delph, 1996; Diggle, 2002) and also whether static integration patterns vary among species or populations in accordance with pollinators (Pérez et al., 2007; Rosas-Guerrero et al., 2010; González et al., 2014). However, to our knowledge no studies have yet examined integration during flower ontogeny and it is not known how ontogenetic integration is related to static integration of mature flowers, and to what extent ontogenetic integration reflects developmental factors and/or functional associations. In principle, ontogenetic integration might reflect a constraint imposed by developmental processes in the production of variant floral morphologies. Alternatively, ontogenetic integration may result from selection, but given that pollinators are able to exert direct selection only on mature flowers, static and ontogenetic integration should differ. However, if we consider that mature morphologies are the result of development and that changes in developmental process can have ecological significance (Gould, 1977; Alberch et al., 1979) with a positive impact on fitness, ontogenetic and static integration could be similar.

The genus *Schizanthus* Ruiz and Pavón (Solanaceae) comprises 12 species of annual and biannual herbs, with bilabiate, zygomorphic, papilionaceous flowers. Evolutionary transitions from bee- to hummingbird- and moth-pollination syndromes have occurred in the genus (Pérez et al., 2006), accompanied by strong changes in function, shape and static integration patterns of mature flowers (Pérez et al., 2007). *S. porrigens* Graham has a bee syndrome; during pollination the lateral parts of the lower lip (wings) are used as a landing platform and the keel (inferior part of the lower lip) releases the stamens in an explosive pollen discharge mechanism (Cocucci, 1989). These structures are reduced and not functional in *S. integrifolius* Phil, with a moth syndrome. Functional differences among species are reflected in the patterns of static integration, indicating that these were shaped by pollinator-mediated selection; in *S.*

porrigens the size of wings and keel in mature flowers is correlated with the size of the upper

lip, whereas in *S. integrifolius* the lower lip represents an independent module (Pérez et al.,

2007). In this study we examined the floral ontogeny of *S. integrifolius* and *S. porrigens*. We

estimated the patterns and magnitude of integration among floral parts during ontogeny

(ontogenetic integration *sensu* Klingenberg, 2014), from the stages in which floral primordia

are recently differentiated (and petals can be distinguished) until bud opening. We also

estimated floral integration at early and late phases of ontogeny. In this way we address the

following questions: Do *Schizanthus* species differ in their level and pattern of ontogenetic

integration? If so, are the differences related to the function of traits during pollination? Is

ontogenetic integration variable throughout ontogeny? If so, is the change during development

linked to static integration? And finally, does ontogenetic integration show developmental

constraints to morphological change?

2. Material and Methods

2.1 Studied species

We studied the flower development of two Chilean endemic species of *Schizanthus*, *S.*

integrifolius and *S. porrigens* (Fig. 1). The former grows in the winter rainfall sector of the

Atacama Desert and only emerges during rainy years associated with El Niño events. *S.*

porrigens grows a little farther south under Mediterranean conditions. The corolla of both

species consists of five petals partially fused in their lower extremes to form a narrow corolla

tube that is short in *S. porrigens* and long in *S. integrifolius*. The three uppermost petals

constitute the upper lip, which in turn is composed of the banner and two lateral sections. The

two lowermost petals are profoundly dissected, and their inner portions are fused to form a keel.

S. porrigens has a pink-purple corolla with nectar guides, non-reflexed lateral sections, the wings extended as landing platform and the keel retains the stamens in an explosive pollen discharge mechanism. *S. integrifolius* has a pure white corolla with a long tube, backward-reflexed and highly dissected lateral sections, reduced lower lip and absence of explosive pollen discharge. This species has a moth pollination syndrome, but receives visits of bees and butterflies, which use the stamens as landing platform instead the wings or keel (which have no evident function during pollination). Although this species receives relatively few visits of pollinators in comparison to *S. porrigens* (Pérez et al., 2007. Table 1), it is highly dependent on pollinators (Pérez et al., 2009). These *Schizanthus* species have contrasting pollination systems (Pérez et al., 2006) associated with functional differences of the lower lip.

2.2 Sampling

We collected inflorescences of five individuals per species in September, 2014 from the same two populations studied by Pérez et al. (2007) (Fig. 1). Inflorescences were preserved in a 70% aqueous ethanol medium. Nine to ten inflorescences of different sizes representing different stages of floral ontogeny were removed per individual, resulting in 51 buds for *S. integrifolius* and 48 buds for *S. porrigens*. Buds were dissected under a zoom stereomicroscope (Nikon SMZ 745T) and photographed. Sepals and/or stamens were removed if necessary for petal visualization. SEM photos were also taken in order to visualize better organ differentiation in early stages. We recorded the length of the following six traits from digital images using the software Image J: banner, lateral section, wing, keel, corolla tube and stamen bud (Fig. 2). All measurements were log transformed before statistical analyses.

2.3 Statistical Analysis

To characterize the levels and patterns of floral integration we estimated the Pearson correlation coefficient among each pair of traits across floral ontogeny, from the stage in which each petal primordial is clearly differentiated until the opening of the bud. We also divided developmental process into two arbitrary phases, 'Early Phase' and 'Late Phase'. To distinguish the phases we considered the time when the lobe petals of the lower and upper lip grow enough to make contact (Fig. 3). We used this event because it has been argued that both spatial proximity and contact of structures can trigger an integration among them (Armbruster et al., 2014), and because the contact among the other petal primordia occurs before wing primordia are well differentiated. Pairwise correlation coefficients were used to construct three 6×6 correlation matrices per species corresponding to Early Phase (CM_{EI} and CM_{EP}), Late Phase (CM_{LI} and CM_{LP}) and Complete Ontogeny. The pattern of significant correlations per species and phase was depicted as a correlation diagram. The magnitude of floral integration (INT) for each species and phase was estimated from the variance of eigenvalues in each correlation matrix. A 95% confidence interval of INT was estimated by bootstrapping the original log-transformed data 10,000 times (Cheverud et al., 1989; Herrera et al., 2002). To compare the integration patterns among species and phases we performed pairwise comparisons among the six correlation matrices using matrix permutation tests (Cheverud et al., 1989). To assess whether ontogenetic integration patterns are related to the function of traits during pollination between species (as was reported for static integration patterns by Pérez et al., 2007), we compared the observed correlation matrices with two hypothetical correlation matrices. Matrix F1 represents the integration of banner, lateral sections, wings, keel and stamens, which are the traits that work together during pollination in *S. porrigens* (upper lip attracts pollinators, wings as landing platform, and the keel retains stamens prior to

explosive pollen discharge), and was constructed by assigning 1 to correlations between them and 0 to the remaining correlations. Matrix F2 represents the decoupling of wings and keel, and the integration of traits that work together during pollination in *S. integrifolius* (upper lip attracts pollinators and stamens as landing platform), and was constructed assigning 1 to the correlations among them and 0 to the remaining correlations. To discriminate if observed data fit better to Matrix F1 or Matrix F2 we used the approach of Herrera et al. (2002): a difference matrix Z=F1-F2 was constructed, then a permutation test was performed between Z and each of the six observed matrices (a positive correlation indicates that observed data fit better to F1 than F2, and a negative correlation indicates that observed data fit better to F2 than F1). All analyses were conducted in R version 3.1.1 (R Development Core Team, 2014).

3. Results

Flowers of *Schizanthus* species showed high levels of ontogenetic integration. The INT values estimated across ontogeny (from primordia differentiation until bud opening) were 4.4 (95%CI=4.2-4.7, n=48) for *S. porrigens* and 4.5 (95%CI=4.3-4.6, n=51) for *S.integrifolius*, representing 73% and 75% of the maximum integration possible (Fig. 4). All correlations among floral traits were positive: r ranged between 0.90 and 0.98 in *S. integrifolius*, and between 0.86 and 0.98 in *S. porrigens*, (Table 2). The level of ontogenetic integration varied during ontogeny in both species, but following opposite patterns; while in *S. integrifolius* INT values decreased significantly from 4.3 to 1.9 (Early phase: 95%CI= 4.1-4.6, n= 34; Late phase: 95%CI=1.5-2.6, n= 17), INT values increased significantly from 2.8 to 4.6 in *S. porrigens* (Early phase: 95%CI=2.4-3.3, n=26; Late phase: 95%CI=4.4-4.7, n=22) (Fig. 4).

Patterns of ontogenetic integration of *Schizanthus* species were more similar in the early phase of ontogeny (CM$_{EI.}$ *vs*. CM$_{EP}$: r= 0.31, p=0.19) than in the late phase (CMLI *vs*. CM $_{LP}$: r=-0.46, p=0.08) (Fig.5). Difference among phases in r values were statically significant (95%CI =0.31-0.46, r=0.77, p=0.02). In both species ontogenetic patterns tend to reflect the functional relationships among traits during pollination. As expected, integration patterns of *S. porrigens* at both ontogenetic phases fitted better to matrix F1 (representing the integration of upper lip, lower lip and stamens) than to matrix F2 (representing the functional decoupling of wings and keel) (CM$_{EP}$ *vs*. Z: r= 0.61, p=0.03; CM$_{LP}$ *vs*. Z: r=0.67, p=0.01). In contrast, the pattern of ontogenetic integration of *S.integrifolius* fitted better to matrix F1 than to matrix F2 at the late phase of ontogeny (CM$_{LI}$ *vs*. Z: r=-0.56, p=0.04), but not at the early phase of ontogeny (CM$_{EI}$ *vs*. Z: r= 0.18, p=0.30), revealing an ontogenetic tendency for corolla disintegration and decoupling of non-functional traits (wings and keel), as is also shown by the floral integration diagrams (Fig. 5).

4. Discussion

The patterns of ontogenetic integration differed among *Schizanthus* species, and the differences become more accentuated at the end of ontogeny. These results have not been reported previously for flowers, but studies in mammals suggest that ontogenetic integration can vary across development (Zelditch, 1988; Zelditch et al., 1992; Zelditch et al., 2006; Goswami et al., 2006). Variation among *Schizanthus* species is congruent with the differences in pollination syndrome, functional relationships among floral traits and static integration patterns observed in mature flowers (Pérez et al., 2007. Fig. 5). *S. integrifolius* has a moth pollination syndrome, but receives visits of bees and butterflies that use the stamens as landing

platform instead of the wings or keel. These traits become decoupled from the remaining corolla lobes (the wing is decoupled to a greater degree than the keel) at the end of ontogeny. We propose that the decoupling of these traits (which arise from the same floral primordia) resulted from a relaxation of pollinator-mediated selection (see also Thompson et al., 1998; Anderson & Busch, 2006). Alternatively, decoupling can result from selection acting against a default co-variation pattern produced by developmental or genetic factors (pleiotropy). *S. porrigens* followed the opposite path of *S. integrifolius*; floral integration increased during ontogeny, but the patterns of integration remained constant, fitting the F1 model better at both early and late phase of ontogeny. This model represents the integration of all the traits that work together during the pollination process in *S. porrigens* (upper lip that attracts pollinators, wings as landing platform, and the keel retaining stamens prior to explosive pollen discharge). Static integration can be explained by pollinator-mediated selection acting on mature flowers (Kudoh et al., 2001; Ushimaro et al., 2003). Nonetheless, ontogenetic integration patterns must be explained in other terms as pollinators are not able to exert direct selection on floral buds. We suggest that developmental factors related to morphological integration may have consequences on the selection of adult morphology and therefore on the developmental programs that are maintained at the population level.

The decoupling of wings and keel in *S. integrifolius* is related to the reduction of the lower lip during development. The reduction of wings and keel probably result from independent developmental processes, and has occurred at least three times in the phylogeny of *Schizanthus* (Pérez et al. 2006). Indeed, although the wings and keel emerge from the same floral primordia they do not co-vary, and therefore they do not comprise a developmental module. Ontogenetic decoupling of lower lip parts probably allowed the independent

evolution of floral parts during the phylogeny of *Schizanthus*. This may be also the case of *S. hookerii*, in which the keel is integrated with the banner and the wings are almost completely decoupled from the rest of the corolla (Pérez, 2007). Also, we found that integration may occur among floral parts that are physically separated. The banner and lateral sections, for example, were always highly integrated even though they arise from different primordia. Lower and upper lips were highly integrated in *S. porrigens* although they are physically separated by two huge stamens (which may be somehow related to the zygomorphic bilabiate morphology present in the genus; Knapp, 2002) that are expected to act as a physical barrier against lip integration (Fig. 3). Nonetheless, these patterns may be affected by other developmental factors not considered in this study.

Finally, we lack enough evidence to understand which mechanisms –if any- are triggered when the lips come into contact (the milestone used to define early and late phases), but surely this is a more gradual process than we could distinguish under a dissection microscope. This event is accompanied by an increase in integration between lips in *S. porrigens* and by a disintegration in *S. integrifolius*. In the latter species more time is required for the lower lip to grow large enough to touch the upper lip, so the interaction occurs in a larger and older bud, which may explain the interspecific differences.

5. Conclusion

Our results show that ontogenetic floral integration varies during ontogeny and among species. Changes in integration across ontogeny are related to the function of traits during pollination, and are more accentuated in *S. integrifolius*, which has a highly divergent and derived morphology. Ontogenetic integration patterns in the late phase of ontogeny are similar

to static integration patterns described for mature flowers, and reflect that evolutionary tendencies shaped by pollinator-mediated selection and developmental tendencies are congruent. Future ontogenetic integration studies will focus on phylogenetic comparisons for a better understanding of evolutionary changes in the ontogenetic patterns that shape tendencies and constraints in floral evolution, as well as ontogenetic studies for a better understanding of the developmental process underlying the changes in integration patterns.

5. Acknowledgements

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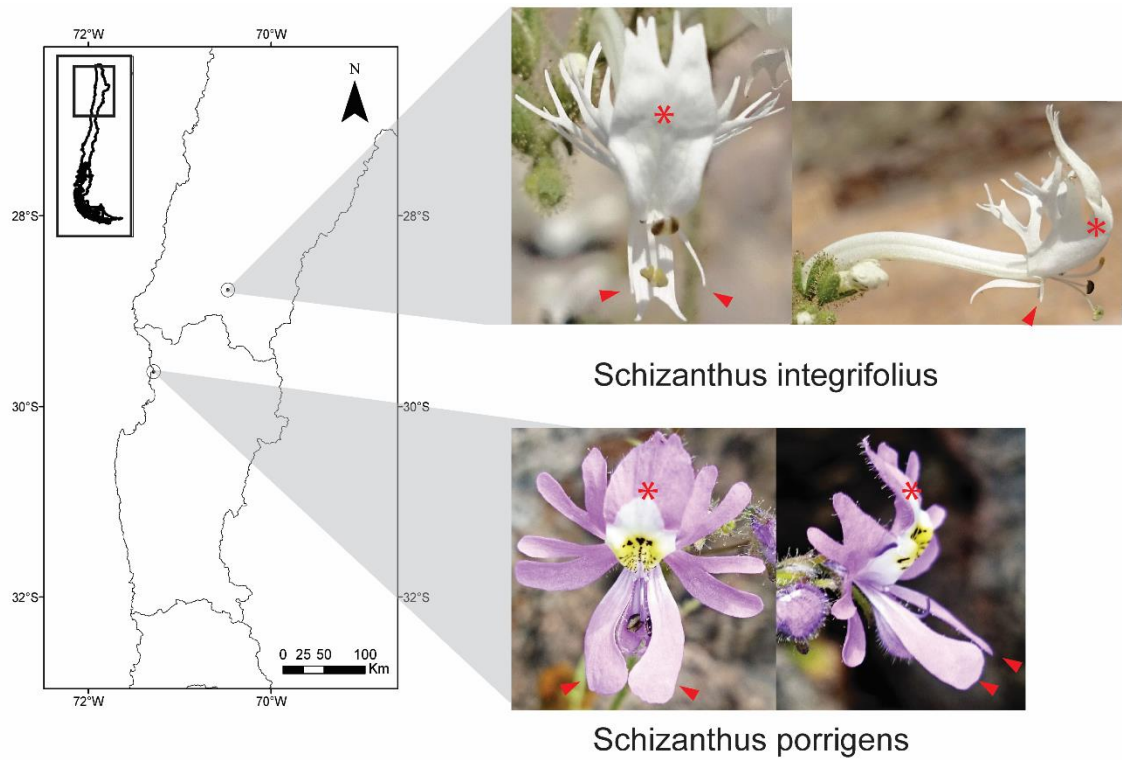
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FIGURES

Fig. 1. *Distribution and morphology of the studied species*. Map of the Atacama Desert in northern Chile showing the sampling sites of *Schizanthus* species. Front and lateral views of mature flowers of *S. porrigens* and *S. integrifolius* are shown. Asterisks show banners and arrowheads show wings. Between the two wings is the keel.
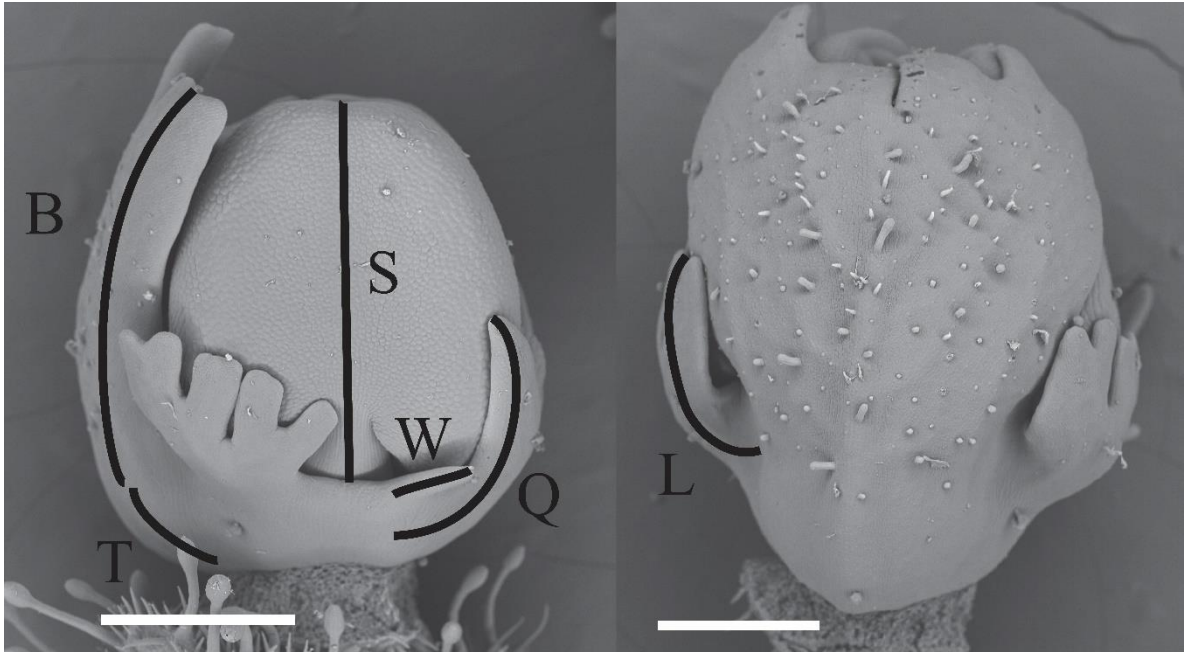
Fig. 2. *Measurements used in this study.* Floral buds of *Schizanthus* showing the measurements used in this study: B, banner; L, lateral section; T, corolla tube; W, wing; K, keel, S, anther. Scale bars: 0.5 mm.
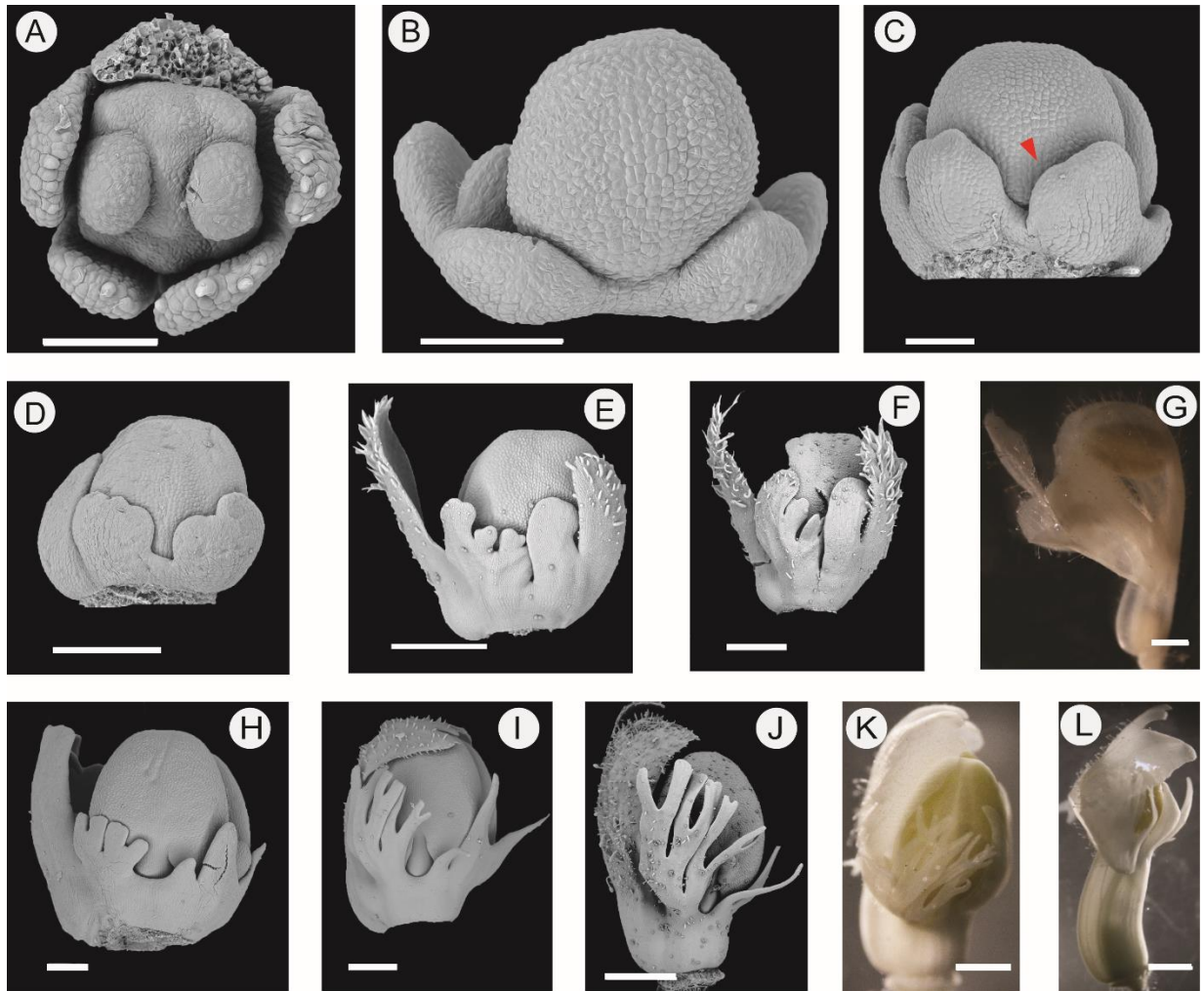
Fig. 3. *Floral ontogeny of Schizanthus species*. A-C early stages. A) View from above. All five petal primordia are differentiated, the two primodia of the lower lip are above. The precocious anthers are shown. B) Lateral view showing the upper lip primordium at the left, which is spatially separated by the anthers from the lower lip primordium. C) Lateral view showing the beginning of the wing differentiation (arrowhead) from the keel primordium. Lateral view of ontogenetic stages used in this study for *S. porrigens* D-G and *S. integrifolius* H-L. The upper lip is always at the left. Scale bars; A-C) 100 um, D) 200 um , E-F) 0.5 mm G) 1 mm, H)0.2 mm, I,J) 0.5mm, K) 1 mm L) 1.5 mm.
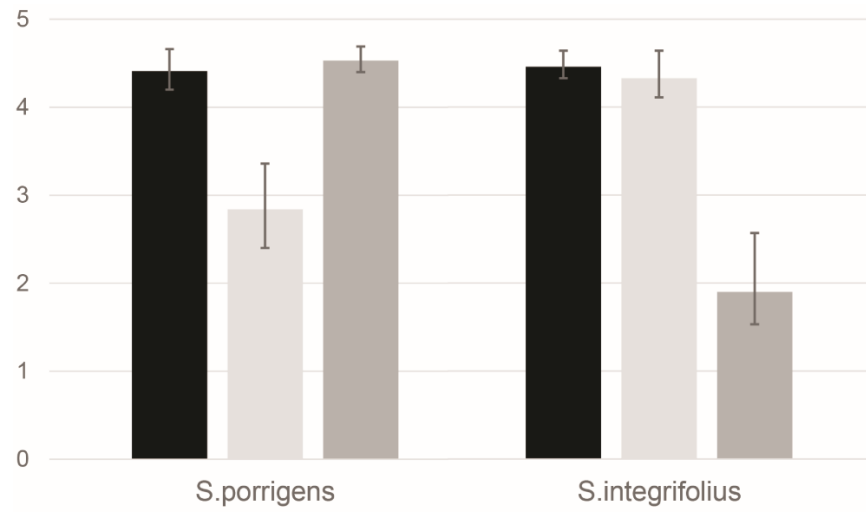
Fig. 4. *Ontogenetic integration in Schizanthus species*. Early phase (light grey), late phase

(dark grey) and complete ontogeny (black). 95% Confidence intervals are shown.
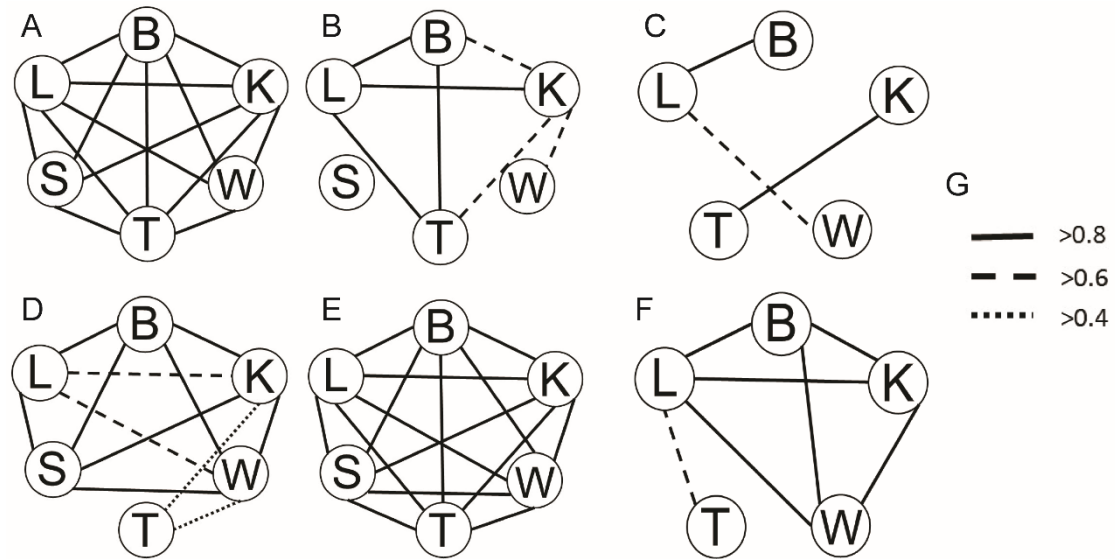
Fig. 5. *Floral integration patterns*. Diagrams of the observed ontogenetic integration pattern for the six characters in *S. integrifolius* (upper line) and *S. porrigens* (lower line) before (A, D) and after (B, E) the two lips become into contact. The patterns of static integration of mature flowers in which the stamens were not considered (C, F) are also shown (modified from Pérez et al. 2007). Different line types represent different levels of correlation as is graphically shown in (G). The absence of lines indicates no significant correlation.

Original article: *In review* at Annals of Botany

Integration and allometric changes during development underlying floral diversification

in Schizanthus (Solanaceae)

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Running title: Growth dynamics and floral diversification in *Schizanthus*

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Abstract

Background and Aims: Zygomorphic flowers origin and diversification rely on the ability of each organ to growth independently, generating different flower proportions. Changes in proportions have been broadly studied regarding pollinator-driven diversification where their developmental basis have been paid less attention. In this work, flower development of six Schizanthus species with contrasting pollination syndromes is studied to explore: 1) how growth dynamics vary along ontogeny and their relation with pollinator-driven diversification, 2) how the coordination of growth among floral organs vary along ontogeny and their relation with pollinator-driven diversification, and 3) how growth dynamics and the coordination among floral organs coordinates along ontogeny.

Methods: Growth dynamics (allometric slopes) and the coordination among organs (floral integration) were measured considering buds from petal differentiation until shortly before anthesis. Changes along ontogeny were studied using overlapping ontogenetic ranges for integration and using an early/late division of ontogeny for allometry. Both were compared with patterns of pollination-driven diversification.

Key Results: Ontogenetic integration and allometry varied through ontogeny and among species. Differences among species were generally congruent with pollinator-driven diversification. Particularly, reduced structures that lost functionality in the pollination process tended to be decoupled and have lower allometric slopes during development, while functional traits tended to be integrated. However, this occurred only for two of the three organs studied. Changes of integration and allometry along ontogeny tend to occur coordinately: allometric slopes among traits tended to vary to a greater degree at low integration levels. However, this

tendency was not found in all species, suggesting a more complex relationship between these growth dynamics.

Conclusions: comparative quantitative analysis of growth dynamics along a broad range of ontogenetic stages is crucial to understand the role of ontogenetic allometry and integration shaping patterns of pollinator driven diversification.

Keywords: Ontogenetic integration, ontogenetic allometry, morphological evolution, flower evolution, flower development, Schizanthus, Solanaceae.

Introduction

Changes in the proportion of floral organs are associated with rapid evolutionary changes in higher Angiosperms, which are maintained mainly by pollinator mediated selection (Darwin 1859; Stebbins 1970; Armbruster *et al.* 2009; van der Niet and Johnson 2012; Van Der Niet *et al.* 2014). Particularly, changes in petal proportions underlie the transition from actinomorphic to zygomorphic flowers, which have occurred many times independently in angiosperm evolution (Reyes *et al.* 2016). Zygomorphy is considered a key innovation as it favored more specialized flower-insect interactions (Leppik 1974; Neal *et al.* 1998; Westerkamp and Claßen-Bockhoff 2007; Ushimaru *et al.* 2009) leading to higher fitness and higher speciation rates than actinomorphic flowers (Sargent 2004; Fenster *et al.* 2009; Vamosi and Vamosi 2010). Zygomorphic flowers can easily change their proportions by allowing each floral organ to growth independently (Moyroud and Glover 2017). Moreover, cell growth parameters and their underlying genetic control can vary not only among petals but also along ontogeny (Amelia A. Green *et al.* 2010). However, it remains unclear 1) how growth dynamics vary along ontogeny at the organ level and their relation with pollinator-driven diversification, 2) how the coordination of growth among floral organs vary along ontogeny and their relation with pollinator-driven diversification, and 3) how growth dynamics at the organ level related to the coordination among floral organs along ontogeny.

Growth dynamics along ontogeny are usually studied under the conceptual framework of ontogenetic allometry, which describes how a trait size changes with the size of the organism during ontogeny (Pélabon *et al.,* 2013). In this approach, growth rates are defined as allometric slopes obtained from log–log scatterplots of trait size against organism size (Mosimann 1970). Conservation of floral allometry between ancestors and descendants

(ontogenetic scaling) is expected to result in proportional changes in shape and size relationships, which in turn might constrain the response of plants to pollinator-mediated selection (Feng *et al.,* 2009; Olson *et al.,* 2009). However, comparative studies show that allometric curves can vary among closely related species (Hazle and Judith, 2005; Summers *et al*., 2015; Strelin *et al*., 2017). For example, the variation of traits suited to hummingbird pollination, such as the length and width of the corolla tube, can result from variation in the growth rate of the corolla tube with respect to other traits, reflected in distinct allometric slopes or intercepts. Despite these studies strongly links ontogenetic allometry with patterns of pollinator-driven diversification, they did not explore how allometry varies along ontogeny. In addition, the ontogenetic variation of allometry has been described in flowers (Kampny *et al.* 1993), but it has not been linked to patterns of pollinator-driven diversification.

Growth coordination among floral traits is usually studied under the conceptual frameworks of morphological integration (Hallgrímsson *et al*., 2009; Haber, 2011; Armbruster *et al*., 2014). Floral integration is the tendency of traits to co-vary and is usually measured as the pattern and strength of correlations among traits in a population (Hallgrímsson *et al*., 2009; Haber, 2011; Armbruster *et al*., 2014). Floral integration is thought to result from the action of natural selection on traits functionally linked during pollination, which displays higher correlations among themselves than with others (Diggle, 2002; Ordano *et al.,* 2008; Armbruster *et al.,* 2009). Nevertheless, floral integration can also result from developmental and genetic factors, which in turn might evolve to match functional relationships or may affect adaptive evolution as a constraint or bias (Armbruster, 1999; Herrera, 2001; Herrera *et al.,* 2002; Armbruster *et al.,* 2004). Regardless of the mechanisms underlying phenotypic integration patterns, separation of phenotypes into modules would enable rapid and specific

adaptations, as traits highly integrated into modules are independent of other traits.

Developmental factors shaping floral integration are usually studied at a particular

developmental stage (developmental integration, Armbruster *et al.*, 2014). In contraposition,

integration measured across ontogenetic stages (ontogenetic integration sensu Klingenberg,

2014) remains poorly studied. Accordingly, a key issue to understand the developmental basis

of floral diversification is to examine how floral traits become divided into functional units in

ontogeny.

Changes in the growth rate of a floral organ are expected to affect its coordination with

the growth of the rest of the flower. Accordantly, ontogenetic integration is considered to be

conceptually related to ontogenetic allometry (*Reviewed* in Armbruster *et al.* 2014;

Klingenberg 2014). Theoretically, proportional size variations are produced by the same

developmental and functional process (Huxley *et al.* 1932; Klingenberg 1998) regardless if it

affects two or more traits (integration) or one trait in relation to the overall size (allometry). In

addition, in systems where growth parameters can vary independently among each floral

organ, it is expected that any change in the allometric slopes of one organ will brake its

coordination with the remaining organs, therefore, decreasing overall flower integration.

Despite its theoretical relationship, how integration and allometry variations are related along

ontogeny in natural systems remains unclear. Studies considering both ontogenetic integration

and allometry are expected not only to describe better growth dynamics during ontogeny but

also may shed light on the relationship between them.

The genus *Schizanthus* (Solanaceae) has strongly zygomorphic bi-labiated flowers with

huge morphological diversity, with bee-, hummingbird- and moth-pollination syndromes in

only twelve species (Pérez *et al.*, 2006). In bee-pollinated species (e. g. *S. porrigens* and *S.*

alpestris, the ancestral form. Fig. 1A,B), the lateral parts of the lower lip (wings) are extended as landing platforms for insects, whereas the interior part of the lower lip (keel) retains the stamens prior to explosive pollen discharge (Cocucci, 1989; Pérez *et al.,* 2006). Reduction of wings and keel and the loss of the explosive pollen discharge mechanism co-occurred with the transition from bee- to hummingbird pollination syndrome only in *S. coccineus* (Fig. 1D) and with the transition to moth-pollination syndrome in a clade composed of three species (including *S. lacteus* and *S. integrifolius*, Fig. 1E,F). Extremely long corolla tubes are found in the transition to moth-pollination in *S. integrifolius* and *S. hookeri*, the only species with a mixed pollination syndrome characterized by reduced wings, extended functional keel, representing an intermediate stage in the bee-to-bird transition (Fig. 1C). The evolution of autonomous selfing accompanies increased specialization in pollination systems in some species (including *S. lacteus* and *S. coccineus*).

Differences in pollination syndromes and trait functions among *Schizanthus* species are reflected in the integration patterns of mature flowers; in pollinator-dependent syndromes, integration between non-functional reduced wings and keel and the rest of the corolla was disrupted (Pérez *et al.,* 2007). In addition, a preliminary study showed that ontogenetic integration varied reflecting functional relationships among traits in two contrasting pollination syndromes and in accordance with static integration patterns (Chinga and Pérez, 2016). However, it is still unclear (1) to what extent ontogenetic integration and allometry vary along with development and among *Schizanthus* species, (2) how ontogenetic changes among species relate to pollinator driven floral divergence, and (3) whether changes in integration and allometry along development occurs coordinately during development in all species. To explore these questions, we estimated integration and allometry of floral traits

along ontogeny and among six *Schizanthus* species with different pollination systems. We predict that (1) allometry and integration should vary along ontogeny and among species; (2) in species with wing and/or keel reduction, these petals should present lower allometric slopes and being more decoupled from the rest of the corolla at discrete ontogenetic stages; (3) in long-tubed species, the tube should be less integrated and present steeper allometric slopes at discrete ontogenetic stages; (4) ontogenetic integration and allometry should vary along ontogeny co-ordinately, that is, reductions in floral integration (i.e., phases of low coordination among traits) should be associated with greater variation in allometric slopes.

Methodology

Species

The genus *Schizanthus* Ruiz and Pavón (Grau and Gronbach, 1984) comprises 12 species of annual plants endemic to the southern South American Andean region (between 22-40°). All species are found in Chile, with only two species reaching the Argentinean side of the Andes. The present study focuses on flower development of six species of *Schizanthus* endemic to the northern and central regions of Chile. The northern species, *S. integrifolius* and *S. lacteus*, grow in the winter rainfall sector of the Atacama Desert and only emerge during rainy years associated with El Niño events. In contrast, the Mediterranean climate species *S. porrigens* and *S. alpestris* and the high Andean species *S. grahamii* and *S. hookerii* emerge during the austral spring-summer. *Schizanthus* has strongly zygomorphic bilabiate flowers, which are unusual in the family Solanaceae (Walters, 1969; Grau and Gronbach, 1984; Knapp, 2010). The pentamerous corolla has two lips fused into a tube. Three petals compose the upper lip (the banner and the lateral sections), while the lower lip is composed of two asymmetrical, but

mirror symmetrical petals forming together the keel and the wings (Fig. 1).

Sampling

Five to ten floral inflorescences from five to ten individuals per species were collected in the field during 2015, a year characterized by a strong El Niño event (Bozkurt *et al.,* 2016). The following populations were considered: *S. porrigens*; Puente Juan el Soldado, La Serena. *S. grahamii*; La Parva, Santiago. *S. hookerii*; Valle Nevado, Santiago. *S. alpestris*; Vicuña, La Serena. *S. integrifolius*; Alto del Carmen, Huasco. *S. lacteus*; Paposo, Antofagasta. Samples were fixed in the field in 70% aqueous ethanol. Nine to ten floral buds were removed per inflorescence, from 0.1 mm to shortly before anthesis. Due to different availability of flower material, total bud number used differed among species (*S. porrigens* n=49, *S. hookerii* n=85, *S. grahamii* n=86, *S. alpestris* n=86, *S. integrifolius* n=53, *S. lacteus* n=75). The buds were dissected under a zoom stereomicroscope (Nikon SMZ 745T, China) and photographed. Sepals and/or stamens were removed for petal visualization, if necessary. SEM photos were taken using a Scanning Electron Microscope (Hitachi TM3000, Japan) in order to visualize organ differentiation better in early stages. Since the wings appear as a lateral prolongation of the keel primordia during development, only buds with all traits clearly differentiated were measured. In the final analysis, floral buds were considered from the moment in which each petal primordium is clearly differentiated and the wings and tube are already initiated (Fig. 2A) until shortly before anthesis (Fig. 2C). We recorded the length of the following six traits from digital images using the software Image J: banner (B), lateral section (L), wing (W), keel (K), corolla tube (T) and bud length (S) (Fig. 3). All measurements were log-transformed before statistical analyses using the software R.

Ontogenetic Integration Analysis

To estimate the patterns of floral integration, 5×5 correlation matrices were built using the Pearson correlation coefficient between each pair of traits (without considering bud size) over floral ontogeny. Significant correlations were depicted as correlation diagrams. The magnitude or level of floral integration (INT) was estimated from the variance of the eigenvalues of each correlation matrix and the 95% confidence interval was estimated by bootstrapping the original log-transformed data 10,000 times (Cheverud *et al.,* 1989; Herrera *et al.,* 2002).

To explore how ontogenetic integration changes during development, a "rolling/moving" approach was used. This approach uses a moving average methodology and confers the advantage of calculating integration level during ontogeny without defining any previous ontogenetic stages. We performed this approach by ordering the data by increasing bud size and defining ontogenetic overlapping windows of *k* points. The first window will be the first *k* points, the second uses the second data point and the following *k* values and so on, until n-*k* windows are obtained (n being the sample size of point). The INT and the 95% confidence interval were estimated for each window. Several *k* values between 10 and 20 were tested in this methodology. For *Schizanthus* species, a value of *k*=15 was optimum to depict ontogenetic differences among species. As changes in the level of integration do not necessarily represent changes in the patterns of integration (a coordinated decrease/increase of integration among all traits may occur), the pattern of integration was calculated at each change of the INT. Only ontogenetic stages characterized by a decoupling pattern were depicted, thus the range of windows not depicted had homogeneous integration among traits. Finally, as adult flower size varies among species, integration values were plotted against relative bud size (average bud size at each window/final bud size).

Phylogenetic and morphological relatedness effects on integration patterns

        To evaluate whether patterns of ontogenetic integration vary independently of the phylogenetic relationship among species, a matrix of phylogenetic distances among the species (PDM) was compared with a matrix of integration curves distances among the species (CDM). The PDM was constructed using the sum of the branch lengths separating each pair of species in the phylogeny, which were extracted from Pérez (2004). The CDM was constructed using the distance between integration curves for each pair of species using a Dynamic Time Warping (DTW) algorithm that calculates the inherent difference between two given time series. The minimum global dissimilarity or "DTW" distance between curves was calculated as the sum of the diagonal elements of the minimum distance matrix of the best match between the curves. The best match between points of each curve was calculated allowing the ends of the curves to be open (i.e., ends were not forced to match). The dynamic time warping analysis was performed using the package dtw in R. In addition, to test if the patterns of ontogenetic integration correlated with patterns of morphological similarity among species, CDM was compared with a morphological distance matrix (MDM). The MDM was built using the Euclidian distance among PCA scores obtained from a morphological matrix of the species studied. The morphological matrix contained four continuous characters (Corolla size, Relative size of the lower lip, Relative length of the corolla tube, Corolla complexity or area/perimeter ratio) and five discrete characters described as binary characters (Corolla colour, Lateral sections orientation, Presence of the explosive discharge mechanism, Stamens length, Anther dehiscence). All morphological characters were obtained from Pérez (2004). Comparisons among matrices were performed using a Mantel test using the package vegan in R.

Ontogenetic allometry analysis

To describe how the size of traits varies with changes in bud size, allometric slopes were calculated from linear regressions of the traits on bud size (Mosimann, 1970). To explore general tendencies during development, we calculated allometric slopes for each trait using all ontogenetic data. Additionally, ontogeny was divided into early and late stages, under the assumption of different cell behaviour occurring at each stage. The early stage (Fig. 2A) was characterized by "blade-shaped" primordia without secondary differentiation of the lower lip and the late stage (Fig. 2B) by secondary differentiation in the shape of lower lip structures such as the appearance of angles, curves, outgrowths or segmentation of the primordia, etc. In all species, the transition between these two stages was around 10% of the final bud size. In order to analyse whether allometric changes during ontogeny are related to inter-specific differences, ontogenetic allometric slopes for complete, early and late ontogeny were plotted against the relative size of the trait in the adult form (tube + banner adult length as a proxy of adult flower lateral size). All statistical tests were performed using the software R.

Phylogenetic relatedness effects on allometry patterns

To test the effect of phylogenetic relatedness on patterns of ontogenetic allometry, the fit of a linear model that takes into account phylogenetic relatedness was compared with the original model between the allometric slope of the trait at early and late ontogeny and the relative size of the trait in the open flower. In all cases, lambda was determined for each trait. The analysis was performed using the package caper in R.

Ontogenetic integration and allometry relationship

To test if allometric slopes differed more at low integration levels, the coefficient of variance among allometric slopes of each trait (CV) and integration values (INT) were calculated using the rolling/moving approach (k=15) for each ontogenetic window and fitted under a linear model. All tests were performed in R.

Results

Changes in integration during ontogeny and pollinator driven diversification

When the patterns and levels of floral integration were estimated for all ontogeny (from petal primordia differentiation until shortly before anthesis), no significant differences were detected between species. INT values ranged from 4.29 to 4.76, representing 85%-95% of the maximum integration possible; and all correlations among floral traits were positive and above 0.88 (Table 1). On the contrary, the rolling/moving approach showed that the level of floral integration varied during ontogeny (Fig. 4B). Except for *S. integrifolius* in which integration declined with bud size, all species started with low integration levels, which tended to increase with bud size. Pronounced declines of INT values in the middle stages of ontogeny were observed in *S. alpestris* and *S. grahamii*.

Patterns of ontogenetic integration were only partially congruent with patters of pollinator driven diversification (Fig. 4C). The expectation of reduced traits that lost its function in the pollination process being decoupled from the rest of the corolla along ontogeny was true mostly for wings and only slightly for the keel. All species presented some degree of wing and tube decoupling from the rest of the corolla: correlations between corolla tube and remaining traits were the most variable across ontogeny ($\sigma_r = 0.10$), followed by those

involving wings (σ_r =0.064). Therefore, all species with wing reduction presented either a more intense decoupling in early ontogeny (*S. hookeri* and *S. lacteus*) or extra events of decoupling during late ontogeny (*S. coccineus* and *S. integrifolius*) compared to bee pollination species. In contrast, only two of three species with keel reduction (*S. integrifolius* and *S. lacteus*) presented a slight decoupling of the keel from the rest of the corolla along ontogeny. Interestingly, these two species present the most reduced keels in the genus, which were reduced in shape and form. *S. coccineus*, with a keel reduced only in size, presented no clear decoupling of the keel along ontogeny. Finally, there was no correlation between long tubed species and more decoupled wing along ontogeny. The correlation between the distance matrix among curves (CDM) and the phylogenetic distance matrix (PDM) did not differ from zero (r=0.028, p=0.48). These results showed that phylogenetic relatedness does not determine the patterns of ontogenetic integration. Similarly, the correlation between CDM and the morphological distance matric (MDM) was higher, but still not significantly different from zero (r=0.49, p=0.071), suggesting that patterns of ontogenetic integration may be at least slightly determined by morphological similarity among species.

Changes in ontogenetic allometry and pollinator-driven diversification.

Allometric relationships between corolla traits and bud size, estimated across all ontogeny [**Supplementary table 1**], fitted better to a quadratic than a linear model for most species and traits (data not shown), suggesting that allometric slopes vary during development. Accordingly, to explore whether variations in floral shape among species result from changes in allometric slopes along ontogeny, we analyzed early and late stages of ontogeny separately. Allometric slopes varied along ontogeny but not for all traits considered: while allometric

slopes tend to be higher in early than in late ontogeny for the wings and the keel, they did not change between early and late ontogeny for the tube.

Morphological differences produced by pollinator-driven diversification were congruent with ontogenetic allometry patterns only when changes along ontogeny were considered [**Supplementary table 1**]. Allometric slopes measured along ontogeny had no significant correlation with the relative size of the trait in the open flower for most petals (r^2: 0.10-0.48, except for the banner with r^2: 0.67 and p=0.02), and, similarly to patterns of ontogenetic integration, the wings and the tube display higher allometry slopes than the other traits along all ontogeny. On the contrary, allometric slopes for keel and wings were positive for most species during early ontogeny and correlated positively with the relative size of each floral part (wings: r=0.91, p=0.003; keel: r=0.94, p= 0.001, Fig. 5A,B). Particularly, all species with wing reduction had significantly lower allometric slopes during early ontogeny (Fig. 5A), while only two of the three species with keel reduction had significantly lower allometric slopes compared to bee-pollinated species (Fig. 5B). In contrast, during late ontogeny allometric slopes did not correlated with the relative size of floral parts (wings: r=0.20, p=0.34, keel: r=0.23, p=0.32). Finally, species with longer corolla tubes were not significantly correlated with higher allometric slopes either at early or late ontogeny (Fig. 5C, early: r=0.62, p=0.11; late: r=0.68, p=0.08). These patterns were not determined by phylogenetic relatedness as all traits had lambda=0 at both, early and late ontogeny and linear models considered phylogenetic relatedness showed the same pattern than when phylogenetic relatedness is not considered **[Supplementary table 2]**.

Relationship between ontogenetic integration and allometry

In all *Schizanthus* species except *S. lacteus*, the CV of allometric slopes among traits correlated negatively with INT values (range r^2=-0.71 to -0.98, Fig. 6), indicating that acceleration/slowing in the growth rate of some traits with respect to other traits is associated with a reduction in the level of integration during ontogeny. In *S. lacteus,* CVs correlated positively with INT values. Global correlation considering all species between INT and CV of the allometric slopes had r=-0.78 (p <0.001).

Discussion

Changes in proportions associated with pollination driven diversification in *Schizanthus* are produced by changes in both petal growth dynamics (ontogenetic allometry) and the coordination among petals growth along ontogeny (ontogenetic integration), which occurred at discrete ontogenetic stages and corresponded to interrelated ontogenetic processes.

Changes in integration and allometry during ontogeny and pollinator-driven diversification

Ontogenetic integration and allometry varied along ontogeny and among petals, showing changes at specific ontogenetic stages, emphasizing the importance of considering a broad range of developmental stages in this kind of study. This evidence is in agreement with tendencies found at the cellular and molecular level, where growth parameters tend to vary in time and space each petal ontogeny (Amelia A Green *et al.* 2010) and vary independently among petals in zygomorphic flowers (Moyroud and Glover 2017).

Contrasting the growth dynamics of the wings and the tube compared to the other corolla traits are key to produce the proportions needed in the bee-pollinated syndrome. Both traits appear once the other petal primordia are already differentiated. Only by higher allometric slopes allowed by a decoupling from the rest of the corolla along all ontogeny, the wings and the tube can reach similar size than the other petals. In this scenario, morphological reduction should be reached by a comparative decrease in allometric slopes and either steeper or more events of the coupling from the rest of the corolla. Compensation among ontogenetic processes such as the time of the onset and their posterior growth dynamics has been previously described in comparative quantitative ontogenetic studies (Naghiloo and Regine 2017) and are key to disentangle the relative role of each process shaping patterns of morphological evolution.

Ontogenetic allometry and integration also varied among species. Pollinator-driven patterns of lower lip reduction in *Schizanthus* strongly correlated with growth dynamics at early ontogeny, mostly in relation to ontogenetic allometry and only partially for ontogenetic integration. Differences during early ontogeny are expected to be maximized during the exponential growth phase having a higher impact in adult morphology (Alberch *et al.,* 1979), suggesting a crucial role shaping differences among species. Despite many studies have correlated ontogenetic allometry with pollinator driven diversification (Ushimaru and Nakata 2002; Temeles *et al.* 2002; Summers *et al.* 2015) and it is known that ontogenetic allometries can vary along ontogeny in flowers (Kampny *et al.* 1993), no correlation has been made before between changes in ontogenetic allometry and pollinator-driven diversification. Similarly, integration measured across ontogeny was expected to be considerably high, as development is usually considered as the coordinated growth of organs, and therefore to be

more related to growth process rather than with pollinator-driven diversification (CP Klingenberg 2014). Past studies have already shown that ontogenetic integration can vary along ontogeny in accordance with adult morphologies (Chinga and Pérez 2016). The present study considered more species and found that changes in ontogenetic integration related to pollinator-driven diversification, but with considerable variations among species. Particularly, only species with a reduction in shape and size presented clear patterns of decoupling of those traits along ontogeny, a pattern not found in species with only size reductions. Finally, differences among species not explained by patterns of ontogenetic allometry and integration (in this study the corolla tube), suggesting that these morphologies are produced by changes even earlier in ontogeny, such as an earlier onset of the tube in long-tubed species.

Relationship between ontogenetic integration and allometry

Although it is broadly accepted that ontogenetic integration and allometry are conceptually related (Armbruster *et al.*, 2014; Klingenberg, 2014), few studies have tested experimentally how they relate during development. In this study, low integration levels correlated with higher variation (CV) among allometric slopes of traits in all but one species. This evidence suggests that in species in which ontogenetic allometry and integration did not vary as expected, the relationship between these two parameters is more complexly regulated or even not regulated at all, allowing them to vary independently. Regardless of the mechanisms causing this variation among species, this result is in agreement with patterns of pollinator driven diversification being less explained by ontogenetic integration than ontogenetic allometry, despite its expected close relationship.

Concluding remarks

Corolla traits have independent growth dynamics that can vary along ontogeny and among species, explaining changes in corolla proportions associated with shifts in the pollination syndrome in *Schizanthus* zygomorphic flowers. Particularly, the growth dynamics of each trait (ontogenetic allometry) and the coordination among traits (ontogenetic integration) varied along ontogeny and among species. Identifying discrete changes of these growth parameters along ontogeny and their possible compensatory effects is key to disentangle their relative role shaping patterns of pollinator-driven diversification. Finally, the regulation of organ growth and the coordination of growth among organs is more complex than theoretically expected, as changes in ontogenetic allometry strongly correlated with changes in ontogenetic integration, but not for all species. More research is needed to understand the relationship between these two growth parameters in experimental systems.

Supplementary material

Supplementary table 1 show allometric slopes for complete, early and late ontogeny of floral traits, and the relative size of each trait in adult individuals.

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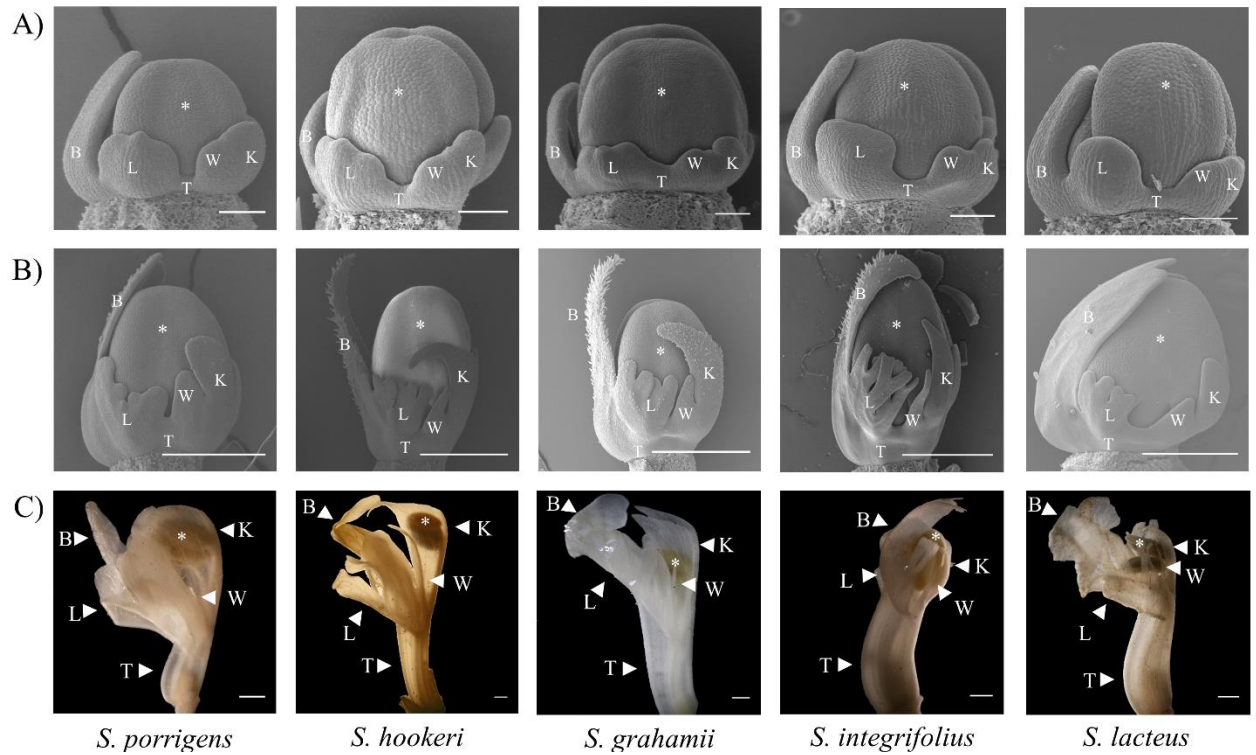
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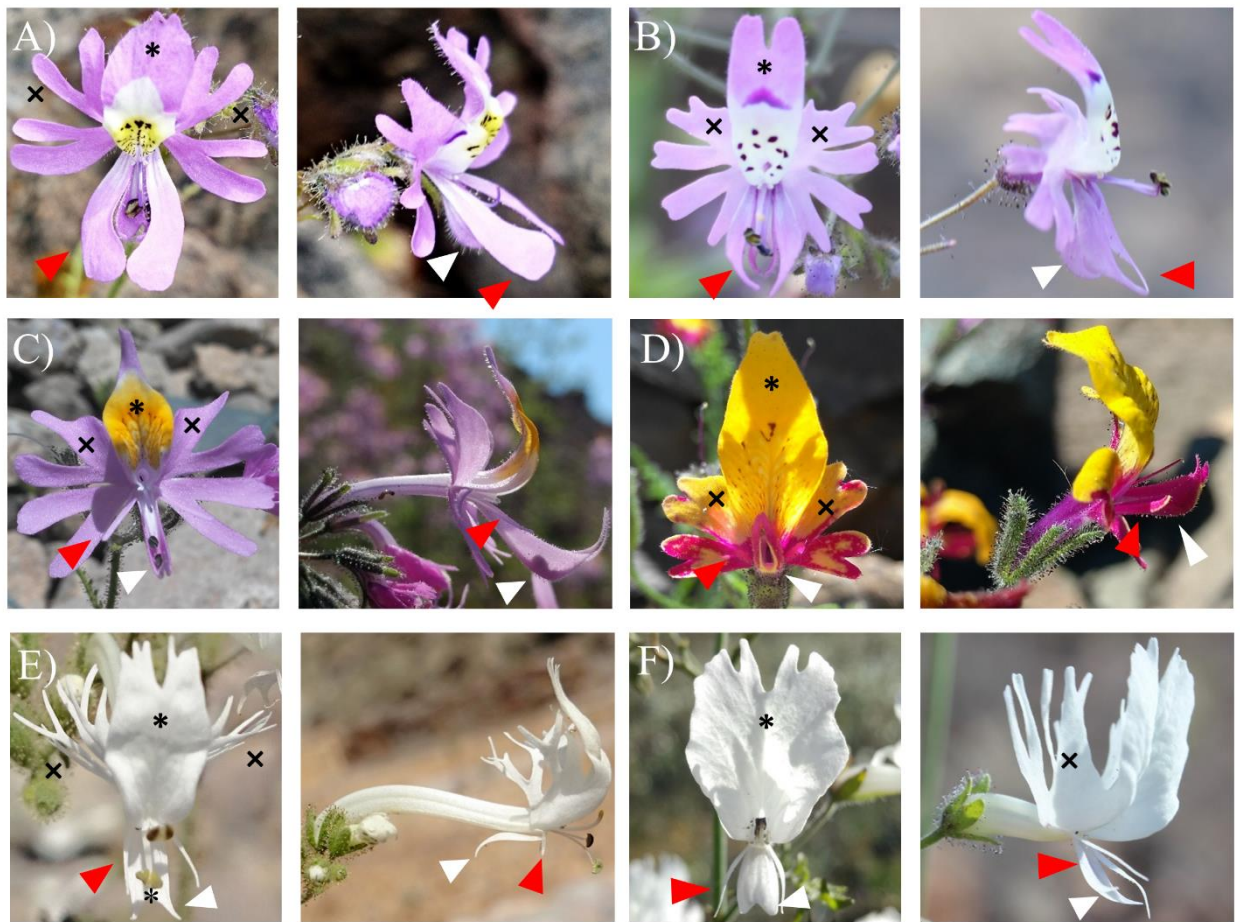
Figure 1: *Morphological diversity in the genus Schizanthus.* Front and side views showing the upper lip at the top (asterisk: banner, cross: lateral sections) and the lower lip structures at the bottom (red arrows: wings, white arrows: the keel). A) *Schizanthus porringens*, B) *S. alpestris*, C) *S. hookerii*, D) *S. grahamii*, E) *S. integrifolius*, F) *S. lacteus.*
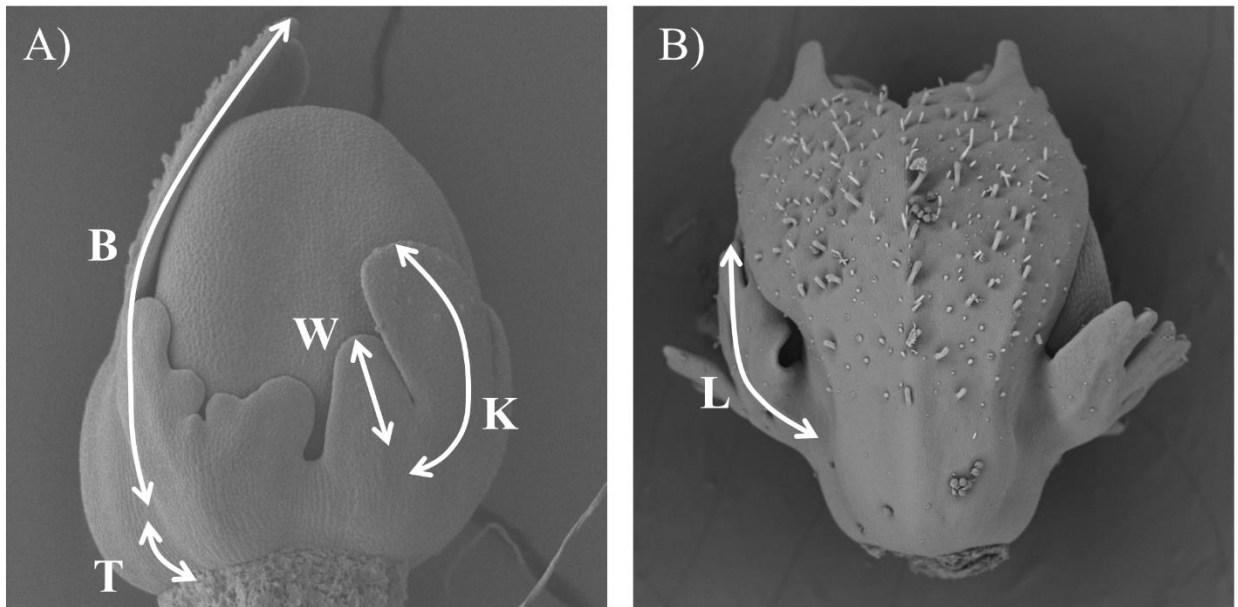
Figure 2: *Key ontogenetic stages considered in this study.* A) Floral buds at wing initiation, which most characterized the early stage (scale bar: 100µm), B) Floral buds during the beginning of the late stage (*S. porrigens* and *S. lacteus*: 0.5mm, all the others: 1mm) and C) floral buds just before anthesis (scale bar: 1mm). B; Banner, L; lateral parts, K; keel, W; wing, T; tube.

Figure 3: *Measurements of the petals in the bud.* A) Side view of the bud showing measurements for the banner (B), the corolla tube (T) and the lower lip (W; wings, K; keel). B) Rear view of the bud, showing the measurements for the lateral parts (L).
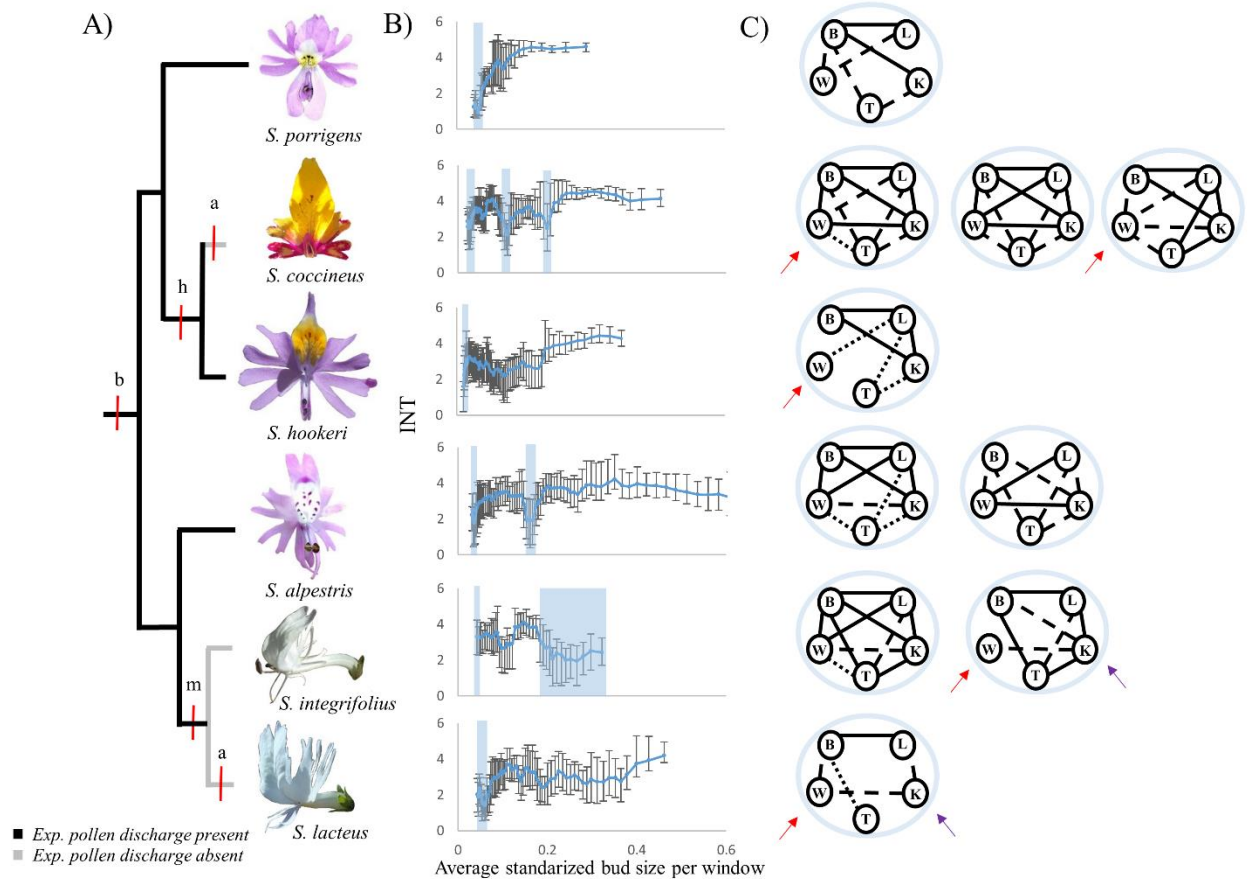
Figure 4: *Ontogenetic integration using the rolling/moving approach.* A) Phylogenetic relationships among species showing the transition from bee- (b) to hummingbird- (h) and moth-(m) pollination syndromes, as well as the presence of autonomous delayed selfing (a) and the explosive pollen discharge mechanism in black and grey (Pérez et al. 2006). B) their corresponding changes in the level of integration at different standardized bud sizes (bud size/final bud size before anthesis), relatively lower INT stages associated with changes in the pattern of integration are marked (light blue), and C) Integration patterns of stages marked at B (filled the same colour as in B), wing decoupling is shown by red arrows and keel decoupling by purple arrows. B, Banner; L, Lateral section of upper lip; W, wings; K, keel; T, Corolla tube.
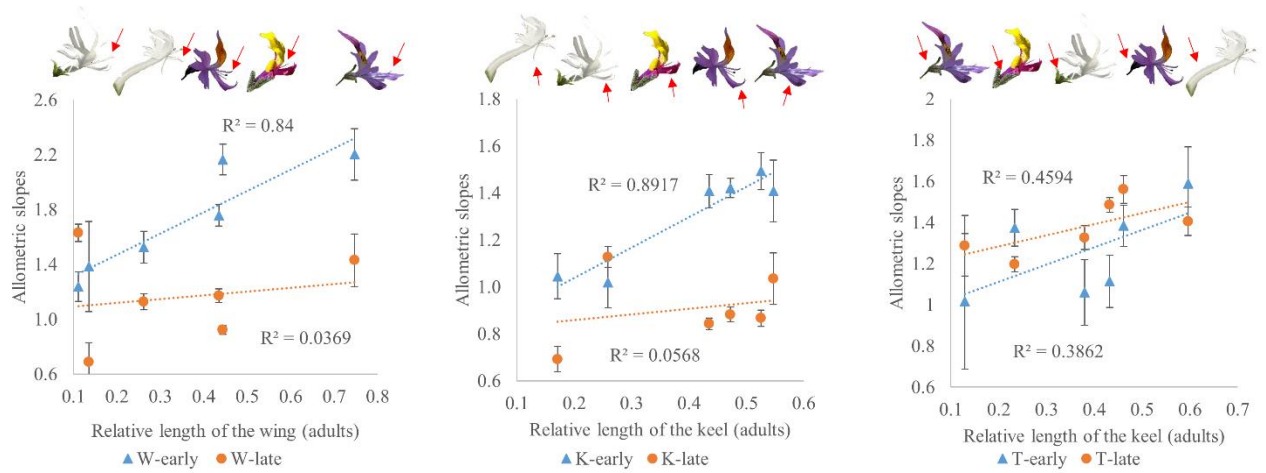
Figure 5: Ontogenetic allometry for traits against bud size. Allometric slopes in early and late ontogeny for the wings A), the keel B) and the corolla tube C) against the trait size relative to the banner in adults for each species. The shortest and longest trait are the extremes of each graph and pictures of flowers on the top of each graph are order by trait relative size as a guide. Pearson correlations with p-values <0.001 are shown for early and late ontogeny.
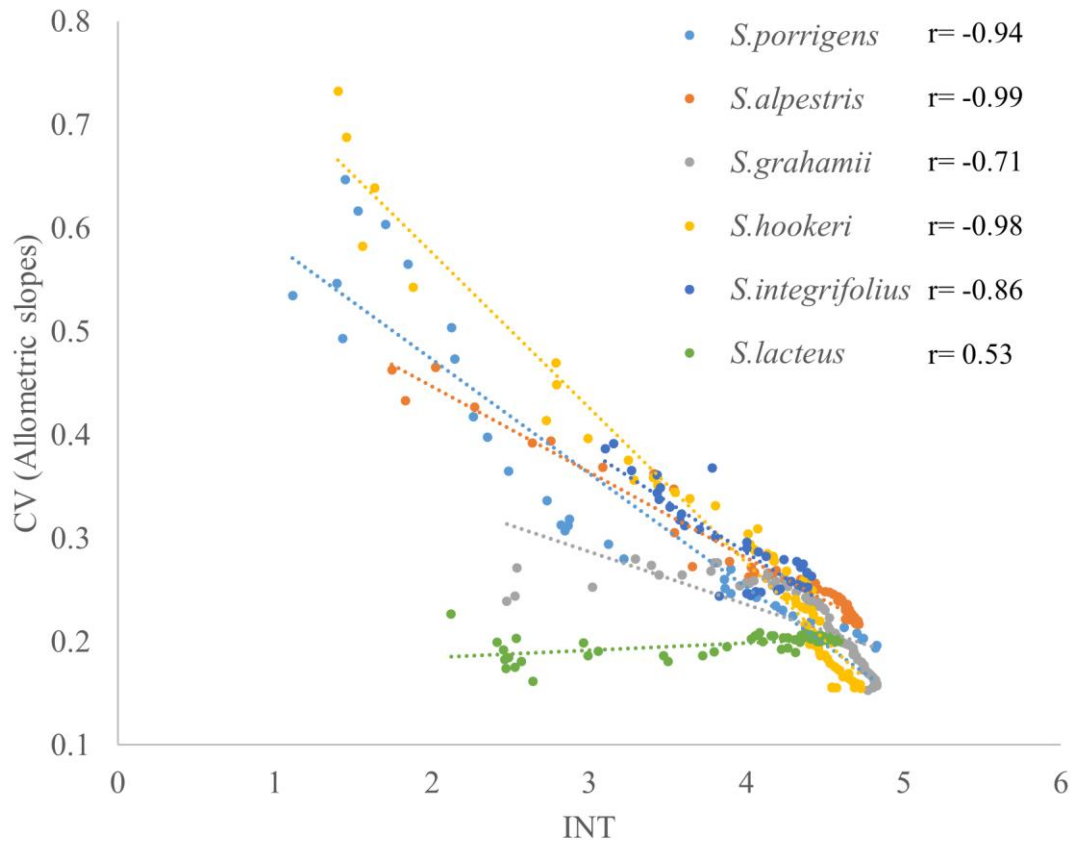
Figure 6: Relationship between ontogenetic integration and allometry. INT values are plotted against the coefficient of variance of the allometric slopes of traits calculated using the rolling/moving approach (k=15) for each species. Pearson r for each individual correlation are shown for each species. P-values were <0.001 for all the correlations.

Table 1: Correlation coefficients for each pair of floral traits: B, Banner; L, Lateral section of upper lip; W, wings; K, keel; T, Corolla tube. All values had a statistical significance of <0.001. The integration level (INT) for each species is also shown

	S. por	*S. hook*	*S. grah*	*S. alp*	*S. int*	*S. lac*
B-L	0.97	0.96	0.98	0.98	0.97	0.98
B-K	0.96	0.98	0.99	0.99	0.98	0.98
B-W	0.97	0.97	0.99	0.98	0.91	0.97
B-T	0.86	0.96	0.97	0.96	0.96	0.96
L-K	0.96	0.94	0.98	0.98	0.96	0.98
L-W	0.98	0.95	0.98	0.97	0.92	0.98
L-T	0.88	0.94	0.98	0.98	0.93	0.97
K-W	0.98	0.94	0.99	0.98	0.94	0.99
K-T	0.9	0.95	0.98	0.97	0.96	0.97
W-T	0.89	0.94	0.98	0.94	0.9	0.97
INT	4.29	4.59	4.76	4.70	4.37	4.69

	S.porrigens		S. alpestris		S. hookeri		S. grahamii		S. integrifolius		S. lacteus	
Complete ontogeny	m	SE	m	SE	m	SE	m	SE	m	SE	m	SE
B	1.16	0.056	0.94	0.015	1.07	0.020	1.08	0.017	0.92	0.021	0.97	0.016
L	1.18	0.052	0.95	0.017	1.06	0.019	1.07	0.015	1.01	0.042	1.04	0.018
K	1.28	0.052	1.07	0.021	1.20	0.033	1.16	0.021	0.98	0.035	1.20	0.025
W	1.89	0.087	1.42	0.040	1.44	0.042	1.45	0.030	1.40	0.101	1.60	0.031
T	1.32	0.100	1.44	0.027	1.37	0.037	1.43	0.027	1.39	0.048	1.37	0.034
Early ontogeny												
B	1.58	0.151	1.21	0.044	1.21	0.049	1.29	0.042	0.98	0.070	1.21	0.055
L	1.20	0.135	0.99	0.057	0.96	0.045	0.93	0.038	0.64	0.124	0.84	0.076
K	1.41	0.131	1.41	0.071	1.49	0.079	1.42	0.041	1.04	0.096	1.02	0.107
W	2.20	0.189	2.16	0.112	1.52	0.115	1.76	0.079	1.38	0.328	1.24	0.108
T	1.02	0.329	1.11	0.127	1.38	0.101	1.37	0.090	1.59	0.178	1.06	0.159
Late ontogeny												
B	0.88	0.079	0.84	0.024	0.95	0.040	1.02	0.034	0.80	0.036	0.82	0.023
L	1.03	0.115	0.83	0.035	1.04	0.044	1.10	0.032	0.95	0.061	1.10	0.030
K	1.03	0.109	0.84	0.025	0.87	0.035	0.88	0.031	0.69	0.054	1.13	0.044
W	1.43	0.193	0.92	0.031	1.13	0.058	1.17	0.050	0.69	0.140	1.63	0.064
T	1.29	0.148	1.48	0.036	1.56	0.068	1.20	0.037	1.40	0.069	1.33	0.058

Table 1S: Allometric slopes for complete, early and late ontogeny of floral traits, and the relative size of each trait in adult individuals. "m": allometric slope, SE: standard error. B, Banner; L, Lateral section of upper lip; W, wings; K, keel; T, Corolla tube.

	Wings EO	Wings LO	Keel EO	Keel LO	Tube EO	Tube LO
Lambda	0	0	0	0	0	0
intercept (std.error)	1.16 (0.14)	1.06 (0.29)	0.78 (0.09)	0.81 (0.21)	0.94 (0.21)	1.18 (0.12)
slope (std.error)	1.55 (0.33)	0.29 (0.70)	1.29 (0.22)	0.25 (0.49)	0.85 (0.52)	0.53 (0.29)
multiple r-squared	0.84	0.17	0.89	0.06	0.40	0.46
p-value	0.009	0.70	0.004	0.63	0.18	0.14

Tabla S2: Summary of linear regression between the ontogenetic allometric slopes of the trait against its relative length in the open flower among *Schizanthus* species. EO: Early ontogeny, LO: Late ontogeny.

The role of paedomorphosis and peramorphosis in *Schizanthus* flower evolution- a quantitative analysis.

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ABSTRACT

Premise of the study – Heterochrony, the phyletic change in the timing of development, is considered one of the most important factors shaping floral diversification. Few heterochrony studies have included comparative quantitative measurements in a broad range of ontogenetic stages to detect the effect of different ontogenetic changes in morphological diversification. In this study, quantitative heterochronic changes related to morphological evolution are explored in the genus *Schizanthus* underlying shifts from 1) bee- to hummingbird- and moth-pollination, 2) specialized to generalized species and 3) outcross to self-pollinated species.

Methods – Bud traits of five species of *Schizanthus* were measured at early (organ appearance) and late (organ growth) ontogeny. During early and late ontogeny, changes in size and shape were traced in key ontogenetic stages. To better explore changes in late ontogeny a geometric morphometrics approach was used.

Key Results –Transition in the pollination system occurred by a shift to paedomorphic lower lips: bud and lower lip shape is reached at higher bud sizes in species with lower lip reduction. However, lower lip shape changes were significant only for the desert clade, were the ancestral final morphology is never reached. Wing reduction can occur by a late onset of the organ alone, a late onset enhanced by a growth rate decrease or even an early onset compensated by a growth rate decrease. In contrast, keel reduction occurred by either post-displacement or neoteny. Shift from specialized to generalized systems is associated with a shift to a peramorphic keel produced by an early increase in its growth rate. Finally, species that evolved autonomous selfing showed no shared pattern of heterochronic changes.

Conclusion – Different ontogenetic process underlie paedomorphosis and peramorphosis associated with pollinator-driven floral diversification in *Schizanthus*. These processes occurred in different combinations through ontogeny having either an additive or a compensatory effect.

Key words: *Schizanthus*, Solanaceae, Heterochrony, Pollinator-driven diversification, Flower evolution, Paedomorphosis, Post-displacement, Neoteny, Peramorphosis, Acceleration.

INTRODUCTION

Floral morphologies present different patterns of diversification. While basal angiosperms tend to vary in organ number and arrangement, in more advanced groups with highly synorganized flowers diversification originates from flexibility in shape and the appearance of new structures (Endress, 2001). Shape diversification occurs usually by rapid evolutionary changes in the proportions among floral organs and tends to be closely associated with both shift in the pollination system and heterochrony changes. Evolutionary shifts in the pollination system related to changes in proportions among floral organs has been largely documented (Armbruster et al., 2009; van der Niet and Johnson, 2012; Van Der Niet et al., 2014). In comparison, heterochrony, understood as the phyletic change in the timing of development (Gould, 1977; Alberch et al., 1979), has been only recently considered as a major evolutionary process in plants (Endress, 1990; Box and Glover, 2010; Buendía-Monreal and Gillmor, 2018; Ronse de Craene, 2018). As plants are complex developmental systems characterized by indeterminate growth and modular organization, heterochrony has been mostly studied in flowers, which constitute determinate units similar to animals. Particularly, heterochrony is considered a widespread driver of speciation and saltatory morphological changes in flowers (Bateman and DiMichele, 1994; Box and Glover, 2010; Barfod, 2017). Some authors even claimed that heterochrony was the basis of floral shape evolution (Kampny and Harris, 1998).

Heterochrony in flowers – Heterochronic process were firstly defined for animals, based mostly on the separation among juvenile, adult and reproductive stages, which tend to be clearly differentiated (Gould, 1977; Alberch et al., 1979). In contrast, plants present ephemeral reproductive structures and somatic growth continues while flowering. Therefore, heterochrony has been defined differently for flowers. Paedomorphosis is defined as an

abbreviation of trait development, retaining traits found in the bud of the ancestor in the open flower of the descendant, and peramorphosis is defined as an extension of trait development, adding new morphologies in the descendant which were not present in the ancestors (sensu Box and Glover, 2010). Paedomorphic structures in flowers may result either due to a later onset of the organ (post-displacement), a reduction in its growth rate (neoteny) or by a premature cessation of its growth (progenesis). Similarly, peramorphic structures in flowers can occur either due to an early onset of the organ (pre-displacement), an increase in its growth rate (acceleration) or by a prolongation of the growth period (hypermorphosis) (sensu Bateman and DiMichele, 1994; Box and Glover, 2010; Barfod, 2017). Regardless of the underlying process, paedomorphosis is expected to produce more generalized morphologies by the retention of juvenile traits, while peramorphosis is expected to produce more specialized morphologies by the appearance of new traits (Alberch et al., 1979).

Several studies have documented heterochronic changes underlying floral diversification (Box et al., 2008; Guo, 2015; Bull-Hereñu et al., 2016; Naghiloo and Claßen-Bockhoff, 2017; Vasconcelos et al., 2017). Moreover, heterochronic changes has been showed to underlie morphological changes related to shifts in the pollination system in several species. Most studies focus on changes from cross to self-pollinated species, mainly related to paedomorphosis (Lord, 1982; Minter and Lord, 1983; Gallardo et al., 1993; Stewart and Canne-Hilliker, 1998; Pedersen and Ehlers, 2000; Porras and Muñoz, 2000; Li and Johnston, 2001, 2010; Georgiady and Lord, 2002; Box and Glover, 2010). However, heterochronic changes also underlie transition in the pollination system from specialized to generalized (Laurent et al., 1998; Box et al., 2008; Box and Glover, 2010), specialized to specialized (Guerrant Jr., 1982), and generalized to specialized species (Armbruster et al., 2013). Few of

these comparative studies have included quantitative measurements of ontogenetic size changes among species (Bull-Hereñu et al., 2016; Naghiloo and Claßen-Bockhoff, 2017). However, quantitative analysis are of key importance determining the role of heterochrony in floral morphological evolution, as distinct type of heterochrony can occur at discrete stages of a developmental process (Li and Johnston, 2000) and the effect of early heterochronic events can be enhanced, reduced, or eliminated by later changes of the growth rate during development (Naghiloo and Claßen-Bockhoff, 2017).

Flower morphological evolution in Schizanthus – The genus *Schizanthus* Ruiz and Pavón (Grau and Gronbach, 1984) comprises 13 species of annual plants endemic to the southern South American Andean region (between 22-40°). Morphological and molecular studies indicate that *Schizanthus* diverged early from the rest of the family Solanaceae, probably related to Gondwana fragmentation (82-86 ma, Wikström et al., 2001) and constitutes a monogeneric tribe: Schizanthoideae (Olmstead and Palmer, 1992; Knapp, 2002; Olmstead et al., 2008).

The flowers of *Schizanthus* are morphologically unusual in the Solanaceae family (Walters, 1969; Grau and Gronbach, 1984; Knapp, 2002), with oblique zygomorphy and resupination (Cocucci, 1989b). The calyx and the corolla are pentamerous, but while the calyx is actinomorphic, the corolla is highly zygomorphic: five sympetalous lobes are arranged in two lips (Fig. 1). The upper lip is produced by the fusion of three petals (the banner and the two lateral sections), while the lower lip is produced by the fusion of two petals forming the keel and two outer lateral sections from each petal forming the wings (Fig. 1D-H). In species with an extended lower lip, the keel retains the exerted stamens forming an explosive pollen discharge mechanism (Cocucci, 1989a) and the wings act as a landing platform for insects

(Fig. 1B,D). The lower lip is completely reduced in some species in which the pollen

discharge mechanism is lost (Fig. 1C, E-H). However, in *S. hookeri*, in which only the wings

are reduced, the pollen discharge mechanism is conserved and the keel also act as a landing

platform (Pérez et al., 2006, Fig. 1E). The petals of the upper lip can present different degrees

of septation, specially the lateral sections, which can be deeply dissected in some species. The

androecium is highly zygomorphic: five stamens are fused to the corolla tube, but only the two

transverse stamens will develop fertile bithecate anthers, the other will remain as staminodes.

The two staminodes associated to the upper lip (between the banner and the lateral sections)

are considerably longer than the sole lower lip's staminode, remaining visible in the open

flower. The coralline tube encloses the bicarpellate-syncarpous gynoecium. The ovary is

superior, with several ovules, a filiform style and a thin stigma.

Extant *Schizanthus* species are phylogenetically divided into three clades that diverged

relatively recently, about 5 million years ago (Pérez et al., 2006). They present a huge

diversity of flower morphologies associated with ecological changes in the pollination

systems. Ancestral reconstruction analysis show that lower lip morphological changes strongly

correlate with changes from bee- to hummingbird- and moth- pollination syndromes (Pérez et

al., 2006). Bee-pollinated species are mostly grouped in one clade and represent the ancestral

morphology in the genus, with a fully functionally and extended lower lip. Reductions in the

lower lip appeared independently in two clades: in the high Andean clade in coordination with

bee-to-hummingbird transition (in this study *S. coccineus* and *S. hookeri*¸ Fig. 1E,F) and in the

desert clade in coordination with bee-to-moth transition (in this study *S. integrifolius* and *S:

lacteus*, Fig. 1G,H). Additionally, lower lip reduction is accompanied by the shortening of

stamens in some species (in this study *S. coccineus* and *S. lacteus*, Fig. 1F,H), in which

autonomous selfing has evolved (Pérez et al., 2009). Finally, the only generalist species of the genus, *S. hookeri* , has reduced wings but an elongated keel, which is considered an intermediate state in the bee- to hummingbird-pollination transition (Pérez et al., 2006).

Aims of the present study – In this study, quantitative heterochronic changes were traced along ontogeny in five species of *Schizanthus,* representing morphological diversity closely associated with shifts from 1) bee- to hummingbird- and moth- pollination, 2) specialized to generalized species and 3) outcrossed to self-pollinated species. Particularly, size and shape changes will be traced during both key developmental events by ontogenetic curve analysis and along ontogeny using a geometric morphometric approach. Altogether, comparative quantitative analysis of growth during development in the genus may help to answer the following questions: which heterochronic changes are underlying shift in the pollination and breeding system? Do these changes occur by the same heterochronic processes and at the same time in independent reductions of the lower lip? In general, how does heterochrony contribute to the diversification of *Schizanthus* species?

MATERIAL AND METHODS

Species used and plant material –The study includes five species of *Schizanthus* (Fig. 1). One representative of the coastal/Mediterranean clade, *S. tricolor*, was selected to represent the ancestral bee-pollinated morph (Fig. 1A). Two species of each clade with lower lip reductions were selected: for the high Andean clade *S. hookeri* and *S. coccineus*, and for the Desert clade *S. integrifolius* and *S. lacteus*. *S. coccineus* and *S. lacteus* present autonomous delayed selfing and inserted stamens.

S. coccineus has been recently validated as a new species different from *S. grahamii* (Rodríguez et al., 2018). In some studies, cited in this work, this species is considered as *S. grahamii coccineus* or even just as *S. grahamii*. We collected material from the population from where *S. coccineus* was described as a different variety/species in past works. All species are found in Chile, with only two species reaching the Argentinean side of the Andes. All plant material was collected in the field from chilean populations (*S. tricolor*, Quebrada de Córdoba, El Tabo, Región de Valparaíso; *S. hookerii*, Valle Nevado, Santiago, Región Metropolitana; *S. coccineus*, La Parva, Santiago, Región Metropolitana; *S. integrifolius*, Alto del Carmen, Huasco; Región de Atacama; *S. lacteus*, Paposo, Taltal; Región de Antofagasta).

In this work, only floral development of the bee-pollinated *Schizanthus porrigens* is described in detail. For the other species, only aspects related with lower lip and stamen reductions will be described. Despite the resupination described for the genus, in the following description the upper lip will be consider in an adaxial position and the lower lip in an abaxial position.

SEM techniques – Inflorescences were collected and fixed in 70% ethanol in the field. The specimens prepared for SEM were dissected under a stereomicroscope and critical point dried (CPD 030; BAL-TEC, Pfäffikon, Swizterland). If necessary, samples were dissected again in order to improve floral traits visualization, mounted on stubs and sputter-coated with gold in an argon atmosphere (SCD 005; BAL-TEC). The samples were viewed and photographed at 15 kV in a scanning electron microscope (ESEM; Philipps, Eindhoven, the Netherlands). Ten to eleven inflorescences for each species were dissected.

Ontogenetic stages definition and measurements – Ontogenetic stages were defined to identify key changes in size and shape during ontogeny. Early ontogeny was defined as the

phase of organ appearance (from septal to gynoecium) and late ontogeny was defined as the phase of organ differentiation (from petal primordia appearance until the petals reach their final adult form). In order to explore changes in organ appearance, bud size was measured in early ontogeny considering only top view images and setting five landmarks on vertices corresponding to the five petal primordia (Fig. 2A). Changes in growth dynamics only of the lower lip (keel fusion and wing appearance) were measured using five landmarks in side view images of the bud in the following positions: two in the base, one in the top, one in the highest of the lower lip and one in the highest point of the banner (Fig. 2B). Regardless of the ontogenetic stages, the top landmark is placed considering the highest point of the banner and the two lateral points were always at the same height in the floral bud. Growth dynamics during organ differentiation was measuring using the he length of the two structures of the lower lip (wing and keel) and the length of the bud (Fig. 2C).

In total, five stages were defined a priori for early ontogeny: (1) sepal appearance, (2) petal appearance, (3) stamen appearance, (4) staminode appearance (5) gynoecium appearance (Fig. 3). Additionally for the lower lip, two extra stages were defined for early ontogeny: (1) keel primordia fusion (Fig. 8, stage 1) and (2) wing appearance (Fig. 6, stage 2). Regarding the lower lip late ontogeny, stages were defined for the wings and the keel separately and a after a detailed analysis of their development (Fig. 6,8). Stages were defined based on key changes in shape, such as a change in the angle of an organ respect to the bud, curve appearances, elongations, etc. For both the keel and the wing, if the last stage was not present in any specie, pictures showing equivalent ontogenetic stages were used. For early stages, 7-30 floral buds per stage were measured for each species depending on their frequency of appearance.

Similarly, for late stages, 5-32 floral bud per wing stages and 5-29 floral buds per keel stages were measured.

Geometric morphometric analysis – Landmarks were used to measure the overall shape of the bud (19 landmarks) and lower lip shape (first 9 landmarks) (Fig. 2D). For each species, 20 photos from different ontogenetic moments were selected being representative of bud size diversity through development for each specie. Landmarks were scaled, translated and rotated against the consensus configuration by GLS Procrustes superimposition method (Bookstein, 1991; Rohlf, 1999). The variability in the shape space was assessed using the scores obtained for each specie obtained by a procuter tangent coordinates analysis (i.e., all procrustes residuals). As this analysis is a PCA, the first axis was used to describe shape change for each species. The trajectory of shape change relative to size for each species was quantified plotting the first PCA axis obtained from the procuster analysis against the natural log of the bud length separately for each species. All morphometric analysis were performed using the geomorph package in R(R Core Team, 2018). To test significant differences among the regressions, ANCOVA test were performed using the following model: shape~budsize*species for both, the bud and the lower lip shape. Significant differences among species were explored performing a Tukey test on the ANCOVA model. Both, ANCOVA and Tukey analysis were performed in R.

Ontogenetic curves – For early ontogeny, each ontogenetic stage was plotted against its averaged bud size with their correspondent standard deviation. In strongly synorganized systems as *Schinzathus*, changes in reproductive meristem size may affect organs proportionally, leading to smaller or larger flowers, or nor proportionally (reviewed in Ronse De Craene, 2018). *Schizanthus* species differ in corolla size (*S. tricolor*: 2.75 cm^2, *S.*

coccineus: 4.26 cm^2, *S. hookeri*: 2.82 cm^2, *S. integrifolius*: 2.3 cm^2, *S. lacteus*: 1.44 cm^2, extracted from Pérez et al., 2006). As in our study only no proportional changes are evaluated (i.e., reductions of the lower lip), bud size was standardized by the size of the flower at anthesis for each species in the early ontogeny analysis, in order to discard proportional changes affecting flower size.

For late ontogeny, each ontogenetic stage was plotted against its averaged bud length to describe the growth dynamic of each organ. To evaluate proportional change of each lower lip organ, ontogenetic stages were plotted against the proportion at bud (averaged organ length at each ontogenetic stage/bud length at each ontogenetic stage) in relation to the proportion at the open flower (organ length at open flower/ flower length when open).

Allometry analysis – In case that growth dynamic cannot be deduced directly by linearization of the ontogenetic curves due to the y-axis election, allometric slopes will be calculated directly from the raw data of ontogenetic stages that differ in their growth dynamics. In all cases, allometric slopes were calculated from linear regressions of the traits on bud size (Mosimann, 1970) and 95% confidence intervals were calculated by bootstrapping the original log transformed data using the package MASS in R.

RESULTS

Early ontogeny – In the ancestral bee-pollinated *Schizanthus tricolor*, the pentamerous calyx is initiated in a helix sequence. The abaxial sepal is formed first and considerably earlier than the rest of the sepals, remaining bigger during all early ontogeny (data not shown). Five petal primordia, alternating with the sepal primordia, originate almost simultaneously with two stamen primordia, which were only slightly delayed in comparison with the petals. The two

stamens initiated on either side of the floral apex will develop into fertile anthers, while the following three smaller androecial primordia (2 adaxial, 1 abaxial) will develop into staminodes. Two carpel primordia are initiated on the dorso-ventral axis of the remaining meristem. Deviations of this pattern were only found in *S. coccineus*, where staminode appearance (stage 4) occurred simultaneously with gynoecium appearance (stage 5) (Fig. 3).

Quantitative analysis showed that the desert species were constantly delayed through early ontogeny, as sepal, petal, stamen, staminode and gynoecium appearance occurred at significantly higher bud sizes in comparison with the bee-pollinated *S. tricolor* (*S. integrifolius*: 1.9-2.2, *S. lacteus*: 2.6-2.8 times bigger, Fig. 4, stages 1-5). On the contrary, different tendencies are found in the high Andean species: while *S. hookerii* showed a delayed appearance of petals and staminodes regarding *S. tricolor* (1.3-1.4 times higher), *S. coccineus* only presented a delay in staminode's appearance (less than one time higher) (Fig. 4, stages 1-5).

Late ontogeny of the lower lip– The first axis of the PCA explained the 63% of the variance in shape and has a lineal relationship with the natural logarithm of the bud length ($R^2_{S.\ tricolor}$: 0.96, $R^2_{S.\ coccineus}$: 0.97, $R^2_{S.\ hookeri}$: 0.94, $R^2_{S.\ integrifolius}$: 0.93, $R^2_{S.\ lacteus}$: 0.98, Fig. 5A). The linear relationships differed in the intercept as shown in ANCOVA results (size: F= 2196.274, Df=1, p= <2e-16, species: F= 38.362, Df=4, p= <2e-16). Therefore, the same form is reached at significantly different bud lengths among species. Particularly, the intercept of *S. tricolor* is significantly higher than for all the other species, as confirmed by Tukey test results. This imply that species with lower lip reduction reached the same bud shape at significantly higher bud lengths, being delayed in comparison with *S. tricolor*.

When morphometric analysis were performed only on lower lip landmarks, the first axis of the PCA explained 56% of the shape variance and has a linear relationship with the natural logarithm of the bud length ($R^2_{S.\ tricolor}$: 0.97, $R^2_{S.\ coccineus}$: 0.94, $R^2_{S.\ hookeri}$: 0.94, $R^2_{S.\ integrifolius}$: 0.91, $R^2_{S.\ lacteus}$: 0.86, Fig. 5B). Significant differences were found in both the intercept and the slopes, as shown by ANCOVA results (size: F= 978.397, Df=1, p= <2e-16; species: F= 7.043, Df=4, p= 5.6e-5; size:species: F=8.737, Df=4, p=,5.2e-6). This result imply that lower lip shape is reached at significantly higher bud lengths for all species with lower lip reduction. Notably, none of the four species with lower lip reduction reached the shape of *S. tricolor* at the biggest bud length. However, differences were significant only among *S. tricolor, S. integrifolius* and *S. lacteus* as shown by Tukey test of the ANCOVA, suggesting that lower lip reduction has a stronger shape component than in the Andean clade being a truly paedomorphic clade.

Late of ontogeny of the wings – In *S. tricolor*, wing primordia started as a lateral outgrowing of the keel primordia. At first, the wing appears as two small lobes in the outer border of the keel primordia (Fig. 6, stage 2). Later, only one lobe is distinguishable of similar size than the tip of the keel and forming and acute angle with the base of the bud (Fig. 6, stage 3). In the following stage, the angle between the wing and the base of the bud will become more perpendicular (Fig. 6, stage 4). After a stage of wing elongation the wing becomes more long than wide (Fig. 6, stage 5) and, finally, reaches its final form characterized by the appearance of a lobe in its tip (Fig. 6, stage 6). Deviations of this pattern are found in all the other four species with wing reduction: wing primordia tend to be smaller (at least in width) from stage four onwards and none of the species reaches the final form with the lobe in the tip (Fig. 6).

Quantitative analysis showed that changes in wing growth occurred even before. All species with lower lip reduction tend to had higher bud size at wing appearance than *S. tricolor*, except for *S. coccineus* (Fig. 4, stage 7). This delay was more intense in the desert clade (*S. lacteus*: x2.5, *S. integrifolius*: x1.7) than for *S. hookeri* (x1.3), being only statically significant for *S. integrifolius*. The remaining stages of wing development occurred at a significantly bigger bud size than *S. tricolor* for all species with wing reductions (Fig. 7A, B). This delay was more pronounced for *S. hookeri* during the first three stages (x1.3-1.7, Fig.7A), but did not increase in the following stages (x1.5-1.6, Fig.6, B). In contrast, the other three species became more delayed trough ontogeny (Fig. 7B). While the desert clade became the delayed since stage 4 (from x1.3-1.4 in both species to x2.1-2.5 in *S. integrifolius* and x2.5-2.9 in *S. lacteus*), *S. coccineus* showed a strong delay only in the last stage (x2.7, Fig. 7B). This change in the pattern of delay among species between wing onset and the following ontogenetic stages suggest that all species with wing reduction except for *S. hookeri* pass through a neotenic process.

Patterns of wing appearance and its growth dynamics are congruent with changes in their relative proportions in the bud. In the desert clade, wings were proportionally smaller through all ontogeny compared not only with *S. tricolor*, but also with the Andean clade (Fig. 7D), being congruent with its strong delay in wing appearance (more marked in *S. integrifolius*) and its decrease in allometric slope (more marked in *S. lacteus*). For the Andean clade, wings were proportionally smaller constantly through ontogeny for *S. hookeri*, congruent with its slight delay in wing onset and decrease in allometric slope (Fig. 7D). In contrast, wings were proportionally smaller only in late stages for *S. coccineus*, in which there was no delay in wing onset, only a decrease in the allometric slope in late stages (Fig. 7D).

Late ontogeny of the keel – Keel development in *S. tricolor* started with two primordia separated by the abaxial staminode (Fig. 8, stage 1) which are subsequently fused forming the keel (Fig. 8, stage 2). Later, the keel primordia gets curved (Fig. 8, stage 3). This curve is maintained in the following elongation of the keel (Fig. 8, stage 4). The final form of the keel is characterized by a curved end with elongated tips (Fig. 8, stage 5). Deviations of this pattern are found in *S. integrifolius*, *S.lacteus* and *S. coccineus*: the angle of the keel is less conserved since stage 3 onwards and the angle of the adult form is never reached in the desert clade. In *S. hookeri*, the keel is not reduced and present an even stronger angle than in *S. tricolor* since stage 4 (Fig. 8).

Quantitative analysis shows that, only for *S. lacteus* and *S. integrifolius*, keel appearance occurred at significantly bigger bud size compared to the *S. tricolor* (x1.3 and x2, correspondently. Fig. 4, stage 6). Interestingly, keel fusion tend to occurs at smaller bud size for *S. coccineus*. Growth dynamics of the keel were congruent with the final organ size instead of its proportion in relation to the corolla, thus reflecting corolla size tendencies among species (Fig. 9A,B). When allometric slopes were analysed, contrasting patterns were found among clades (Fig. 9C). *S.tricolor* and *S. hookerii* presented an increase in allometric slopes among ontogenetic stages, but it was significant only for *S. tricolor* (Fig. 9C). Species with keel reduction presented either a non-significant decrease (*S. coccineus*) or no change at all in the allometric slopes (*S. integrifolius* and *S. lacteus*) (Fig. 9C).

Similarly to the wings, patterns of keel appearance and growth dynamics are congruent with changes in its relative proportion in the bud (Fig. 9D). The desert clade, characterized by a significant delay in keel appearance and constant allometric slopes through ontogeny, had a proportionally smaller keel constantly through all ontogeny compared with the other species

(Fig. 9D). The Andean clade showed a contrasting pattern; both species had a proportionally bigger keel in the first stages, which remains relatively constant in *S. hookeri* due to its increase in keel allometric slope, but changes for *S. coccineus* due to its decrease in keel allometric slope in the last two stages (Fig. 9D).

DISCUSSION

Heterochrony shifts are commonly related with mayor pollination mediated morphological changes (Box and Glover, 2010; Barfod, 2017; Ronse de Craene, 2018). In general, temporal changes in ontogeny are an importance source of morphological variation, promoting rapid evolutionary shifts due to changes in structural proportions among species. This is the case in *Schizanthus*: the three clades diverged relatively recently (5 mya, Pérez et al., 2006) mostly based on pollinator-driven changes in lower lip proportions produced by different heterochrony processes. By definition, heterochrony changes are defined in relation to an ancestral ontogenetic trajectory (Gould, 1977; Bateman and DiMichele, 1994; Box and Glover, 2010), therefore, heterochrony changes in *Schizanthus* are defined in relation to bee-pollination morphology (*S. tricolor* in this study) considered to be the ancestral state in the genus (Pérez et al., 2006). When the proper ecological correlations have been stablished (e.g., as the shift in the pollination system in *Schizanthus*), heterochronic changes can be considered as an ontogenetic adaptation (Gould, 1988).

In several studies in which Paedomorphosis leads to shifts in the pollination system neoteny is the most common underlying ontogenetic process documented (Li and Johnston, 2001; Whittall and Hodges, 2007; Armbruster et al., 2013). However, this tendency may be produced because few studies consider early ontogeny stages, and therefore does not analyse

changes in the onset of the organ. In this study, a broad range of ontogenetic stages were considered, and lower lip reduction underlying shifts in the pollination systems represent Paedomorphic morphologies that can be produce either by changes in the onset of organs, their growth rates or both. Proportionally smaller wings can be produced by either a delayed appearance of the organ (post-displacement, *S. integrifolius*), a decrease in its growth rate (neoteny, *S. coccineus*) or both (*S. hookeri* and *S. lacteus*) (Fig. 10). Similarly, ontogenetic processes producing keel reductions were post-displacement process in the desert clade and neoteny in the Andean clade (Fig. 10). The sharp post-displacement process affecting the keel and the wings mainly explains the stronger lower lip reduction (affecting size and shape) in the desert clade, as changes in early ontogeny are maximized through ontogeny having a bigger impact in adult morphologies (Alberch et al., 1979). Therefore, quantitative analysis considering early stages is key to disentangle if founding the same form at bigger bud sizes than in the ancestral morph implies a decrease in the growth rates or it is just the result of a post-displacement process maximized through ontogeny.

Heterochronic shifts underlying lower lip reductions in *Schizanthus* can involve a combination of both a change in the onset of an organ and its following growth dynamics. In *S. coccineus*, a later neotenic process compensates the slight pre-displacement. In contrast, neoteny in *S. lacteus* enhances the effect of the post-displacement process. This evidence is in accordance with other quantitative ontogenetic studies showing that the effect of a heterochronic process can be eliminated or enhanced by later changes of growth rates (Naghiloo and Claßen-Bockhoff, 2017). Of our knowledge, few studies have also analysed shape quantitatively. In this study this approach allow us to prove that in species with lower lip reduction overall bud shape appearance is delayed through ontogeny for all species and the same occurs for the

lower lip shape but strongly for the desert clade. In addition to ontogenetic curve analysis, this evidence implies that the desert clade is more reduced in both shape and size than the Andean clade. Therefore, quantitative analysis are very useful to describe additive and compensatory effect between changes in the onset of an organ and their subsequent growth dynamics and how this processes related to shape.

Gould (1977) described paedomorphosis as an escape from specialization, as generalized juvenile forms are conserved in the adult phase, and peramorphosis as a mechanism to reach new and more specialized structures. However, in plants, several studies have found that paedomorphosis can lead to pollination changes without affecting the specialization of the system (Guerrant Jr., 1982; this study) or even increasing its specialization (Armbruster et al., 2013). Similarly, peramorphosis in plants has been found to underlie shifts from specialized to generalized systems (Laurent et al., 1998; Box et al., 2008; Box and Glover, 2010). This same pattern has been found in this study: the proportionally big keel in *S. hookerii* correspond to a case of peramorphosis by acceleration. This proportional increase in size co-occurred with a shift to a generalized pollination system, where the keel gains a double function acting as an explosive pollen discharge mechanism (Cocucci, 1989a) and as a landing platform (Pérez et al., 2006). Finally, morphometric analysis for the lower lip in *S. hookerii* showed a slightly non-significant higher slope compared with *S. tricolor*, suggesting an acceleration process affecting the keel shape. However, shape scores of *S. hookerii* did not showed new morphologies compared to *S. tricolor*. The opposite process underlying their paedomorphic wings and peramorphic keel may produce this trend.

It has been broadly documented that paedomorphosis and, in less degree, peramorphosis, underlies the ecological transition from cross- to self-pollination (Lord, 1982; Minter and

Lord, 1983; Gallardo et al., 1993; Stewart and Canne-Hilliker, 1998; Pedersen and Ehlers, 2000; Porras and Muñoz Álvarez, 2000; Georgiady and Lord, 2002; Box and Glover, 2010; Li and Johnston, 2010). In this study, species that evolver autonomous selfing showed no shared ontogenetic patterns of lower lip reduction. Despite included stamens and pistil also characterize the selfing syndrome, these organs presented no delayed onset, therefore, morphological changes in androecium and gynoecium should appear in later development. The lack of shared ontogenetic pattern in selfing species of *Schizanthus* may be related to the fact that in these species autonomous delayed selfing evolved in association with an increased specialization in the pollination system (Perez et al, 2009). Consequently, selfing species in *Schizanthus* can present big and sometimes colourful corollas, despite their high levels of autonomous delayed selfing. These characteristics do not fit the "classic" selfing syndrome, with reduced, un-attractive corollas and low herkogamy, which is usually used to explore the transition from cross- to self-pollination in the studies cited above.

Quantitative heterochronic analyses are in general (Alberch et al., 1979) and particularly in physico-dynamic studies of floral development (Ronse de Craene, 2018) of key importance to better understand phenotypic changes and their relations to morphological evolution. In this study, pollination-driven evolution in *Schizanthus* is found to be explained by phaedomorphosis and peramorphosis. Paedomorphic morphologies can be reached by both changes in the onset of an organ and its growth dynamics, which can occur with different intensities between organs and among species. These processes can also occur in combination, having either an additive or a compensatory effect through ontogeny. Peramorphic morphologies are reach by an acceleration process and underlie the shift from specialized to generalized systems. Finally, there was no common ontogenetic pattern among selfing species,

which may be due to the highly specialized corolla shape of species with autonomous delayed selfing in the genus. Overall, heterochronic changes through ontogeny are the mayor process explaining morphological evolution in *Schizanthus*.

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AUTHOR CONTRIBUTIONS

JC and FP collected the samples in the field, JC and RCB made the analysis and JC wrote the manuscript, reviewed by RCB and FP.

DATA ACCESSIBILITY STATEMENT

Numerical data such as those used in statistical analyses and modeling, should be organized in an editable file (e.g., as a tab-delimited text file), and included as a supplement or deposited in an archive such as Dryad (http://datadryad.org) or figshare (https://figshare.com), along with an additional text document that provides summary information about the meaning of each column in the data set.

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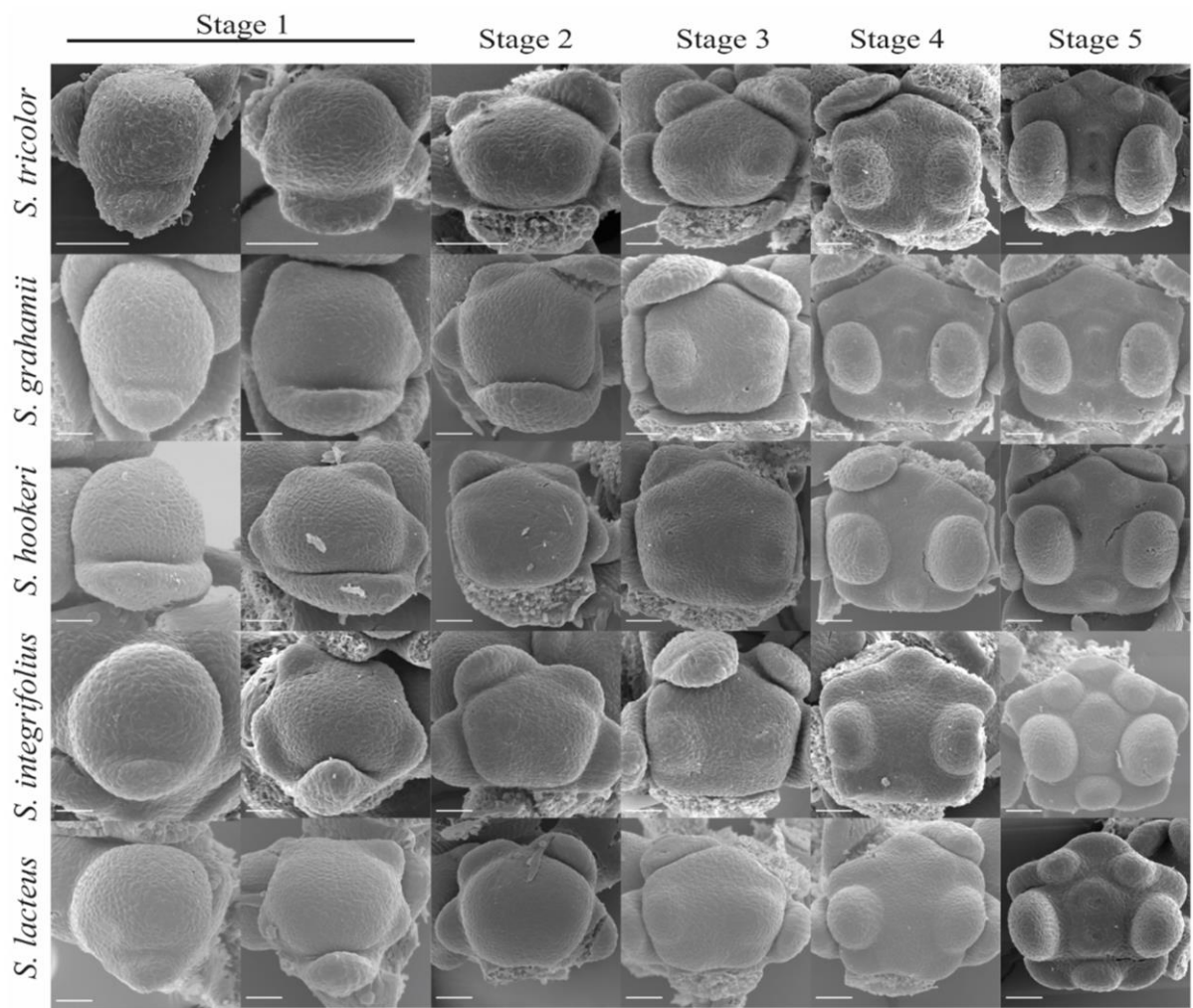
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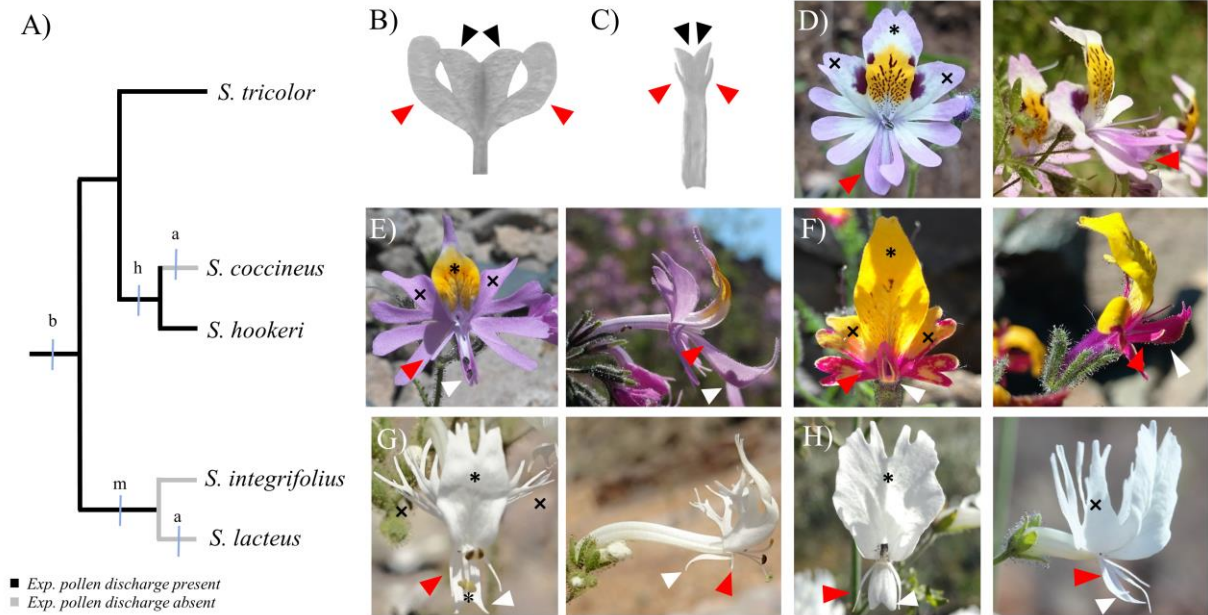

Figure 1: *Species used and their phylogenetic relationships*. A) Phylogenetic relationships among species according to Pérez *et al*.(2006) and the major ecological changes regarding shift from bee (b) to hummingbird (h) and moth (m) pollination, the appearance of autonomous selfing (a) and the loss of the explosive pollen discharge mechanism (grey lines). B-C) Floral morphology of the lower lip showing the keel (black arrows) and the wings (red arrows) for the extended B) and reduced lower lip C). D-H) Floral morphology of open flowers showing the upper (*:banner, x: lateral sections) and lower lips (white arrow: keel, red arrow: wings) D) *S. tricolor*, E) *S. hookeri*, F) *S. coccineus*, G) *S. integrifolius* and H) *S. lacteus*.
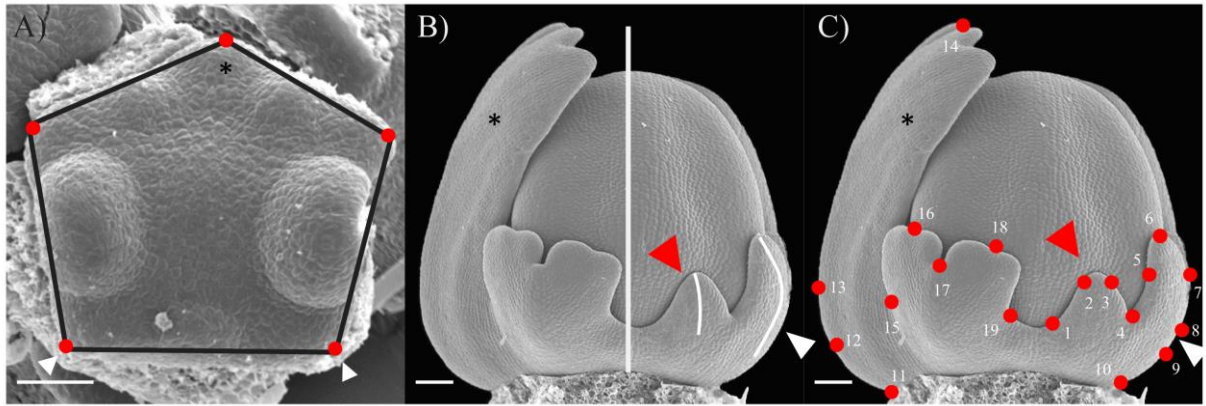
Figure 2: *Morphogenetic measurements*. A) meristem size measured at early development, B) bud size measured at late development, C) wing and keel sizes measured at late development and D) landmarks used in the morphometric analysis. For reference, the banner is indicated by an asterisk (*), the lower lip primordia and the keel by a white arrow and the wing by a red arrow. Scale bar = 50 μm in all images.

Figure 3: *Early stages for all species studied*. Stage 1: sepal appearance (showing the early initiation of the abaxial sepal). Stage 2: petal appearance. Stage 3: stamen appearance. Stage 4: staminode appearance. Stage 5: gynoecium appearance. Scale bars for all pictures: 50 μm.
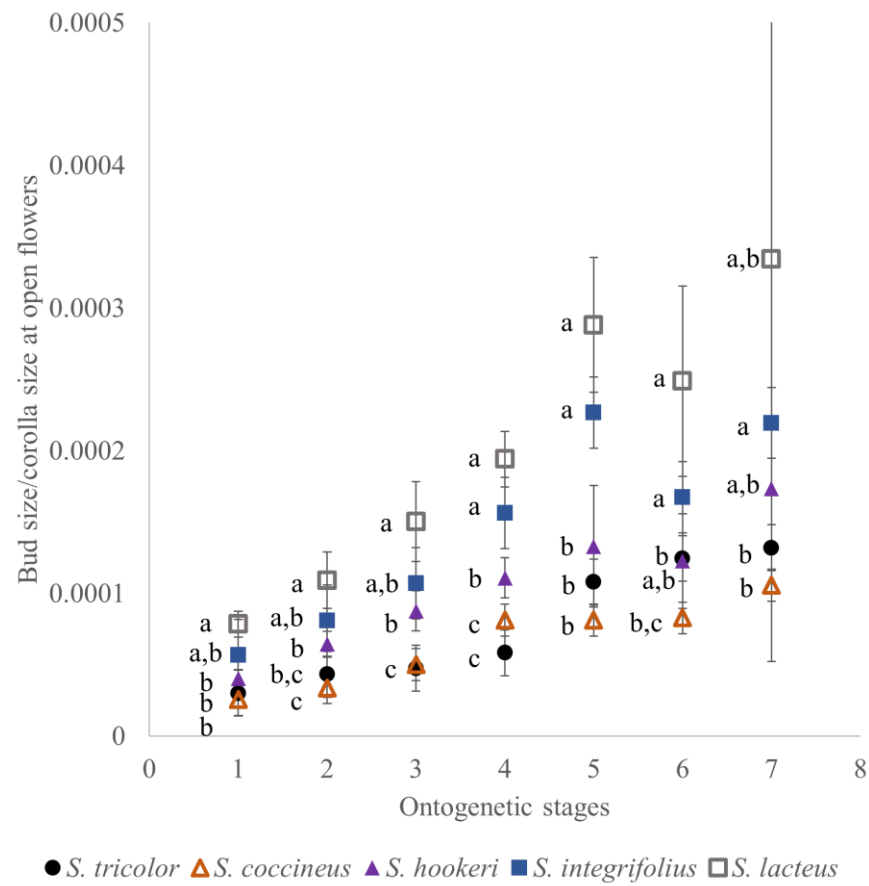
Figure 4: Quantitative comparison of growth during early development among species. Ontogenetic stages describe the appearance of 1) sepals, 2) petals, 3) stamens, 4) staminode and 5) gynoecium. Letters show the significant differences among species at each stage.

A)

B)
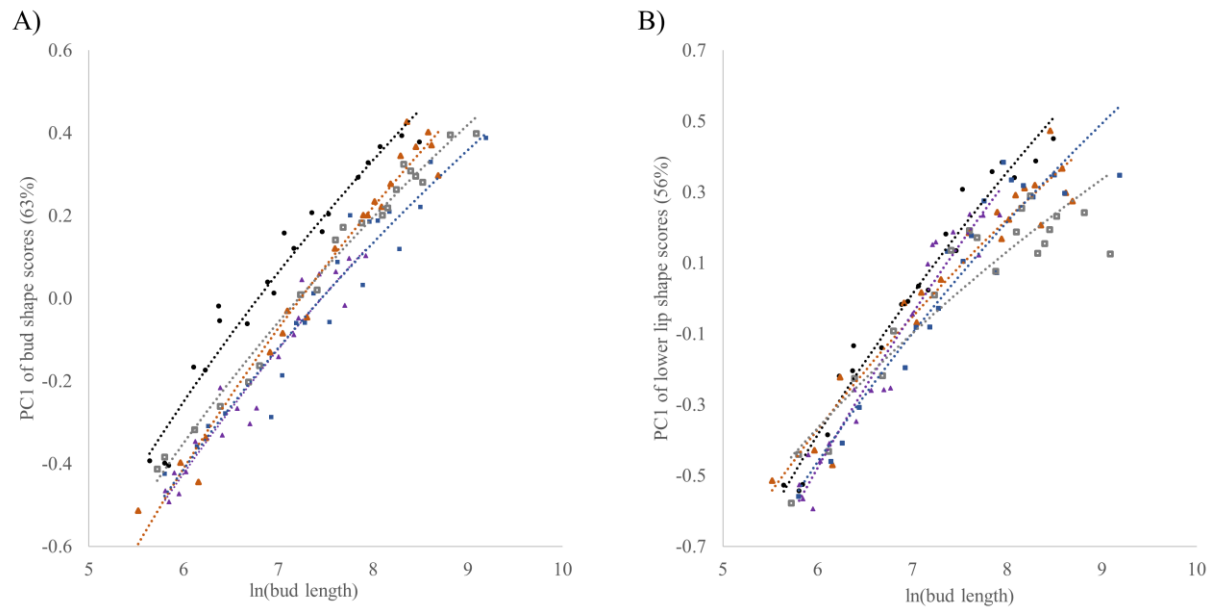
Figure 5: Shape trajectories related to size for the entire bud shape A) and for the lower lip shape B).

Figure 6: *Late ontogeny stages for the wings*. Stage 1: no wings. Stage 2: wing initiation. Stage 3: firs angle. Stage 4: second angle, Stage 5: elongation. Stage 6: final form. For detailed description of the stages, refer to the text. Scale bars: *S. tricolor*, stages 1-5: 50 μm, stage 6: 100 μm; *S. coccineus* and *S.hookeri*, stages 1-3: 50 μm, stages 4-6: 200 μm; *S. integrifolius* and *S. lacteus*, stages 1-4: 50 μm, stages 5 and 6: 200 μm.
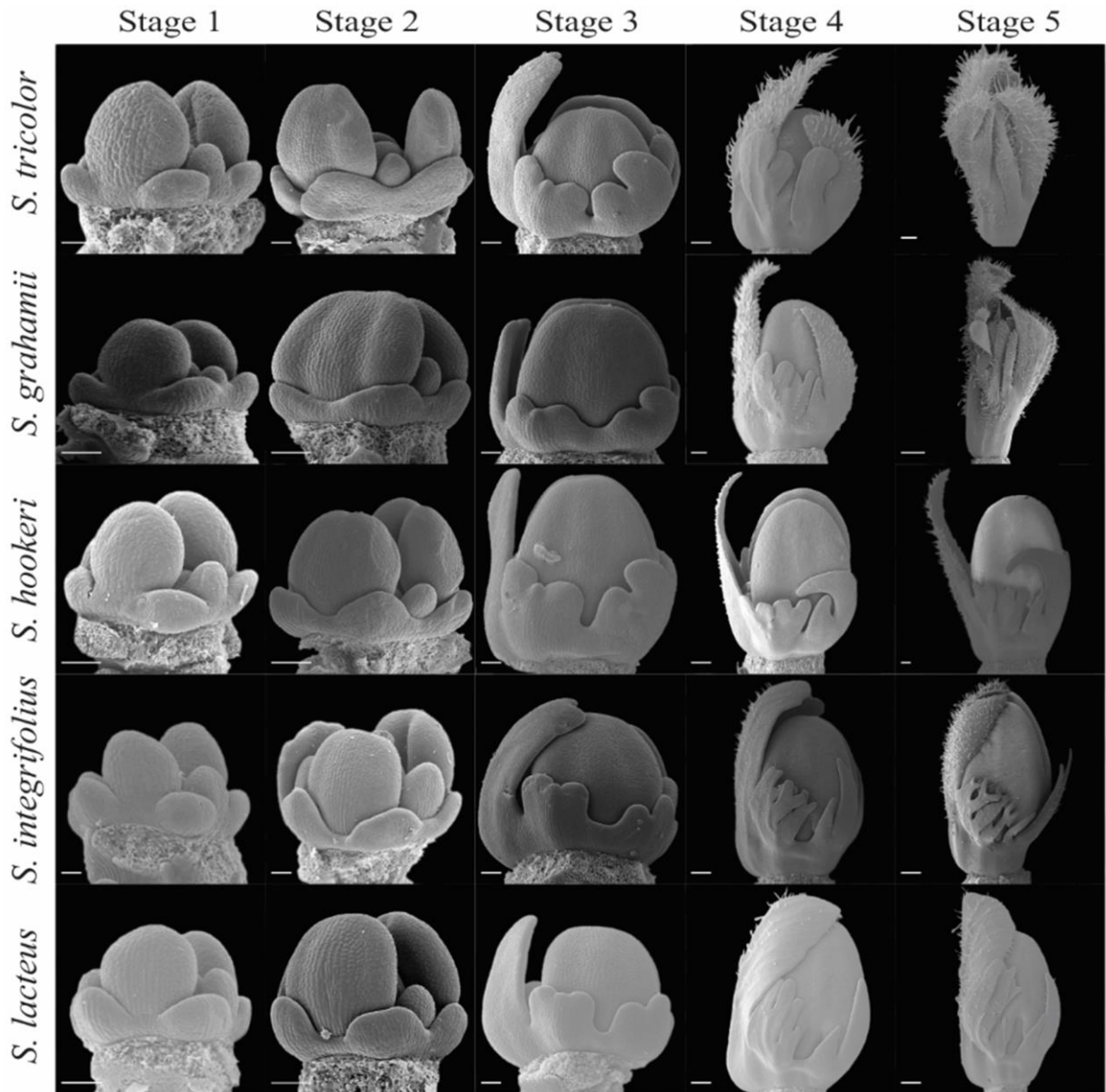
Figure 7: *Late ontogeny stages for the keel*. Stage 1: unfused keel, Stage 2: fused keel, Stage 3: angle, Stage 4: elongation, Stage 5: final form. For detailed description of the stages, refer to the text. Scale bars: *S. tricolor*, stages 1-3: 50 μm, stage 4: 100 μm, stage 5: 200 μm; *S. coccineus*, stages 1-3: 50 μm, stage 4: 200 μm, stage 5: 300 μm; *S.hookeri* stages 1-3: 50 μm, stages 4 and 5: 100 μm; *S. integrifolius* and *S. lacteus*, stages 1-3: 50 μm, stages 4 and 5: 200 μm.
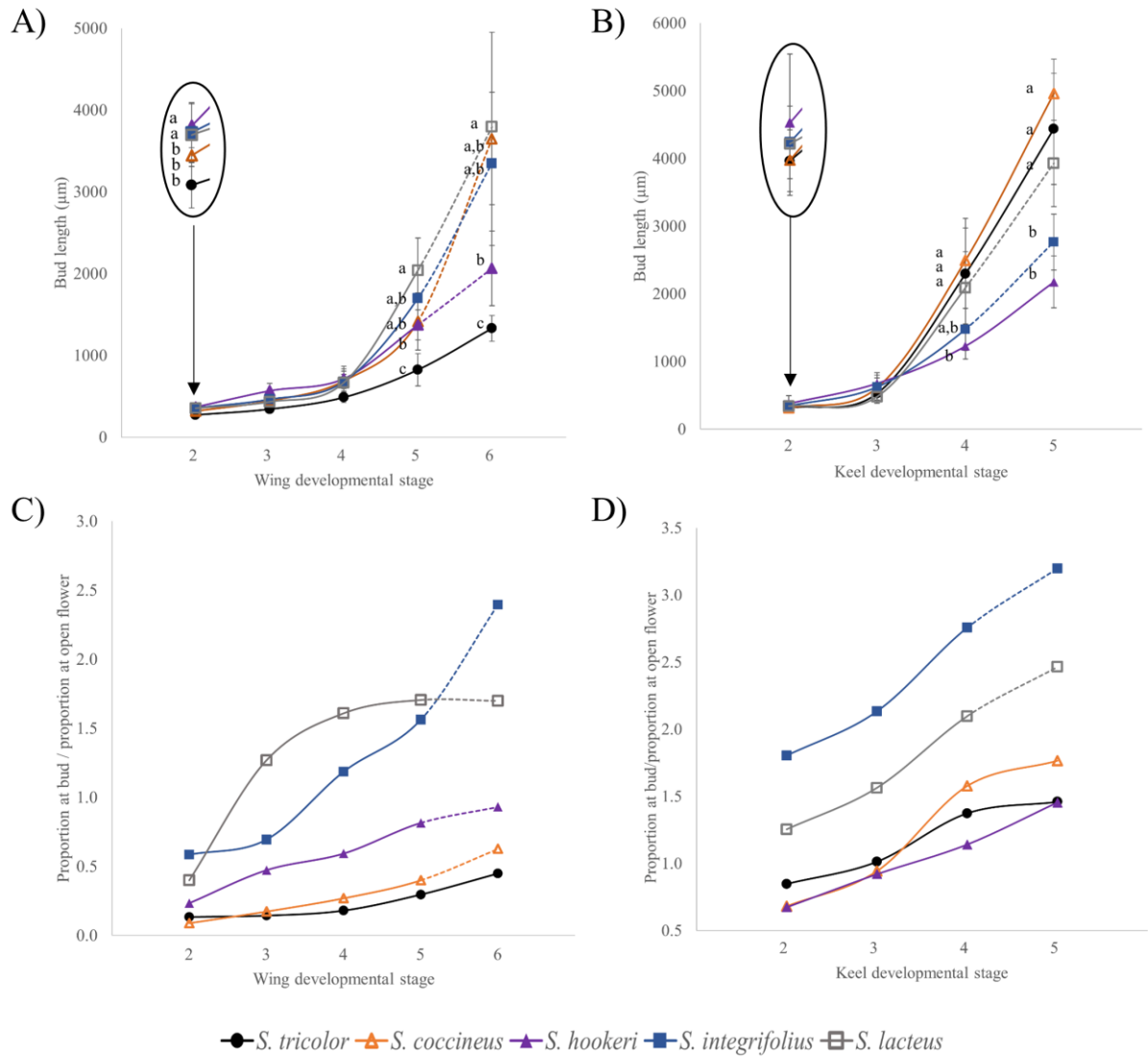
Figure 8: Quantitative comparison of growth dynamics of the wings A,C) and the keel B,D).

Bud length at which each developmental stage is reach for the wings A) and the keel B). Relative

size of the wing C) or keel D) at which each developmental stage is reach.
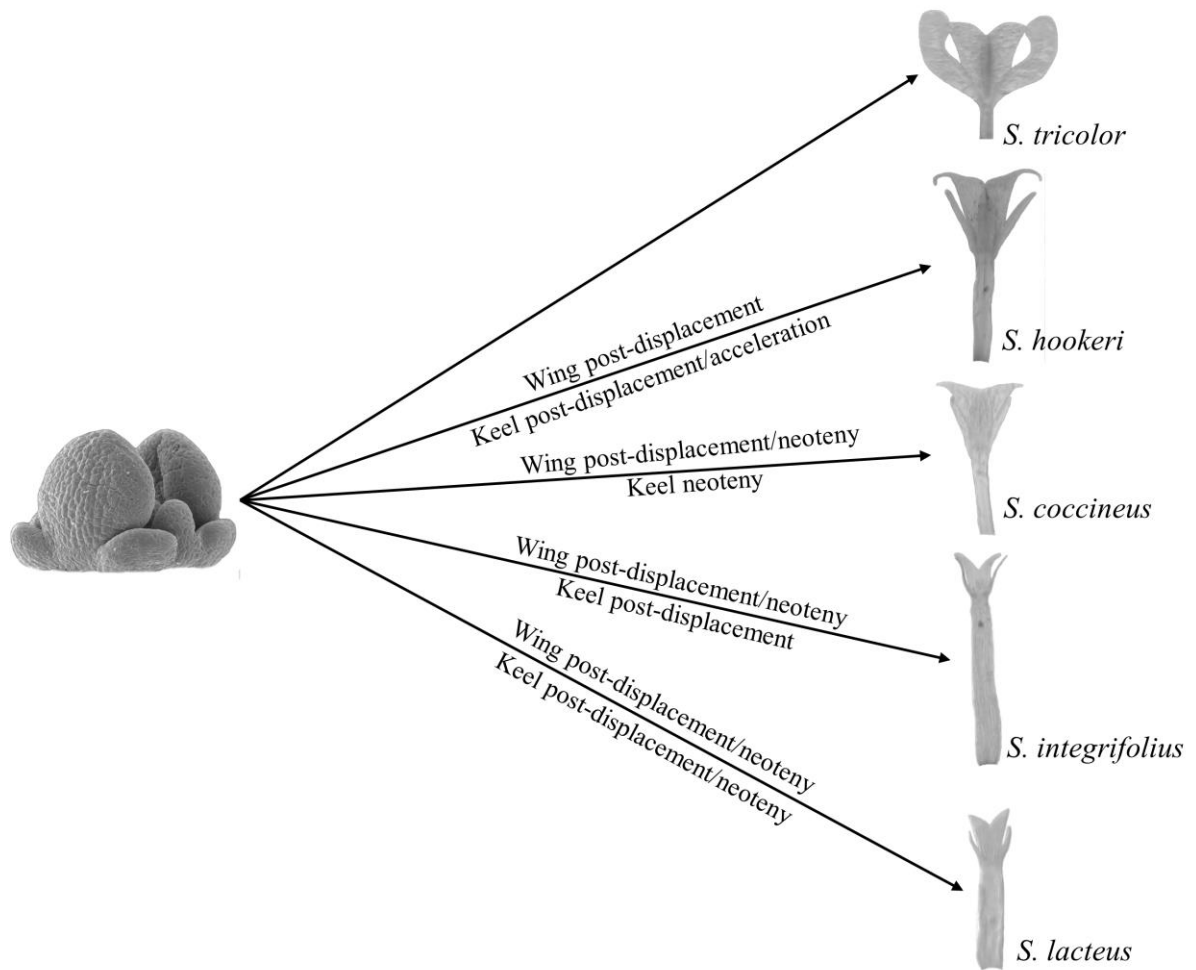
Figure 9: *Ontogenetic process underlying heterochronic shifts in pollinator-driven morphological evolution in Schizanthus*. The ancestral lower lip morphology is represented by *S. tricolor*. Lower lip reductions are reached by different ontogenetic process in the Andean clade (*S. hookeri* and *S. coccineus*) and in the desert clade (*S. integrifolius* and *S. lacteus*). The desert clade present strong size and shape reduction probably associated with changes in early ontogeny (the onset of the organ).

CONCLUSIONS

Comparative quantitative analysis of changes in size and shape of flower organs along ontogeny depicted how integration, allometry, and heterochrony are related among them and with pollinator-driven diversification in the *Schizanthus* genus.

1. Does ontogenetic integration and allometry vary through development and among species?

Flower morphological integration varied along ontogeny and among species, occurring at discrete stages of ontogeny. This tendency was found considering either a two-stage division of ontogeny (early and late) or a rolling moving approach with no a priori definition of ontogenetic stages. However, in the former case, changes occurring at a narrower range of bud sizes (that the one considered for each ontogenetic stage) might be obscured due to its underrepresentation in the data set. Accordantly, when integration was measured using a rolling-moving approach, there was a broader diversity of integration changes along ontogeny and among species. Similarly, ontogenetic allometry also varied along ontogeny and among species. These changes were traced using a two-stage approach, resulting in changes during either early ontogeny or both early and late ontogeny, which varied among species. Overall, ontogenetic integration and allometry varied along ontogeny and among species. Consequently, considering a broad range of ontogenetic stages is key to describe integration and allometric dynamics along ontogeny and their change among species, which can occur at discrete events in ontogeny.

2. Are changes in ontogenetic integration congruent with changes of ontogenetic allometry in all species?

A close relationship between ontogenetic integration and allometry was expected, as these two parameters describe different aspects of growth dynamics. Accordantly, ontogenetic integration and allometry tend to vary coordinately along ontogeny and among species. Particularly, low integration events were related to a greater difference among the growth rates (allometric slopes) of each floral organ. However, this relationship varied among species, as low integration events correlated with different levels of variation in the allometric slopes of the traits among species. This evidence suggests that there is a complex biological relationship between these two parameters and that another biological process regulating these growth dynamics may exist (e.g., compensatory or additive effects).

3. How does changes in ontogenetic integration and allometry among species relate to pollinator-driven morphological diversification in the genus?

Changes in ontogenetic integration were congruent with patterns of pollinator-driver diversification in the genus: reduced structures that lost their functionality in the pollination process tended to be decoupled from the rest of the corolla. This pattern was found when integration was traced between developmental stages and when the rolling-moving approach was used, but in the latter, changes were found in a greater variation along ontogeny and among species. Similarly, allometric changes along ontogeny calculated in a two-stage approach were congruent with pollinator-driven morphological diversification either during early ontogeny (for lower lip structures) or during both early and late ontogeny (tube). Particularly, larger structures corresponded with greater ontogenetic allometric slopes.

Although patterns of ontogenetic integration and allometry were congruent with pollinator-driven diversification, the degree of change along ontogeny was congruent with adult morphologies only for ontogenetic allometry. For example, while more reduced structures in the open flower tend to have lower allometric slopes, they did not necessarily present a higher degree of decoupling. This evidence suggests again that the relationship between ontogenetic integration and allometry are more complex than previously thought. Finally, integration and allometry presented a shared ontogenetic tendency among species: traits that appeared later during development tended to be more decoupled and had higher allometric slopes during ontogeny. This ontogenetic pattern did not constrain pollinator-driven diversification, as it can be compensated by other changes of integration and allometry along ontogeny.

4. How does heterochrony contribute to the diversification of Schizanthus species?

Pollinator-driven morphological evolution in *Schizanthus* is closely related with transitions to paedomorphic and peramorphic morphologies. These transitions occurred by different combinations of ontogenetic processes along ontogeny (changes in the onset of traits and their posterior growth dynamics), which can present compensatory effects. Therefore, earlier changes in the timing of development does not necessarily produce a greater impact on adult morphologies. The combination of ontogenetic processes varied among and within clades, but also among lower lip structures. Particularly, different ontogenetic processes underlying wing reduction were found within clades, while ontogenetic processes underlying keel reduction were congruent within clades. Overall, quantitative analysis of heterochronic changes in a broad range of ontogenetic stages is key to disentangle the relative role of those ontogenetic processes shaping adult organ morphologies and their proportions in the open flower.

5. Which is the relationship between ontogenetic integration, ontogenetic allometry, and heterochrony changes underlying pollinator-driven morphological diversification in Schizanthus?

Changes in ontogenetic integration and allometry were measured in a range of ontogenetic stages equivalent to the ones measured at late ontogeny for heterochrony, showing congruent results among them. Particularly, neoteny playing a role in wing reduction in all derived species and post-displacement being the most common process underlying keel reduction are congruent with wings being more decoupled than keel in the integration study. This evidence suggests that integration patterns along ontogeny are more affected by changes in the growth dynamics (i.e., allometry changes) of the floral organs than by changes in their onset. In some species, this pattern is produced by the compensation of changes in the onset of a trait by following changes in their growth dynamics.

Altogether, ontogenetic integration, ontogenetic allometry, and heterochrony are interrelated processes of size and shape variation of organs along ontogeny. This interaction is more complex than expected by their mathematical relationship, as compensatory effects may occur along ontogeny. Thus, comparative quantitative analyses of shape and size along ontogeny are key to disentangle the relative role of ontogenetic changes and their relationship with pollinator-driven diversification.