



## Immunocompetence of breeding females is sensitive to cortisol levels but not to communal rearing in the degu (*Octodon degus*)



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### HIGHLIGHTS

- No evidence that communal rearing enhances female reproductive success and survival
- No evidence that communal rearing enhances offspring immunocompetence or survival
- Females with high fecal glucocorticoids (FGC) increased lymphocytes and monocytes
- Females with low FGC experienced increases in N:L ratios, neutrophils, and total IgG
- Immunocompetence of females is sensitive to FGC but not to communal rearing

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### ABSTRACT

One hypothesis largely examined in social insects is that cooperation in the context of breeding benefits individuals through decreasing the burden of immunocompetence and provide passive immunity through social contact. Similarly, communal rearing in social mammals may benefit adult female members of social groups by reducing the cost of immunocompetence, and through the transfer of immunological compounds during allonursing. Yet, these benefits may come at a cost to breeders in terms of a need to increase investment in individual immunocompetence. We examined how these potential immunocompetence costs and benefits relate to reproductive success and survival in a natural population of the communally rearing rodent, *Octodon degus*. We related immunocompetence (based on ratios of white blood cell counts, total and specific immunoglobulins of G isotype titers) and fecal glucocorticoid metabolite (FGC) levels of adults immunized with hemocyanin from the mollusk *Concholepas concholepas* to measures of sociality (group size) and communal rearing (number of breeding females). Offspring immunocompetence was quantified based on circulating levels of the same immune parameters. Neither female nor offspring immunocompetence was influenced by communal rearing or sociality. These findings did not support that communal rearing and sociality enhance the ability of females to respond to immunological challenges during lactation, or contribute to enhance offspring condition (based on immunocompetence) or early survival (i.e., to 3 months of age). Instead, levels of humoral and cellular components of immunocompetence were associated with variation in glucocorticoid levels of females. We hypothesize that this covariation is driven by physiological (life-history) adjustments needed to sustain breeding.

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### 1. Introduction

Group-living or sociality involves spatial and temporal proximity among individuals that results from the mutual attraction rather than from attraction to the same resource or physical condition [55,72].

Relevant attributes of sociality include group size, group stability, and the extent to which group members display cooperative or competitive interactions [16,72,100]. Thus, sociality is thought to increase with group size, but also with the extent to which group members cooperate to attain food, avoid predators, or rear their offspring, among other aspects [27]. Functionally, sociality is thought to evolve when fitness benefits, namely decreased predation risk, enhanced access to resources, or decreased thermoregulatory costs [19,28,55], outweigh inherent costs

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to group-living, including increased transmission of parasites, pathogens, and competition over resources [2,55]. While fitness benefits of sociality derived from ecological variation have been well identified, proximate mechanisms remain less understood [55].

Determination of proximate underpinnings to these fitness effects remain critical to develop more integrated research programs that facilitate new insights into the physiological causes and consequences of social variation [8]. In particular, it is important that we understand the impact of immunocompetence – the physiological ability of individuals to develop an immune response following exposure to an antigen – on the reproductive success of social species. Biomedical studies on lab or domesticated rodents generally support that social conditions (e.g., individually versus group housing) influence immunocompetence [4]. For instance, it is well known that individuals experiencing low ranking status when housed in groups generally suppress immunocompetence, a time-dependent effect mediated by the stress response [4,83]. In contrast, the influence of other aspects of group-living on immunity such as cooperative behavior remains less understood.

Compared with laboratory species, few studies in natural populations of social species have examined how immunocompetence varies within and across social groups and how this influences reproductive success and survival [4,37]. This lack of information limits our ability to establish the extent to which immunocompetence is a driver of the evolution of sociality or the consequence of challenges operating within groups [48,91]. Achieving this goal has been complicated by the fact that a connection between sociality and immunocompetence is not straightforward [10,41,103]. On the one hand, horizontal pathogen transmission within groups is thought to increase in social species as a result of relatively more frequent physical contact, shared use of space, crowding, and the build-up of waste products [15,18,48]. This hypothesis is generally supported by the observation that, across studies and species, prevalence of contact-borne pathogens and parasites increases with the size of host social groups [15,77], and that greater investment in immunocompetence is associated with individuals in larger (or more cooperative) social groups, presumably to counteract these costs [63,64,68,88,92]. An alternative view is that individuals in social groups reduce their risk of horizontal pathogen transmission through decreasing contact with members of other social groups (i.e., a condition referred to as “social clustering”; [103]). This hypothesis is supported indirectly by within [11] and among [45,98] species comparisons documenting negative or no association between prevalence of pathogens and parasites and the size of host social groups. More direct support comes from across species comparisons reporting lower investment in immunocompetence in more social hosts [85,103]. Taken together, both theoretical and empirical evidence support a complex association between pathogen transmission and sociality, modulated by the parasites' main mode of transmission (e.g., contact-borne vs. mobile vector-borne), or by the extent of social clustering [10,103].

Several social mechanisms (referred collectively to as “social immunity”) have been suggested to reduce the burden of individual immunocompetence, including antimicrobial secretions, socially transmitted immune compounds, hygienic behavior, or mutual grooming [17,18,59,69,71]. This possibility has been supported by single species studies in bumble bees (*Bombus terrestris*) and termites (*Zootermopsis angusticollis*), two eusocial insects where social contact enhances survival and ability to resist infection [54,91]. The social immunity hypothesis also is supported by a comparative study where more social species of thrips exhibit higher antimicrobial strength [92]. For other social insects however, some components of individual immunocompetence have been shown to decrease while others decrease with group size [81]. Evidence from social vertebrates is meager on this point. Similar to eusocial insects, some mole-rats are singularly breeding rodents in which most group members delay breeding to help raise the offspring of breeders [61]. Immunocompetence based on spleen mass has been shown to be similar in breeding and non-breeding Natal mole-rats (*Cryptomys hottentotus natalensis*), suggesting that help from non-

breeders allows breeders to invest in their own immunocompetence [60]. Natal mole-rats from larger colonies decrease their metabolic costs and parasite abundance, implying energy savings from social living can be diverted into parasite defense [61]. Most intriguingly, immunocompetence based on the phytohemagglutinin-P test has been shown to increase with the number of nonbreeders (helpers) in singularly breeding magpies, suggesting a social effect on immunity [93]. Taken together, available studies on insects support social effects in terms of enhanced immunocompetence, yet different components of individual immunocompetence may be affected differently. Results from the few studies conducted on singularly breeding mammals and birds are consistent with immunocompetence benefits derived from social living, yet evidence remains largely indirect.

In contrast to singular breeders, most group members of plural breeders produce offspring [86]. In species that rear offspring in communal litters, females may nurse non-filial offspring, a form of parental care referred to as allonursing [42,79]. Allonursing may provide different benefits, including an enhancement of repertoire and total amount of immunoglobulin and immune cells available to offspring in colostrum and milk [3,80]. As a result, offspring raised in groups with more females may benefit through enhanced passive immunity and from increased early survival. However, producing better quality offspring in terms of ability to defend from pathogens may come at a cost to breeding females. Females may be required to invest more heavily in individual immunocompetence to provide offspring with immunoglobulins and immune cells, and possibly to defend them from enhanced pathogen transmission from allonursing [80]. Additionally, maternally transmitted immunoglobulins may indirectly enhance or inhibit the offspring's humoral immunocompetence [41]. Thus, it is far from clear how sociality and communal rearing may be beneficial to adult group members and their offspring in communally rearing mammals.

### 1.1. Model species and hypothesis predictions

Based on previous theory and empirical evidence we examined three hypotheses that are pertinent to degus (*Octodon degus*) as a study model. Degus are diurnal, herbivorous, and social rodents where multiple adult male and female group members share underground nests [31,43]. All female members of groups rear their litters communally [31], engaging in several forms of communal care, including huddling, retrieving and nursing non-descendent offspring [30,32,33,50]. In contrast, male degus huddle over and groom the pups, yet these direct forms of care have no fitness consequences to the offspring [33]. These observations suggest that communal rearing may benefit adult female degus directly through decreasing the burden of individual immunocompetence. Under these conditions, females may divert these savings into other maintenance or reproductive functions. Conversely, females exhibiting greater immunocompetence may be less able to attain such savings, and thus, less capable to divert them into fitness enhancing processes. On the other hand, two observations seem preliminary consistent with the non-mutually alternative hypothesis in which communal rearing and total group size benefit adult female degus indirectly through their offspring by means of enhancing offspring immunocompetence and survival. First, females transfer immunoglobulins to their own offspring during pregnancy and lactation [6]. Second, multiple female and males communally nest during lactation [34,43], implying that offspring are exposed to pathogens and parasites from contact with all adults, including males and females. Finally, we considered hypothesis 3 in which the relatively low inter-year survival of degus resulted in an “all or none” strategy and where any social effects on communal rearing would be weak or absent. We examined eight predictions relevant to validate these hypotheses (Table 1).

**Table 1**

Hypothesis and predictions examined. We used “Not relevant” to highlight predictions not critical to a particular hypothesis.

Prediction	Hypothesis		
	(1) Communal rearing decreases the burden of individual immunocompetence of breeding females	(2) Communal rearing enhances offspring immunocompetence and survival	(3) Degus use an “all or none” strategy where social effects on immunocompetence are weak or absent
(i) Association between the number of breeding females and adult female immunocompetence	Negative	Not relevant	No association
(ii) Association between female immunocompetence and per female offspring produced (reproductive success)	Negative	Not relevant	No association
(iii) Association between female immunocompetence and female survival immunocompetence	Negative	Not relevant	No association
(iv) Association between group size and offspring immunocompetence	Not relevant	No association	No association
(v) Association between the number of breeding females and offspring immunocompetence	Not relevant	Positive	No association
(vi) Association between female and offspring immunocompetence	Not relevant	Positive	Positive
(vii) Association between female immunocompetence and offspring early survival	Not relevant	Positive	Positive
(viii) Association between offspring immunocompetence and offspring early survival	Not relevant	Positive	Positive

## 2. Materials and methods

### 2.1. Study population

The study was conducted between 2009 and 2011 on a natural population of degus located at the Estación Experimental Rinconada de Maipú (33°23' S, 70°31' W), a field station of Universidad de Chile. This study area is characterized by a Mediterranean climate with cold, wet winters and warm, dry summers [26]. The site consisted of open areas with scattered shrubs (*Prostris pungens*, *Acacia caven*, and *Baccharis* spp.) that on average covered 14.5% of ground [29].

### 2.2. Determination of social groups

Social groups were determined in June–July (winter, mating time) and in September through October (a time encompassing parturition, lactation, and offspring weaning) of 2009, 2010, and 2011. Degus are diurnally active and remain in underground burrows overnight [31]. Thus, the main criterion used to assign degus to social groups was the sharing of burrow systems during night time. The sharing of burrow systems was established by means of (i) night-time telemetry, and (ii) burrow trapping in August–October. During burrow trapping, a burrow system was defined as a group of burrow openings surrounding a central location where individuals were repeatedly found during night time telemetry and usually spanning 1–3 m in diameter [38,44]. Eight traps (Tomahawk model 201, Tomahawk Live Trap Company, Tomahawk, Wisconsin, USA) were used per day at each burrow system. The total area examined at Rinconada was nearly 2 ha and did not vary across seasons or years of study.

Traps were set prior to the emergence of adults during morning hours (06:00 h). After 1.5 h, traps were closed until the next trapping event. The identity, location, sex, body mass (to 0.1 g) of all degus and reproductive condition of all caught females (perforated, pregnant, or lactating) was determined. Every degu was marked at the time of first capture with same ID coded tags on each ear (Monel 1005-1, National Band and Tag Co., Newport, USA). Adults weighing greater than 170 g were fitted with 6–7 g (BR radio-collars, AVM Instrument Co., Colfax, California, USA) with unique pulse frequencies. Analyses based on 2008–2011 data from the Rinconada population indicate radiocollars do not decrease physical condition or survival (L.A. Ebensperger unpublished results).

During night-time telemetry, females were radio-tracked to their burrows. Previous studies at Rinconada confirmed that night time locations represent nest sites where degus remain underground [31]. Locations were determined once per night approximately 1 h before sunrise

using an LA 12-Q receiver (for radio collars tuned to 150,000–151,999 MHz frequency; AVM Instrument Co., Auburn, California, USA) and a hand held, 3-element Yagi antenna (AVM instrument Co., Auburn, California, USA). The number of burrow systems monitored, the number of days that each burrow system was trapped, the number of radiocollared degus, and the number of nighttime telemetry locations per radiocollared degu per season and year of study are given in Table S1 of Electronic supplement. This effort has been shown to be sufficient in determining group membership [34,35,43].

The determination of group composition during each study year required the compilation of a symmetric similarity matrix of pairwise association of the burrow locations of all adult degus during trapping and telemetry [101]. The association (overlap) between any two individuals was determined by dividing the number of early mornings that these individuals were captured at or tracked with telemetry to the same burrow system, by the number of mornings or evenings that both individuals were trapped or tracked with telemetry on the same day [31]. To determine social group composition, a hierarchical cluster analysis of the association matrix in SOCPROG software [102] was conducted. The fit of data was confirmed with the cophenetic correlation coefficient, a correlation between the actual association indices and the levels of clustering in the diagram. In this procedure, values above 0.8 indicate that hierarchical cluster analysis has provided an effective representation of the data [101]. The maximum modularity criterion [66] was used to cut off the dendrogram and define social groups.

### 2.3. Assessment of immunocompetence

The adaptive immune system of rodents comprises the activation of antigen-specific T and B lymphocytes, which confer cellular and humoral (antibodies) immunity, respectively [24,95]. The antibody-mediated immune response is initiated during the first exposure to an antigen and is characterized by the slow secretion antibodies of the IgM isotype and the generation of specific memory B-cells, when the antigen has a protein nature. During a second exposure to the same protein based antigen, a rapid secondary, memory response occurs, predominantly in the form of specific IgG antibodies, the principal isotype found in the blood [23]. Subsequent immunizations raise specific titres of antibodies by specific memory B cells. Thus, we recorded changes in cellular and humoral immune components of adult females that were previously challenged with a novel antigen, an approach recommended for field settings [24]. We selected a mollusk (*Concholepa concholepa*) hemocyanin (CCH) because these proteins are strongly immunogenic natural antigens, which make them ideal for long-term repetitive immunizations, eliciting cellular and humoral responses in vertebrates without

known toxic effects [22]. The ability of animals to develop an immune response following exposure to hemocyanin is indicated by the production of specific antibodies against hemocyanins. Due to its superior solubility and stability [21,47], CCH has replaced the hemocyanin from the traditionally used keyhole limpet hemocyanin (KLH) from *Megathura crenulata*.

IgG levels and white blood cell counts were used to quantify immunocompetence because both can be determined from blood samples, a procedure compatible with field studies. Components of humoral immunocompetence included levels of total natural IgG in the sera, and specific IgG titers against CCH. Counts of white blood cells included neutrophils and monocytes both of which have roles during the innate immunological response. Neutrophils and monocytes are phagocytic cells that increase in circulation in response to bacterial and protozoan infections [7,20]. Lymphocytes play a major role during the adaptive immunological response [24,95], and circulating levels of these cells are indicators of investment in immunocompetence [7].

One potential limitation of our procedure is that elevated antibody levels may reflect recent sickness or pathogen exposure instead of baseline measurements [7,24,67]. To counteract these limitations, we used a longitudinal, prospective analysis that span over three years. Regarding the use of white blood cell counts, these may also be affected by acute stress [7,95]. While we were unable to prevent degu subjects from experiencing acute stress from restraint while inside the traps, we made an effort to avoid a bias due to time inside traps. Blood samples from captured degus were obtained randomly with respect to burrow locations (i.e., social group), or sex.

While degu females are known to provide offspring with both IgG (through the placenta and lactation) and IgA (through lactation) [6], we monitored IgG exclusively. Given that IgA is typically transferred almost exclusively during early lactation [13], this immunoglobulin would not be detectable in sera when offspring are first trapped (i.e., after weaning). Thus, levels of IgG are likely to provide a more inclusive measure of immunocompetence than IgA at this time. Due to the impossibility of quantifying the level of total IgG in the sera of each degu, we used the total IgG titer and its correlation with specific anti-CCH IgG titer to evaluate an overall state of immunological competence [13,95]. This allowed us to discriminate whether an eventual lack of response to hemocyanin in a degu occurs as an antigen specific immunodeficiency or represents an immunocompromised individual. Yet, all degus examined so far responded to CCH [6].

#### 2.4. Source of hemocyanin and immunization schedule

Hemocyanin from *Concholepas concholepas* in PBS (0.1 M sodium phosphate, 0.15 M NaCl; pH 7.2), purified under sterile and pyrogen-free conditions was provided by Biosonda Corporation (Santiago, Chile). All solutions were prepared using water for human irrigation (Baxter Healthcare Corp., USA) and filtered through a 0.02  $\mu\text{m}$  membrane filter (Millipore, USA).

At first capture of adults during the early austral spring of each year, a blood sample of 500  $\mu\text{L}$  was taken from the saphenous vein to determine baseline levels of immunocompetence measures (i.e., a control condition). Then, and for degus that were not immunized during previous years we took a pre-immune blood sample and were immediately immunized them with a 1 mg CCH /100  $\mu\text{L}$  PBS intraperitoneal injection, without additional adjuvants. These animals were similarly immunized a second time (2 mg CCH/100  $\mu\text{L}$  PBS) within 10–20 days (determined by recapture date) after first immunization. Within 10–20 days after second immunization, a blood sample (500  $\mu\text{L}$ ) was taken. Then, a booster was done (2.5 mg CCH/100  $\mu\text{L}$  PBS) to increase the specific anti-CCH IgG titers. After 10–20 additional days, a final blood sample was collected. These sera represented the secondary and tertiary immune responses, respectively. Degus that were immunized during the previous year were subjected to the same procedures, yet without the

first immunization. All blood samples were taken within 2 h of capture and between 9:00–11:00 h.

At first capture of offspring each year, a single blood sample of 300  $\mu\text{L}$  was similarly taken from the saphenous vein. All blood samples were transported to the laboratory, kept at 5 °C for 24–48 h, and then centrifuged at 3000 rpm during 10 min. The serum was extracted and stored at –20 °C until IgG determinations.

#### 2.5. Determination of white blood cells

We complemented our humoral immune analysis with measures of the cellular component of immunocompetence. Differential white blood cells were quantified from blood smears, and followed methods similar to those used by previous studies on small mammals [12,97], including degus [49]. Blood smears were prepared from a single drop of blood obtained from the saphenous vein of every degu. Samples were air dried, stained with the May–Grunwald–Giemsa dye, and then examined at magnification 450 $\times$  under a light microscope [49]. Cell types (lymphocytes, neutrophils, eosinophils, basophils and monocytes) were counted until the cumulative total reached 100 cells. Of these, eosinophils and basophils were very infrequent and were excluded from subsequent analysis. The use of basal white blood cell counts has been criticized on the basis that values may be influenced by numerous factors, including cell migration and circadian rhythms, which are not implicated in immunocompetence [95,99]. However, these measures are useful when measures examined are ratios calculated from animals that had been challenged with an exogenous antigen (i.e., combined with functional assessments) [24,95]. Under these circumstances, changes in white cell profiles before and after immunization estimate the readiness and ability of individuals to defend against pathogens and parasites.

#### 2.6. Determinations of total IgG

Total IgG titer was determined by an indirect enzyme-linked immunoabsorbent assay (ELISA). To complement our use of a crossed reaction between degu's and *M. musculus* IgGs, we developed an anti degu-IgG rabbit sera. The antiserum to degu IgG was obtained by repeated immunization of two New Zealand rabbits with highly purified pool of *O. degus* IgG and Freund's adjuvants (Thermo Scientific, USA). The purified degu IgG was prepared from a pool of whole sera of non-immunized degus by affinity chromatography with Protein A from Thermo Scientific [14]. The specificity of the antisera was assessed by Western blot [90]. The ELISA for IgG total was conducted as it follows: 96-well polystyrene plates (Thermo Scientific) were incubated overnight at 4 °C with 100  $\mu\text{L}$ /well of 10  $\mu\text{g}/\text{mL}$  solution of goat anti-rabbit IgG sera (Thermo Scientific) in PBS. Plates were blocked with 1% PBS-casein, during 2 to 3 h at room temperature and then incubated with rabbit anti-degu IgG sera diluted 1:1000 in 1% PBS-casein. After being washed with 0.02% PBS-Tween-20, serial two-fold dilutions of the sera of degu in blocking buffer were performed and incubated 3 h at 37 °C or over night at 4 °C. Plates were washed and then 100  $\mu\text{L}$ /well of goat anti-mouse IgG serum alkaline phosphatase (ALP) conjugated (Thermo Scientific) diluted 1/2000 in blocking buffer was added to the wells and incubated for 1 h at 37 °C. Then plates were washed and developed during 30 min at 37 °C by adding 100  $\mu\text{L}$  well of 1 mg  $\mu\text{L}$  pNPP in ALP-buffer ( $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$  0.2 M, pH 9.6). The reaction was stopped and read spectrophotometrically at 405 nm.

The titer of total IgG was defined as the reciprocal of the serum dilution showing half of the maximum absorbance at 405 nm. In all experiments the omission of the degu serum was used as negative control.

#### 2.7. Determination of anti-*Concholepas* hemocyanin antibodies

The specific antibody titer against the gastropod *C. concholepas*' hemocyanin was determined by an indirect ELISA, as previously reported [6], and minor modifications. Briefly, 96-well plates were incubated



overnight at 4 °C with 100  $\mu$ L/well of a solution of 10  $\mu$ g CCH in PBS. Plates were blocked with 200  $\mu$ L well of 1% PBS casein, during 2–3 h at room temperature. Then, serial two-fold dilutions of the immune sera of degu in blocking buffer were incubated for 3 h at 37 °C. Plates were washed with 0.02% PBS Tween-20, and then 100  $\mu$ L/well of rabbit anti-degu IgG sera diluted 1:1000 in 1% PBS-casein was added and incubated during 1 h at 37 °C. After being washed, goat anti-rabbit IgG serum ALP conjugated (Thermo Scientific) diluted 1/2000 in blocking buffer was added to the wells. After incubating for 30 min at room temperature, plates were washed and developed as described before. The titer specific IgG was defined as the reciprocal of the serum dilution showing half of the maximum absorbance at 405 nm. The omission of the degu serum was used as negative control during all these determinations.

## 2.8. Measures of immunocompetence

The ability of adult degus to mount an immune response or immunocompetence was quantified for every humoral (based on titers) and cellular (based on differential leukocyte counts) component as the ratio between values after second and prior to first (secondary response), and as the ratio between values after third and after second (tertiary response) immunization with CCH. Thus, ratios higher than unity would signal increases in a particular humoral or cellular component. We obtained these measures during the austral spring period in 2009, 2010 and 2011. To minimize discomfort to the developing young, offspring immunocompetence was based on titers and leukocyte counts (%) taken only once immediately after weaning during 2010 and 2011.

## 2.9. Glucocorticoids and immunity

Numerous endogenous and exogenous events may be stressful to organisms and induce changes on circulating glucocorticoid hormones, a major component of the hypothalamic–pituitary–adrenal axis [65]. These changes may be immunostimulating or immunosuppressive, depending upon the timing and length of time over which organisms continue to experience stressful conditions [62]. Thus, we quantified levels of fecal glucocorticoids to all adult females (in ng per g of feces), and used this measure as an estimate of adult female condition during our examination of immune response. We used a noninvasive fecal glucocorticoid analysis to extract fecal glucocorticoid metabolites (thereafter FGC) as a means of assessing secretion of cortisol in degus. Fecal samples were obtained from all adult females at first capture by placing Tomahawk traps on paper towels during processing. Thus, FGC measures were obtained immediately before measures of immunocompetence. Details on subsequent fecal pellet processing and quantification of FGC have been extensively described in Ebensperger et al. [36], an approach that has been validated by a previous study on degus [87].

## 2.10. Fitness measures

Three parameters were relevant components of fitness, including the number of offspring produced by females in each social group, offspring survival, and adult female survival.

### 2.10.1. Number of offspring weaned

The number of offspring weaned by each social group during spring (2009, 2010 and 2011) was determined by quantifying the number of offspring captured for the first time at active burrow systems used by social groups during burrow trapping in September–October. Per capita offspring weaned was determined by dividing the number of offspring captured at burrow systems by the number of breeding female group members known to live in groups using the burrow systems. This index has been used in the past as an estimate of direct fitness for plural breeding hystricognath rodents [56] and degus [34,43]. Most importantly, preliminary data from one year of study (2011) indicate that

measures of reproductive success based on demographic and microsatellite DNA estimates of maternity are correlated (L.A. Ebensperger & L.D. Hayes unpublished results).

### 2.10.2. Offspring survival

An index of offspring survival was calculated based on the recapture of offspring. We focused on offspring that were recaptured during the following austral summer (i.e., early survival). Burrow trapping conducted in January and June–July of 2010, 2011 and 2012 was used to estimate the number of offspring from the previous year that were alive in the area. The protocols used and the area sampled during trapping were identical to those used during September–October. The trapping effort per year is given in Table S1 of Electronic supplement. Given that dispersal in degus is not sex biased and animals settle relatively close to their burrows of origin (i.e., within 30–40 m; [75]), this index is likely to be a realistic estimate of survival. Per capita surviving offspring was determined by dividing the number of offspring previously assigned to a social group during spring and recaptured in autumn (at any burrow system) by the number of female group members of the social group in spring.

### 2.10.3. Adult female survival

An index of adult female survival based on the recapture of these females was determined. From these data we recorded the females that survived from spring (main lactation) to early summer, a relevant fitness component to degus as adult females may occasionally breed a second time within same year [36].

## 2.11. Statistical analysis

We first used Principal Component Analysis (PCA) to reduce the number of female measures of immunocompetence to a single, main dimension [96]. Secondary and tertiary female response variables were reduced separately. Accordingly, we defined female secondary immunocompetence PCA and female tertiary immunocompetence PCA as the score values of first axis of their corresponding PCA. Regarding offspring immunocompetence, all measures were reduced to produce a single offspring immunocompetence PCA. All forthcoming analyses aimed to test predictions included these PCA axes as proxies for immunocompetence modeled either as response or predictor variables.

Analysis 1 aimed to determine the effect of number of breeding females (i.e., communal rearing) on adult female immunocompetence. Adult female immunocompetence was analyzed using a linear mixed effects model [104]. The response variables were the female secondary (Model 1.1) or tertiary (Model 1.2) immunocompetence PCA. In both cases, the fixed component of the model included the variables group size, number of females, and FGC. However, initial inspection indicated that group size did not explain any variation, and so this predictor was discarded from the final full model. The full model included group identity as the random component of the model. Year of study was not included as a random component based on preliminary analyses that showed no effects on model fit. The full model was then compared with an intercept only model that also included group identity as the random component. The model comparison was performed with the use of likelihood ratio test [51].

During Analysis 2 we used linear mixed effects model to determine how group identity (the random component), female FGC, female secondary (model 2.1) or tertiary (model 2.2) immunocompetence PCA as fixed effects, influenced per female number of offspring produced. An intercept only with group identity as the random component was used as the null model for hypothesis testing through likelihood ratio test.

We used a generalized linear mixed effects model (GLMM; [104]) to determine how group identity (the random component), female FGC, female secondary (model 3.1) or tertiary (model 3.2) immunocompetence PCA influenced female survival to summer (Analysis 3). The distributional family for this response variable was Binomial. Preliminary results suggested that female FCM did not contribute to model fit, and

was therefore discarded as predictor. Hypothesis testing was performed using the likelihood ratio test.

In Analysis 4 we used linear mixed effects model to examine how offspring immunocompetence PCA relates to offspring body mass, group size, number of females, and female secondary (model 4.1) or tertiary immunocompetence (model 4.2) PCA as predictors. We removed the number of females from model 4.2 to allow model convergence. Similar to previous analyses, group identity was used as the random component of the model. Hypothesis testing was done comparing the full model against the intercept only (plus random component) null model, using the likelihood ratio test.

During Analysis 5 we examined whether offspring survival to summer was related to offspring body mass, offspring sex, and offspring immunocompetence PCA with the use of a GLMM approach. Group Identity was the random component. The response variable was assumed to follow a Binomial distribution. Hypothesis testing was performed using likelihood ratio test of this model against the intercept only model.

We used a GLMM approach to examine how offspring body mass, offspring sex, and female secondary (model 6.1) or tertiary (model 6.2) immunocompetence PCA, predicted offspring survival (Analysis 6). The response variable was declared as Binomial, and group identity was used as a random component. The intercept only model was used as the null expectation to test against the full model by means of likelihood ratio test.

For all models described previously, a Maximum Likelihood approach was used to estimate model parameters for hypothesis testing purposes. However, final models are presented with parameter values estimated using restricted maximum likelihood (REML; [52]) because this method is considered to perform better as the Best Linear Unbiased Estimator (BLUE; [78]). All analyses were done in R statistical environment using libraries lme4 [5] and nlme [74]. Titer analyses were conducted with the GraphPad Prism software (GraphPad Software, La Jolla, California, USA).

### 3. Results

#### 3.1. Descriptive data

Overall, 204 adults and 181 offspring were monitored between early 2009 and early 2012. Of these, 97 adults had sufficient trapping and telemetry records to be identified as members of social groups. The number of breeding females per social group ranged from 2 to 5 in 2009 ( $n = 11$  groups), 1 to 2 in 2010 ( $n = 6$  groups), and 1 to 3 in 2011 ( $n = 16$  groups). The number of female offspring per social group ranged from 1 to 14 in 2009, 1 to 4 in 2010, and 0 to 10 in 2011. The number of male offspring per social group ranged from 1 to 16 in 2009, 1 to 5 in 2010, and 0 to 12 in 2011.

Measures of immunocompetence based on the secondary and tertiary response to *Concholepas* hemocyanin were recorded for a subset of adult female group members, a sample determined largely by our ability to recapture animals during sufficiently short and similar time intervals. In total, we collected data from 17 adult females from 11 social groups monitored in spring 2009 (58% of group members), 4 females from 6 groups in spring 2010 (58%), and 9 females from 16 groups in spring 2011 (50%). Mean ratios ( $\pm$  SE) of immunocompetence measures recorded to adult females are given in Table 2. Measures of immunocompetence (based on IgG titers and cell counts) were available for 34 offspring in 2010 (71% of offspring assigned to groups), and 77 offspring in 2011 (71%). Similarly, means ( $\pm$  SE) of offspring immunocompetence are included in Table 2.

#### 3.2. Principal Component Analysis performed on adult female and offspring immunocompetence

The Principal Component Analysis (PCA) performed on female secondary immunological responses revealed that 52% of variation was

**Table 2**

Means ( $\pm$  SE) of immunocompetence measures (based on secondary and tertiary responses to *Concholepas* hemocyanin) of adult females and offspring across years of study. Data come from 17 adult females from 11 social groups monitored in spring 2009, 4 females from 6 groups in spring 2010, and 9 females from 16 social groups in spring 2011. Measures of offspring immunocompetence come from 34 offspring in 2010 and 77 offspring in 2011.

Measure of immunocompetence	Secondary response (ratio)	Tertiary response (ratio)
<i>Adult females</i>		
Total IgG ratio	2.1 $\pm$ 0.7	3.2 $\pm$ 2.2
Anti-CCH IgG ratio	19.0 $\pm$ 6.2	6.8 $\pm$ 3.1
Neutrophil ratio	1.0 $\pm$ 0.03	1.0 $\pm$ 0.1
Lymphocyte ratio	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1
Monocyte ratio	2.3 $\pm$ 1.1	2.1 $\pm$ 0.9
Neutrophil to lymphocyte ratio	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2
<i>Offspring</i>		
	Titer or cell count (%)	
Total IgG titer	516.4 $\pm$ 62.7 titer	
Neutrophil count	45.4 $\pm$ 1.5%	
Lymphocyte count	50.1 $\pm$ 1.5%	
Monocyte count	4.1 $\pm$ 0.2%	
Neutrophil to lymphocyte ratio	1.1 $\pm$ 0.1	

accounted for by the first major axis (Table S2 of Electronic supplement). The second axis explained only an additional 19% of variation and was discarded from further analyses. An examination of PCA loadings indicated that positive values of first major PCA axis were associated with individuals where lymphocyte and monocyte ratios increased more than all other measures of immunocompetence. In contrast, negative values were associated with animals where neutrophil to lymphocyte, neutrophil, and total IgG ratios increased more than all other measures of immunocompetence. The Principal Component Analysis (PCA) performed on female immunocompetence measures based on the tertiary response revealed a similar trend in which 53% of variation was accounted for the first major axis (Table S3 of Electronic supplement). Examination of PCA loadings indicated that positive values of first major PCA axis were associated with an increase in lymphocyte, anti-CCH IgG, and total IgG ratios. Instead, negative values were associated with greater neutrophil and neutrophil to lymphocyte ratios.

The Principal Component Analysis (PCA) performed on offspring immunocompetence variables revealed that 42% of variation was accounted for by the first major axis (Table S4 of Electronic supplement). The second axis explained an additional 25% of variation and was discarded from further analyses. An examination of PCA loadings indicated that positive values of first major PCA axis were associated with individuals where neutrophil and the neutrophil to lymphocyte ratios increased more than all other measures of immunocompetence. In contrast, negative values were associated with animals exhibiting relatively higher lymphocyte, monocyte and total IgG titer ratios.

#### 3.3. Predictions (i): association between the number of females and female immunocompetence

Female immunocompetence PCA based on measures that were part of the secondary immune response was not affected by the number of breeding females (Model 1.1, Table 3). Instead, female immunocompetence PCA was affected by FGC (Model 1.1, Table 3), and where females with higher FGC exhibited more positive PCA values (Fig. 1). Thus, females with higher FGC showed an increase in lymphocyte and monocytes after exposure to CCH, a protein based antigen. Females with lower FGC exhibited an increase in neutrophils and neutrophil to lymphocyte ratios. Female tertiary immunocompetence PCA was not affected by any of the predictors examined (Model 1.2, Table S5 of Electronic supplement).

**Table 3**

Linear mixed model results for adult female secondary immunocompetence PCA. The model fit section includes Akaike (AIC) and Bayesian (BIC) information criteria, and the likelihood ratio test.

Model fit						
Model	DF	AIC	BIC	Log likelihood	Likelihood ratio	p-Value
Null model	3	112.01	115.89	−53.00		
Model 1.1	5	110.09	116.57	−50.04	5.92	0.05
Model fixed effects						
Source of variation		Estimate	Standard error	DF	t-Value	p-Value
Intercept		−3.70	1.45	19	−2.56	0.02
Number of females		0.07	0.35	19	0.19	0.85
Fecal glucocorticoid metabolites (FGC)		0.02	0.01	5	3.08	0.03

### 3.4. Prediction (ii): association between female immunocompetence and per female offspring produced (reproductive success)

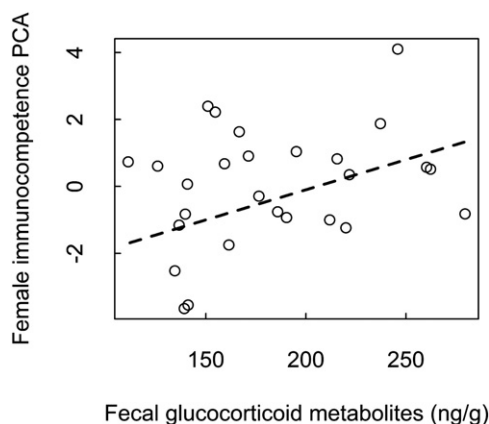
Per female number of offspring produced was not associated with any of the predictors examined, including female secondary (model 2.1, Table S6 of Electronic supplement) or tertiary (model 2.2, Table S7 of Electronic supplement) immunocompetence PCA.

### 3.5. Prediction (iii): association between female immunocompetence and female survival

Similarly, adult female survival was not affected by any of the predictors examined, including female secondary (model 3.1, Table S8 of Electronic supplement) or tertiary (model 3.2, Table S9 of Electronic supplement) immunocompetence PCA.

### 3.6. Predictions (iv) and (v): effects of group size and the number of breeding females on offspring immunocompetence

Neither group size nor the number of breeding females predicted variation in first major axis of offspring immunocompetence PCA (models 4.1 and 4.2, and Tables S10 and S11 of Electronic supplement, respectively).



**Fig. 1.** The relationship between fecal glucocorticoid metabolites of females and female immunocompetence PCA. The regression line represents the relationship between both variables while the number of breeding females was also a predictor in the analysis (Table 3).

**Table 4**

Generalized linear mixed effects model (GLMM) for offspring survival. The model fit section includes Akaike (AIC) and Bayesian (BIC) information criteria, and the likelihood ratio test.

Model fit								
Model	AIC	BIC	Log likelihood	Deviance	Chi-square value	DF	p-Value	
Null model	157.04	162.45	−76.52	153.04				
Model 6.1	80.56	91.28	−35.28	70.56	82.47	3	0.00	
Model fixed effects								
Source of variation		Estimate	Standard error	z-Value	p-Value			
Intercept		1.04	2.16	0.48	0.63			
Offspring body mass		0.00	0.03	0.12	0.91			
Offspring sex		−2.39	0.74	−3.21	0.00			
Female secondary immunocompetence PCA		0.05	0.09	0.56	0.58			

### 3.7. Prediction (vi): association between female and offspring immunocompetence

Similarly, offspring immunocompetence PCA was not associated with female immunocompetence PCA based on secondary or tertiary responses, or with offspring condition (body mass) (models 4.1 and 4.2, and Tables S10 and S11 of Electronic supplement, respectively).

### 3.8. Prediction (vii): association between female immunocompetence and offspring early survival

Offspring survival was not associated with female immunocompetence PCA based on secondary (Table 4) or tertiary responses (model 6.2, Table S12 of Electronic supplement). However, offspring survival was significantly greater for female offspring (67%) than male offspring (37%) when female immunocompetence PCA based on secondary response was a predictor (Table 4). Offspring survival was not influenced by offspring body mass, an estimate of individual condition (Table 4).

### 3.9. Prediction (viii): association between offspring immunocompetence and offspring early survival

Offspring survival was not significantly affected by offspring immunocompetence PCA (model 5, Table S13 of Electronic supplement). Similar to the previous analysis, offspring survival was significantly greater for female offspring than male offspring, and offspring survival was not predicted by offspring body mass.

## 4. Discussion

### 4.1. Main findings

First, our study revealed that the number of breeding females (a proxy of communal rearing in degus) did not affect adult female immunocompetence (prediction i, Table 1). Additionally, the number of offspring weaned per female (a measure of female reproductive success) and female survival seemed unaffected by female immunocompetence (predictions ii and iii, Table 1). Together, these findings do not support hypothesis 1 in which sociality decreases the burden of individual immunocompetence of breeding females. Secondly, total group size and the number of breeding females per group did not co-vary with offspring immunocompetence (predictions iv and v, Table 1). Likewise, female immunocompetence did not predict offspring immunocompetence (prediction vi, Table 1) or offspring early survival (prediction vii, Table 1). Offspring survival in turn was not associated with offspring immunocompetence (prediction viii, Table 1). Thus, the hypothesis that



sociality enhances offspring immunocompetence and survival (hypothesis 2) was not supported either. Only the lack of association between offspring immunocompetence and total group size observed (prediction iv, Table 1) provide some indirect support to this hypothesis. Thirdly, the overall lack of social effects on female and offspring immunocompetence (predictions i, ii, iv, and v, Table 1) and of female immunocompetence on female survival and reproductive success (predictions ii and iii, Table 1) were consistent with degus using an “all or none” immunocompetence strategy. Yet, this hypothesis was also inconsistent with an absence of association between female and offspring immunocompetence (prediction vi, Table 1), or a lack of effects of these two measures of immunocompetence on offspring early survival (predictions vii and viii, Table 1).

#### 4.2. Immunological consequences of sociality and communal rearing

Our study provided original and previously unavailable evidence on the immunological consequences of sociality in a free-living social rodent. There was no support for the hypothesis that communal rearing enhances direct fitness of females mediated by immunocompetence. An absence of communal rearing effects on degu offspring immunocompetence may have different causes. First, current results may reflect a relatively low probability of immunoglobulin and immune cell transfer during lactation. The small intestine of newborn mammals is capable of absorbing macromolecules (e.g., immunoglobulins) during a limited time period after birth, and this time period varies with species [70]. For instance, the transport capacity of newborn guinea pigs (a close relative of degus) decreases rapidly within the first day postpartum, while transport in hamsters ends after day 5 of lactation [58]. Thus, a time-lag in the ingestion of colostrum caused by birth asynchrony within communal rearing groups may result in offspring unable to attain immune cells and antibodies from foster mothers, even though allonursing takes place [6]. Preliminary lab data obtained from a degu breeding colony indicates that uptake of maternal immunoglobulins from milk does not occur after day 6 of lactation in degus (L.A. Ebensperger & M.I. Becker unpublished results). We are currently examining the dynamics of immunoglobulin uptake by newborn degus to a greater extent and relate this to the degree of birth synchrony within social groups in our study population.

Second, the absence of an effect of communal rearing (and potential allonursing) on offspring immunocompetence in degus may be the consequence of the relative importance of prenatal vs. postnatal transfer of maternal immunity. Relatively high prenatal transfer of immune compounds from mother to offspring may be associated with a reduced transfer of these compounds during lactation [57]. We currently lack data to directly compare the extent of prenatal vs. postnatal transfer of maternal immunity in degus. However, prenatal transfer of anti-CCH IgG has been demonstrated in degus [6]. In addition, concentrations of protein in degu milk do not vary during lactation [94], a characteristic of species exhibiting relatively high transfer of prenatal compared with postnatal immunoglobulins [57]. Thus, past selection on maternal investment to favor prenatal over postnatal transfer of immunity may have constrained immunocompetence benefits linked to communal rearing in degus. Finally, the possibility that maternally transmitted immune compounds may suppress offspring immunocompetence cannot be ruled out [41], a possibility that has not been part of the theoretical foundations of communal rearing and other forms of breeding cooperation.

The hypothesis that immunocompetence is one major mediator of the fitness consequences of social behavior remains appealing. The influence of social factors on immune disorders and the existence of life history trade-offs between immune function and fitness support this tenet [4,25,40]. Immunocompetence benefits of group-living have been supported by studies on social insects, and where passive immunity through social contact enhances survival and ability to resist infection [54,91]. Evidence from social vertebrates comes mostly from

comparative studies on birds and carnivores, implying that individual immunocompetence is adjusted in response to a greater risk of parasite and pathogen transmission and represents a cost of sociality [63,64,68,88]. Two studies conducted on singularly breeding mole rats supported immunocompetence benefits to breeders and nonbreeders based on changes in spleen mass and metabolic rate, two effects mediated by enhanced body condition [60,61]. In contrast, cellular immunocompetence was noted to increase with the number of nonbreeders (helpers) in singularly breeding magpies [93], a social effect unrelated to body condition. The extent to which these social effects on immunocompetence translate into net fitness benefits remains to be determined.

Our conclusions are based on the assumption that measures of immunocompetence reflect disease resistance and its effects on fitness components such as survival, an aspect that needs to be confirmed using host resistance tests and experimental challenges to ecologically relevant pathogens [1,24,82]. Survival and other fitness measures might be especially sensitive to more commonly found pathogens and antigens. On the other hand, we verified that the number days between successive measures within same individuals did not have a statistical effect on immunocompetence ratio measures. However, we cannot completely rule out an impact of time spent in a trap on immunocompetence measures. We standardized the extraction of blood samples to within 2 h of capture. It is possible that our immunity measures include the effect of short term changes associated with the acute stress response to trapping [39,46,99]. However, stressful events may be immunostimulating or immunosuppressive, and different immunocompetence measures may be affected differently by acute stressors [9,46,62,84]. Thus, the overall lack of social effects recorded in our study is unlikely to be solely a consequence of acute stress caused by restraint during trapping.

In our study population, mortality is high and many females do not breed multiple times per year or during lifetime [29,36]. One interpretation of our results is that the costs of communal rearing have no effect on female immunocompetence because subsequent breeding is unlikely. In this sense, degus should use an “all or none” strategy where social effects on immunocompetence are weak or absent. However, selection may still favor immunological benefits since females breed multiply during some years [36]. Alternatively, an absence of benefits to females in terms of reduced costs of immunocompetence might be expected if maintenance of immunocompetence is not energetically expensive (e.g., [76,89]). Unexplored in degus, this possibility is supported by the observation that growth and metabolic rate (i.e., a measure of maintenance costs) are not compromised in immunologically challenged (yet ad lib fed) guinea pig offspring [73].

#### 4.3. Immunological effects of physiological stress

Our results revealed that breeding (lactating) females with higher FGC during early spring experienced an increase in lymphocytes and monocytes in circulation relative to other measures of immunocompetence. In contrast, females with lower FGC exhibited an increase in neutrophil to lymphocyte ratios, neutrophils, and total IgG. We hypothesize that this covariation is driven by physiological (life-history) adjustments needed to sustain breeding. Life history trade-offs involving immune function and reproduction are relatively well known [25,40]. For instance, organisms that enhance current investment in immunocompetence generally compromise current reproduction [25,53]. Previously, we observed that (i) females with the greatest reproductive challenges during lactation (based on per capita number of offspring) had the highest levels of FGC [34], and (ii) females with the highest FGC were less likely to breed a second time [36]. Together, these findings imply that females rearing a greater number of offspring during the main annual breeding event of this species do so through keeping high levels of glucocorticoids (necessary to sustain lactation and offspring rearing), relatively higher increases in lymphocytes and



monocytes, but at the cost of not producing a second litter. On the other hand, females experiencing lower reproductive challenges during lactation exhibited relatively low levels of glucocorticoids, relatively higher increases in neutrophil to lymphocyte ratios, neutrophils and total IgG, while retaining the ability to produce a second litter. The observation that female levels of FGC do not vary with communal rearing does not support the hypothesis that sociality influences physiological stress levels under natural conditions in degus [34]. However, the potential effect of other aspects of social behavior (e.g., dominance status) on physiological stress and immunocompetence remains unknown in degus.

## 5. Concluding remarks

As far as we are aware, our current study remains the first to examine the extent to which females and their offspring potentially attain immunological benefits from communal rearing in a wild living, communally rearing rodent. Contrary to main expectations however, (i) communal rearing did not impact adult female immunocompetence, and (ii) variation in adult female immunocompetence did not influence female survival, offspring immunocompetence and survival. These findings imply either no immunological benefits to social and communally rearing degus, or that these are of little biological relevance. Instead, variation in cellular immunocompetence of adult females may be related to varying energetic demands of breeding. The extent to which changes in female immunocompetence result in effects on future reproductive success may depend on how these changes increase the probability of breeding multiply. Multiple breeding of females is tied to the energetic demands of lactation, which is correlated with cortisol levels [36]. Based on this observation, variation in immunocompetence as revealed by changes in lymphocyte, monocyte, and neutrophil ratios likely reflect a cost to females [20]. Subsequent studies are needed to determine how variation in immunocompetence coupled to high glucocorticoid levels are connected to females' low probability of breeding twice and surviving to next main breeding event.

## Ethical standards

All observations and experiments carried out during this study complied with the current laws of Chile. This study was approved by the Institutional Animal Use and Care Committee at the Facultad de Ciencias Biológicas (DFCB-021/2008), and received the required authorization to live trap wild degus by the Servicio Agrícola y Ganadero (1-31/2009 [1956]).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.physbeh.2014.12.028>.

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