Chronic Stress effects on Immune-Neuroendocrine matrix in *Coturnix japonica,* analyses from an ontogenetic perspective

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Abstract

Immune-neuroendocrine characteristics define individual physiological profiles or phenotypes (INPs), which integrated at the population level account for complementary information. In avian models (quail, *Coturnix japonica*; hens, *Gallus gallus*), individuals cluster in three subgroups associated to three INPs: the Lewis-like (pro-inflammatory polarization), the Fischer-like (anti-inflammatory polarization), and an intermediate INP, most represented in the population. This study assesses changes in individuals' physiological configuration, translated in INPs stability/alteration throughout ontogeny (juvenile and adult stages) and evaluates whether an unpredictable and diverse chronic stress (UDCS) modifies this configuration in adults and if this modification persists until senescence. We evaluated corticosterone, pro- (IFN-γ and IL-1β) and anti-inflammatory cytokines (IL-13, IL-4), phytohemagglutinin (PHA-P) lymphoproliferative response, anti-sheep red blood cells antibody (Ab SRBC) response and leukocyte distribution frequency. Individuals expressing Lewis- or Fischer-like profile increased, after UDCS exposure. This trend persisted until senescence. Females showed higher frequency of the Fischer-like INP, while males presented a greater prevalence of the Lewis-like. The variation in the frequency of INPs after UDCS could obey differences in coping strategy, mainly among individuals with intermediate configurations. The distribution after the disruptive event, displaying greater homogeneity among INPs, provides the population with a greater advantage to confronting potential environmental challenges to come.

Key words: Immune-neuroendocrine phenotypes, unpredictable chronic stress, quails, development, environmental challenges, individual scale, population scale, resource allocation

Introduction

During an organism's lifetime, there are compromises in resource allocation among competing traits known as trade-offs (Senner, Conklin & Piersma, 2015; Taborsky *et al.*, 2021). Such trade-offs may lead to individual differences within a population causing immediate or delayed

43 effects on fitness that can lead to changes in individuals' phenotype (Senner *et al.*, 2015;
44 Garland, Downs & Ives, 2022). Coping with a dynamic environment requires the mobilization
45 of the so called allostatic mediators, i.e., glucocorticoids and cytokines, that regulate a variety
46 of processes to ensure the individual's survival by operating at different concentrations in
47 accordance with organism´s demands (Mcewen & Wingfield, 2003). Allostatic mediators work
48 coordinating responses among metabolic pathways which trigger physiological changes in
49 several systems in response to both internal and environmental keys (Cohen *et al.*, 2012;
50 Garland *et al.*, 2022). Certain alterations are linked to natural processes in an animal's life cycle
51 such as sexual maturation, reproduction, or growth which are energetically demanding
52 mechanisms that require changes in resource allocation (Jacobs & Wingfield, 2000; Johnston *et*
53 *al.*, 2012). There are other stimuli known as stressors that can disrupt homeostasis and trigger
54 emergency adjustments in animals' physiology and behavior, such as loss of social rank, severe
55 weather, habitat destruction, extreme climatic events and even human disturbance (Blas &
56 Fairhurst, 2021). Therefore, stress coping induces an emergency life history state that directly
57 impacts energy allocation, thereby reducing the energy available for other essential
58 physiological functions, such as reproduction or growth (Hasselquist & Nilsson, 2012; Wingfield
59 *et al.*, 2017).

60 When the exposure to stressors is prolonged, animals develop a particular and potentially
61 persistent state of physiological imbalance, known as chronic stress (CS). In this state, the
62 physiological response either persists long after the stressor has ceased, or is repeatedly
63 activated (Dhabhar, 2009; Blas & Fairhurst, 2021). CS implies heightened exposure of the
64 organism to glucocorticoids and other allostatic mediators, each with the potentiality to
65 impact several physiological variables such as white blood cell proliferation, cytokine secretion,
66 antibody production, and cytolytic activity, among others which are integrated at the immune
67 neuroendocrine (INE) level (Padgett & Glaser, 2003; Koutsos & Klasing, 2014; Nazar, 2015;
68 Nazar *et al.*, 2017). Consequently, CS can deplete an organism's available resources, reducing

69 its ability to cope with future stressors. Alternatively, CS may provide information to deal with
70 subsequent challenges influencing fitness critical variables like survival or reproductive success
71 (Del Giudice *et al.*, 2018) that can extend beyond the individual, with transgenerational effects
72 that impact subsequent generations (Goerlich *et al.*, 2012; Guibert *et al.*, 2013; Zimmer *et al.*,
73 2017; Giayetto *et al.*, 2020). Even so, coping with challenges will be conditioned by the
74 individual's specific phenotype and its capacity to adaptively shape its response to the
75 environment (Piersma & Drent, 2003; Garland *et al.*, 2022).

76 Differences among sexes can also influence immune and neuroendocrine responses due to the
77 effect of estrogens and testosterone and even some aspects of life history can be related (Klein
78 & Flanagan, 2016; Garcia-Reyero, 2018). It is widely accepted that females tend to allocate
79 more resources to reproduction than males, given the demands of egg development and
80 parental care (Lee, 2006). As a result, females exhibit a Th2-biased immune response, with
81 lower costs in terms of energy budget (Lee, 2006; Vincze *et al.*, 2022). On the contrary, the
82 highest reproduction investment in males is located on mate attraction thus, they have more
83 energy available for an Th1 biased immunological response, i.e., cell mediated response (Lee,
84 2006; Valdebenito *et al.*, 2021). Also, in birds, an anatomical difference is in accordance with
85 this physiological observations, males tend to have a smaller spleen than females (Møller, Sorci
86 & Erritzøe, 1998). This organ is the main leucocytes producer in avians, therefore, differences
87 in size between sexes can be related to variation in parasites sensitivity (Møller *et al.*, 1998).
88 Males tend to have a higher corticosterone response than females, which can influence their
89 survival rate (Jimeno *et al.*, 2018). Furthermore, regarding reproduction, testosterone is the
90 main males reproductive hormones and a precursor of estrogens production in the females'
91 ovary (Lipshutz *et al.*, 2019). This hormone participates in aggression and its effect and
92 integration at the phenotypic levels may vary within an animal's lifetime (Lipshutz *et al.*, 2019).

Ontogeny is another relevant factor to be considered when studying coping mechanisms and its effect will be logically influenced according to the individual's life stage. In general, when juveniles face some kind of challenge this can cause changes in the reaction norm of certain physiological responses causing permanent changes in the phenotype configuration (Stearns, 1989; Piersma & Drent, 2003). On the contrary, facing challenges during adulthood may lead to less permanent responses, for that reason is considered that adults acquire a "reversible state effect" in which they will be able to switch among phenotypes acquiring new configurations in response to the environment (Senner *et al.*, 2015; Burggren, 2018). To differentiate permanent transformation in the reaction norm that happen when challenges occur in juvenile stages from those reversible in response to the environment during adulthood, Piersma and Drent (2003) proposed to name the second ones "phenotypic flexibility". This name refers to changes that occur when environmental conditions vary rapidly and over shorter timescales than a lifetime. The ability of changing and acquiring a "reversible state effect" may turn into an evolutionary advantage because the bigger the diversity of traits in a population, the more prepared it will be to face future unknown challenges (Forsman, 2015).

Modifying phenotype configuration may lead to a reorganization in resource allocation that can have an impact on fitness (Romero, Dickens & Cyr, 2009; Buehler, Tieleman & Piersma, 2010). In some cases, these effects will be immediate and in others will not, driving to carry-over effects that can influence on individual's performance to future situations (O'Connor *et al.*, 2014) and impact on processes at the population level such as survival rate (Saino *et al.*, 2017; Loonstra *et al.*, 2019). In some cases, carry-over effects are not seen until the next generation and the price that individuals pay for facing a challenge are evident in their descendance (Senner *et al.*, 2015; Giayetto *et al.*, 2020). Therefore, understanding how ontogeny and potential carry-over effects influence alterations in their immune and neuroendocrine configuration is crucial. This information is valuable for assessing the impact of challenges in both the short and long term, both at the individual and population levels.

119 In the last decades, immune neuroendocrine phenotypes (INPs) have been described in
120 mammalians including humans (Elenkov *et al.*, 2008), and related species like rodents (Wei,
121 Listwak & Sternberg, 2003). Later on, in representatives of different evolutionary histories such
122 as avian species like hens (*Gallus gallus*; Nazar *et al.*, 2017), or quail (*Coturnix japonica*; Nazar,
123 2015). INPs are a set of characteristics that identify individuals in their neuro-hormonal and
124 immunological responses, similar among them but different from another group within the
125 same animal population (Sternberg *et al.*, 1989; Elenkov *et al.*, 2008; Ashley and Demas, 2017).
126 As a consequence, INPs provide a valuable framework for assessing population responses to
127 chronic and stressful environmental challenges (Sternberg *et al.*, 1989; Nazar *et al.*, 2017). INPs
128 are characterized by neuroendocrine mediator concentrations, activity and expression of
129 hormonal receptors, and cytokine belonging to pro or anti-inflammatory profiles. In quail and
130 hens three INPs have been described (i) the Lewis-like phenotype characterized by low
131 corticosterone levels, high proinflammatory mediators' levels (IFN-γ and IL-1β), high
132 lymphoproliferative response to Phytohemagglutinin-p (PHA-P), high antibody response
133 against to sheep red blood cells (SRBC), low innate : acquired immunity relationship evaluated
134 by the frequency of leukocyte distribution (FLD) and anti-inflammatory mediators' response
135 (IL-13, IL-4); (ii) the Fischer-like phenotype, characterized by high corticosterone levels, high
136 levels of anti-inflammatory mediators (IL-4 and IL-13), high FLD, and low lymphoproliferative
137 response to PHA-P, Ab SRBC, and proinflammatory response (Nazar *et al.*, 2015); and (iii) the
138 intermediate phenotype, in between these two extreme phenotypes (Fig. 1).

139 The ability of individuals to deal with a disease will be related to the INP presented (Sternberg
140 *et al.*, 1989; Wei *et al.*, 2003; Nazar *et al.*, 2015, 2017). As a consequence INPs frequencies in a
141 population can affect its ability to cope with immune neuroendocrine (INE) challenges because
142 a new and differential response will be given to the presence of parasites, and/or other
143 pathogens affecting individuals within the population (Nazar *et al.*, 2017; Nystrand & Dowling,
144 2020). Also, INPs imply an underlying differential INE response, i.e., of the hypothalamus

145 pituitary adrenal axis (HPA), Fischer-like individuals have a higher corticosterone response than
146 Lewis-like individuals (Nazar *et al.*, 2015, 2017). This differential response to corticosterone will
147 impact various other life-history traits, such as metabolic rate (Jimeno et al., 2018b), and the
148 behaviors of the animals (Bókony *et al.*, 2009). Consequently, INE configuration (either
149 synthesized or not in the concept of an INP) could be an indicator of the population's substrate
150 to cope with environmental challenges.

151 As stated before, in stressful situations, the frequency of INPs changes (Nazar *et al.*, 2017).
152 However, the contribution of each phenotype to this new configuration is still not clear. We
153 are not certain whether the majority of individuals altering their phenotype belong to the
154 intermediate INP category, or if those from one extreme phenotype transition to the opposite
155 extreme, or any other potential combination. Understanding the dynamics of change at the
156 individual level can illuminate phenomena even at the population level. It offers
157 complementary information that is currently lacking regarding individuals' physiological
158 integration of environmental and ontogenetic challenges. In this context, we studied INPs
159 configuration during different ontogenetic stages in Japanese quail as an avian model
160 (juveniles and adults). As adults, we conducted further studies to address whether exposure to
161 a disruptive challenging event, characterized by unpredictable and diverse chronic stressor
162 exposures (UCSE), could modify the configuration of INPs at both the individual and population
163 levels, and whether these changes could persist until senescence. We hypothesized that at a
164 population level, INPs will be modulated by UDCS acting as a disruptive force that alters the
165 frequency of the phenotypes within the population. We expect that CS will have effects on the
166 variables that integrate the INE interface, modifying INPs' expression at the population level
167 due to individual´s quail adjustments.

168 **Materials and Methods**

169 *Animals and husbandry*

170 The present study used adult Japanese quail because they are recognized as an useful
171 laboratory model because of their physiology, small size, short life cycle, and low maintenance
172 cost (Huss, Poynter & Lansford, 2008). The quail were raised as described elsewhere (Nazar *et*
173 *al.*, 2012; Caliva *et al.*, 2017). Animal care was provided in adherence to Institutional Animal
174 Care and Use Committee guidelines. The experiment procedures were approved by the ethical
175 committee at Instituto de Investigaciones Biológicas y Tecnológicas (IIByT) in compliance with
176 the legislation regarding the use of animals for experimental and other scientific purposes
177 (ACTA CICUAL N° 27, 09/04/2015) and are in accordance with ARRIVE guidelines.

178 Briefly, from the first day of age (DA), quail were housed in enriched rearing boxes measuring
179 90x90x60 cm (length x width x height) in mixed groups of 40 individuals. A wire-mesh floor (1
180 cm grid) was raised 5 cm to allow excreta passage, and 50% of the total surface was covered
181 with corrugated cardboard. Each box had a feeder arranged in the front, 16 automatic drinkers
182 ("nipple" type), and a cover lid to prevent the escape of the birds and the loss of heat. The
183 breeding temperature was lowered gradually and weekly from hatching to 28 DA, starting
184 from an initial temperature of 37ºC until reaching 24ºC (Nazar *et al.*, 2018). The ambient
185 relative humidity recorded ranged between 50 and 60%. The quail were kept in a daily cycle of
186 14 h of light (330-350 lx) and 10 h of dark (14:10 L:D) during the study, turning on the lights at
187 06:00 h. At 28 DA, quail were sexed using breast plumage coloration and wing banded for later
188 identification. At 35 DA, 48 male-female pairs were randomly re-housed in 39x19x25 cm
189 (length x width x height) pedigree enclosures. The environmental enrichment program
190 included corrugated cardboard and plastic grass on the floor, and rough plastic sheets and
191 wooden boards as enrichment elements that were alternated weekly to minimize the
192 habituation and to avoid the disturbance of a more frequent change (Nazar & Marin, 2011).
193 Quail starter diet (24% crude protein; 2900 kcal metabolizable energy kg−1) was used for the
194 first 4 weeks of life. A laying diet was provided afterward (21% protein, 2750 kcal ME kg−1).
195 The supply of water and food was *ad libitum*.

196 *Experimental design:*

197 The experiment consisted in evaluating selected INE representative variables (previously
198 associated with INPs, see below) in four target stages of analysis (Fig. 2). 1) juvenile quail (6
199 wks of age); 2) adult quail (15 wks of age), when birds evidenced full maturity (fully active
200 foam gland in males and females peaking egg production (~one egg per day) and prior to birds´
201 exposure to a chronic disruptive event (UCSE protocol, for details on the protocol see
202 supplementary material S1); 3) After the UCSE ended (17 wks of age) and 4) during
203 senescence (27 wks of age).

204 *Variables tested:* The set of variables evaluated for characterizing INPs in every individual are
205 briefly described as fully descriptions are provided elsewhere (Nazar, 2015; Nazar *et al.*, 2015,
206 2017).

207 *Response to PHA-P injection:* this is a measure pro-inflammatory potential of the birds. Briefly,
208 after measuring with a caliper the wing web thickness, a 0.05 ml. intradermal injection of a 1
209 mg/ml solution of PHA-P in phosphate-buffered saline (PBS) was administered in the wing web
210 area. On the following day (24 h post-PHA-P), the dermal swelling response was measured as
211 the percentage of increase in wing web thickness at the injection site. Measurements were
212 recorded to the nearest 0.01 mm using a mechanical micrometer (Roberts *et al.*, 2009).

213 *Antibody response to SRBC*: this parameter was used to evaluate the induced humoral immune
214 response. The antibody titer was assessed with a micro agglutination assay (Nazar & Marin,
215 2011). 20 µl of complement-inactivated plasma (through heating to 56°C) were serially diluted
216 in 20 µl of PBS (1:2, 1:4, 1:8, up to 1:512). Next, 20 µl of a 2% suspension of SRBC in PBS were
217 added to all wells. Microplates were incubated at 40°C for 1 h and hemagglutination of the test
218 plasma samples was compared to the blank (PBS only) and negative controls (wells with no
219 SRBC suspension). Antibody titers were reported as $\log_2$ of the highest dilution yielding
220 significant agglutination. The results represent the average of two duplicates for each animal.

221 *Corticosterone and testosterone analyses*: Plasma samples were extracted with diethylether
222 (10 times the volume). Afterwards, the ether phase was transferred into a new vial,
223 evaporated and dissolved in assay buffer (Schöffmann *et al.*, 2009). Aliquots (in duplicate)
224 were quantified in a corticosterone and testosterone enzyme immunoassay (EIA). Details of
225 the EIAs, including cross-reactivities of the antibody, are given in Palme & Möstl (1997) and
226 Auer *et al.* (2020). Steroid concentrations are expressed as nanograms of hormone per ml of
227 plasma (ng/ml).

228 *Frequency of leukocyte subpopulation distribution (FLD)*: First, cell count was done by flow
229 cytometry. To do this, 40 µl of total blood were stained with a fluorescent lipophilic dye (3, 3`-
230 dyhexiloxacarbocyanine iodide; DiOC6, Molecular Probes) to obtain absolute counts of
231 erythrocytes, lymphocytes, monocytes, thrombocytes and granulocytes as described
232 elsewhere (Uchiyama *et al.*, 2005). Then, the FLD number was calculated using the following
233 formula: FLD = number of granulocytes/(number of lymphocytes + number of monocytes) and
234 is an indicator of the innate: acquired immunity ratio.

235 *Cytokines:* To determine cytokine mRNA expression levels, peripheral lymphocytes were
236 isolated and total RNA was extracted from cells according to the manufacturer's instructions
237 and as previously described (Shini & Kaiser, 2009). Then, total RNA was reverse-transcribed.
238 The resulting cDNA was stored at -80°C until used for real-time PCR. For quantitative real-time
239 PCR assays, specific primers for quail IL-1β, IL- 4, IL-13, and IFN-γ genes were used as
240 previously reported (Superina, Carreño & Jahn, 2009). β-actin was used as a reference
241 housekeeping gene. Real-time PCR was performed with a Step One Plus Detection System
242 (Real-Time PCR System- Thermo Fisher Scientific). The results represent the average from
243 three technical replicates for each analyzed animal. The level of expression of each target gene
244 was calculated using the formula: Gene Level = 2-(Target Gene Ct – β-Actin Ct). The value
245 obtained was used to compare the level of expression of each molecule (Nazar *et al.*, 2015).

246 *Statistical analysis:*

247 INPs were formerly described using cluster analysis in order to explore the degree of similarity
248 among the birds in the study on the bases of the analyzed variables. Particularly, with the aim
249 of identifying the best partition of the birds, described by the best orthogonal linear
250 combinations of the factors according to the least-squares criterion, a K-means analysis was
251 performed. The following 9 standardized variables were used in the analysis: plasma
252 corticosterone, testosterone, IFN-γ and IL-4, IL-1β, IL-13, skin-swelling response to PHA-P,
253 antibody response against SRBC and FLD ratio. Based on the theoretical framework previously
254 exposed, the clustering was performed with a K value of 3 (2 subgroups with representatives
255 of potential extreme INPs and 1 intermediate subgroup). Euclidean metrics were used for
256 computing the distance between points and cluster centers. To graphically represent the
257 results of the clustering in a multivariate manner, a Principal Component Analysis (PCA) was
258 used with the clustering variables as explanatory variables (Crawley, 2007; Nazar *et al.*, 2017).
259 Also, on the data from each stage of the experiment, we performed a linear discriminant
260 analysis (LDA) to characterize variation in the population (Crawley, 2007). LDA was used to
261 recognize the variables with more weight to identify INPs during each stage of this study, the
262 predictor power of each variable is represented by the size of a vector in LDA plots. Due to
263 autocorrelation, two of the four cytokines were removed to do LDA analysis. We chose IL-4 as
264 representative of the anti-inflammatory response and IFN-γ as representative of the pro-
265 inflammatory response. To adjust the model, we randomly selected 75% of the data of each
266 experimental stage, and then we tested its predictive power with the remaining 25% of the
267 data. To compare the variation in the frequency of INPs from one experimental stage to the
268 other, we did a proportion analysis. We also compared the proportion of males and females
269 belonging to each phenotype.

270 We evaluated the transition probability from one INP to the others during the four
271 experimental stages using discrete-time Markov chains (DTMC; Spedicato and Signorelli, 2014).
272 This kind of analysis is adequate to evaluate changes in individuals at successive points in time.
273 To do this, we built transition matrices among the four stages of the experiment (Table 3): first
274 a matrix between juveniles and adults, second, a matrix between adults pre-stress and post-
275 stress, and third a matrix between adult post-stress and senescent individuals (Bishop,
276 Fienberg & Holland, 1974). Transition matrices estimate the probability of an individual
277 changing its phenotype. For example, if at the juvenile stage, 16 individuals show a Fischer-like
278 phenotype, we intended to know how many of those individuals remained Fischer-like when
279 they reach adulthood and how many changed to Lewis-like or the intermediate phenotype. We
280 also evaluated the number of individuals that remained in the same phenotype. To estimate
281 probabilities, we divided the number of individuals that had changed by the total of individuals
282 of each phenotype in the previous state (Spedicato & Signorelli, 2014). Since in some cases,
283 the INP could not be established during the four stages, the total number of individuals in each
284 transition may be variable but in the three transitions studied the sample was always more
285 than 70 individuals, thus the results can be considered representative of INPs configuration in
286 the species and of changes during the experiment.

287 **Results**

288 The three groups identified by the cluster analysis are represented in the PCA plots (Fig.3),
289 using biplots as a graphical strategy to represent the phenomena as previously mentioned
290 (separated results of each variable can be seen in supplementary material S3). Cluster analyses
291 was performed as previously described (Nazar *et al.*, 2017). Briefly K-means analysis
292 distributed the birds in 3 main dissimilar groups during the four experimental stages studied.
293 In the first two experimental stages (juveniles and adults pre-stress) the number of individuals
294 belonging to the intermediate phenotype is higher than the number of individuals belonging to

295 extreme phenotypes, i.e., Fischer-like and Lewis-like, as it can be seen in the PCA plots (Fig. 3).
296 After the stress protocol the number of individuals with Fischer and Lewis like phenotypes
297 increased and this change in the representation of individuals with extreme phenotypes
298 remained until senescence (Fig. 3). The three groups, as separate corpus of birds, remained
299 differentiated along the four stages of this experiment.

300 LDA was used to identify the parameters that are more important to recognize INPs during
301 each stage of this study. The results indicated a high influence of corticosterone predictive
302 power during the four stages (Fig. 4). This variable seems to be the main indicator of the
303 variability of the three phenotypes, and it had a negative displacement among canonical axis 2.
304 In juveniles, canonical axis 1 explained 84.46 % of the variability (Fig 4). In pre-stress adults,
305 85.86 % of the variability is explained by canonical axis 1 (Fig. 4). In post-stress adults, 94.36%
306 of the variability is explained by the canonical axis 1. Finally, 85.98% of the variability of
307 senescent individuals is explained by canonical axis 1. Also, in senescent individuals,
308 testosterone was a variable with a high coefficient of linear discriminant, indicated by the size
309 of its vector (canonical axis 1: 0.88, canonical axis 2: -1.49). The complete information of linear
310 discriminant coefficients can be seen in supplementary table S2. Models' predictive power was
311 at least 90% in all the cases (S2-Table).

312 The frequency of the INPs indicated that, in the population studied, the prevailing phenotype
313 during the two first experimental stages (juveniles and adults pre-stress), was the intermediate
314 one, characterized by immune-neuroendocrine responses of intermediate magnitude. After
315 stress, the frequency of the extreme phenotypes Lewis-like and Fischer-like increased
316 significantly (Fig. 5, Table 1; p-value=0.0093 for Lewis-like and p-value = 0.03 for Fischer-like).
317 Also, a reduction in the proportion of individuals with intermediate phenotype was observed
318 in this experimental stage (p-value=0.00005). During senescence, frequencies of the extreme
319 phenotypes seemed to sustain a uniform distribution without significant changes compared to

320 the previous stage (adults post-stress; Fig. 5). Table 1 shows the results of the Proportion test
321 comparing transitions among phenotypes. A significant increase in the proportion of extreme
322 phenotypes and a reduction of the intermediate phenotype was observed only in the
323 transition from adults' pre-stress to adults' post-stress (Indicated in bold). In all the other
324 transitions, no significant differences were found.

325 The proportion analysis comparing the phenotypes among sexes within the same experimental
326 stage (Table 2) indicated that the proportion of females showing a Fischer-like phenotype was
327 significatively higher than males with this INP in adults pre-stress (p-value= 0.00005), in adults
328 post-stress (p-value = 0.001141), and in senescent quail (p-value=0.000003). On the contrary,
329 the proportion of males presenting a Lewis-like INP was significantly higher than the
330 proportion of females in the adults' pre-stress (p-value=0.00135), and in the senescent
331 experimental stages (p-value=0.000000009). In addition, the frequency of males with an
332 intermediate phenotype was significantly higher than the proportion of females in the adults'
333 post-stress experimental stage (p-value=0.00006). Only a trend for females showing a reduced
334 proportion of Lewis-like phenotype compared than males was found during the juvenile
335 assessment (Table 2).

336 The probability of an individual changing its phenotype from one experimental stage to the
337 next one or the probability of remaining in their original phenotype was evaluated using a
338 DTMC analysis (Table 3). As shown, during the transition of juveniles to adults, the probability
339 of changing from Lewis-like or Fischer-like phenotypes to the intermediate was the highest in
340 both cases (0.55 and 0.75 respectively). In addition, most of the individuals belonging to the
341 intermediate type remained in that state (0.64; Table 3; Fig. 6). After the stress protocol, the
342 probability of individuals changing from Lewis-like (0.33) or Fischer-like (0.07) phenotypes to
343 the intermediate phenotypes decreased (Table 3; Fig. 6), and the probability of individuals to
344 change from intermediate phenotype to a Lewis-like (0.29) or a Fischer-like (0.29) increased.

345 Moreover, the probability of Lewis-like individuals changing their phenotype to Fischer-like
346 and vice versa (0.43 and 0.27 respectively) also increased in this transition. Finally, during the
347 transition from post-stress adults to senescence (Table 3; Fig.5), the probability of individuals
348 changing from an intermediate phenotype to a Lewis-like (0.31) remained high. The probability
349 of an individual changing from a Lewis-like to Fischer-like or inversely (0.33 and 0.20
350 respectively) decreased compared to the transition from pre-stress adults and post-stress
351 adults.

352 **Discussion**

353 This study assesses changes in INE variables that lead to the configuration of INPs throughout
354 ontogeny (juvenile and adult stages) evaluating also whether a significant disruptive event can
355 modify previous adult configuration and whether this alteration persists when aging into
356 senescence. In particular, we analyzed whether a chronic unpredictable stress disturbance at
357 the INE level influences INPs proportions, their distributions at the population level and the
358 probability of change (INP assessments in adults pre- and post-stress). It is of outmost
359 importance to highlight that INPs are not considered here a fixed entity, but rather the array of
360 INE responses that a particular individual can deploy in a certain moment of its ontogeny.
361 Thus, each of the evaluated stages provide differential and complementary information. The
362 two first stages i.e., juveniles and adults (Fig. 2), are related to physiological changes
363 associated with regular life processes such as sexual maturation and growth (Garland *et al.*,
364 2022). Lastly, and using the second stage as an adult (before UCDS exposure) baseline, the
365 third and fourth stages i.e., adults after a stress exposure and senescent individuals (Fig. 2), are
366 related to the ability of individuals to cope with external challenges (post-stress sampling) and
367 how challenged individuals reach senescence (last sampling).

368 Findings showed that it is possible to identify three different INPs in the population during the
369 whole study and at the different stages evaluated (Fig. 3). Corticosterone was determined as

370 one of the most important predictors of INPs during the four stages studied. Nevertheless,
371 LDAs showed that the relationship among the variables also changed along the stages (Fig. 4)
372 which may be, at least in part, related to the reorganization in resource allocation due to
373 ontogenetic variations(Senner *et al.*, 2015; Garland *et al.*, 2022). Indeed, findings are
374 consistent with the idea that, throughout their lives, individuals undergo processes influenced
375 by the costs of living, resulting in changes in the relationships between immune and
376 neuroendocrine factors and their respective significance (Jacobs & Wingfield, 2000; Johnston
377 *et al.*, 2012).

378 Some authors proposed that the stress response is influenced by both the life history traits of
379 the species (Schoenle *et al.*, 2021) and the response potential of different tissues (Lattin,
380 Waldron-Francis & Romero, 2013). Furthermore the INPs as a partially new factor to be
381 considered and included in these equations has been suggested (Sternberg *et al.*, 1989b,
382 1989a; Zelazowski *et al.*, 1992; Wei *et al.*, 2003; Elenkov *et al.*, 2008). Fischer-like individuals
383 might be more related to anti-inflammatory and antibody-mediated immune responses than
384 Lewis-like individuals (Mosmann *et al.*, 1986; Nazar *et al.*, 2015, 2017). Therefore, individuals
385 expressing either one of or other of these two extreme phenotypes would have different
386 abilities to cope with diverse environmental conditions, and, as a consequence, they may show
387 distinct environmental sensitivity to stressors understanding them as challenges (Colditz *et al.*,
388 2016). Changes in INP configuration from one life stage to the other would imply a variation at
389 the very individual level in the ability to cope with a variety of challenges (Zelazowski *et al.*,
390 1992; Wei *et al.*, 2003; Nazar *et al.*, 2015, 2017). For example, and from a theoretical point of
391 view, if one individual with an intermediate INP before stress switches to a Lewis-like after a
392 chronic stress disturbance, its ability to develop robust inflammatory responses will be
393 increased, and therefore also their potential to deal with certain types of pathogens (Wei *et*
394 *al.*, 2003; Elenkov *et al.*, 2008; Nazar *et al.*, 2017). On the contrary, if an individual acquires a
395 Fischer-like configuration, the outcome of its response to infections would be opposite to that

396 of a Lewis-like configuration (Wei *et al.*, 2003; Elenkov *et al.*, 2008; Nazar *et al.*, 2017). These
397 examples are just based on some of the shifts observed and, it is worth highlighting, that these
398 and any other shift in INP needs to be understood as a synthesis of a change in the
399 components of the INE system being integrated for analysis.

400 In juvenile and adult pre-stress, the frequency of extreme phenotypes was low, and the
401 frequency of the intermediate phenotype was notoriously higher (Fig. 4), which might
402 resemble a Gaussian distribution. Even so, no significant increase in the frequencies of the
403 transition from juveniles to adults was observed (Table 1). On the contrary, after the chronic
404 stress protocol, the population acquired a new distribution, more uniform, with a significant
405 increase in the proportion of the two extremes phenotypes and reduction of the intermediate
406 representatives during the transition from pre-stress to post- stress adults (Table 1). It is
407 relevant to recall that the stress protocol included not just a variety of different stressors but
408 also a strong unpredictable nature. Hence, as individuals confront pathogens by developing
409 the most effective response allowed by their immunological potential (Buehler *et al.*, 2010),
410 shifting the population to a better-balanced representation of INPs can be viewed as a highly
411 efficient strategy to deal with a broader range of immune challenges. This new distribution
412 may imply different possibilities to deal with environmental challenges, whereas a population
413 "over-expressing" only one INP may find this possibility limited (Forsman, 2015; Nazar *et al.*,
414 2015). Remarkably, this new distribution remained until senescence (Fig. 4) without significant
415 changes in the proportion of individuals belonging to one or other INP (Table 1). Possibly the
416 INP configuration of senescent individuals might be a combination of aging and the long-term
417 effects of the challenge. These results suggest that the INP induced changes, and therefore the
418 population ability to deal with future unpredictable challenges, possibly due to long lasting
419 carry-over effects (Senner *et al.*, 2015; Nazar *et al.*, 2017; Moore & Martin, 2019).

420 Additionally, the proportion of females expressing Fischer-like phenotype was significantly
421 higher than the proportion of males during adults pre-stress, adults post-stress and senescent
422 stages. On the contrary, males expressed a higher frequency of Lewis-like phenotype during
423 the same stages. These results may provide us with information on how both sexes cope with
424 challenges. Our results seem to be clear in indicating that some kind of polarization in the
425 expression of INPs occurs in the population, with males expressing more frequently a Th1
426 response than females, being more prepared for intracellular infections (Kogut, 2022).
427 Possibly, this polarization would be related to different energetic request in males and female
428 in reproduction investment: males tend to invest more energy in matte attraction reducing
429 their investment in immunological response to parasites (Lee, 2006; Valdebenito *et al.*, 2021).
430 Additionally, studies done in zebra finches and broilers, indicated that males have a higher
431 corticosterone response to stress (Marin *et al.*, 2002) and a lower survival rate after a stress
432 induction than females (Jimeno *et al.*, 2018). Considering that Lewis-like individuals will have
433 lower basal corticosterone than Fischer-like or intermediate individuals, increasing the number
434 of males with this phenotype might represent an advantage, because these individuals might
435 be less responsive to future challenges increasing their probability of survival. This proposal
436 requires further study, and the relation between INPs and sex is a factor that should be
437 considered in future research.

438 From the individual's perspective, during the four stages studied, most quail acquired different
439 INPs configuration during the experiment, even without environmental challenges (Fig. 6).
440 Changes in INPs might be related to phenotypic flexibility meaning that some individuals would
441 display a variable expression of INPs within their lifespan (Piersma & Drent, 2003). Changes in
442 the phenotype along an individual's life can progress linearly, affecting the outcome of the
443 next developmental step mainly during juveniles stages, or can occur in a more flexible manner
444 during adulthood when individuals may change adaptively and repeatedly in response to
445 environmental conditions (Jacobs & Wingfield, 2000; Senner *et al.*, 2015). The ability to vary

446 INP in response to the environment will represent an immunological advantage in some cases
447 but, would this change worst the pain? Possibly it would depend on the crosstalk of
448 ontogenetic demands, individuals' energy budget and environmental challenges (Walsberg,
449 2003; Shankar *et al.*, 2019; Garland *et al.*, 2022). Even if in our experiment, individuals were
450 fed all in the same manner and had water *ad libitum*, this does not mean that the use of
451 energy is identical by all of them but at least the access was similar. Some individuals will have
452 a greater capacity to distribute energy to all aspects of their life than others, and they will be
453 able to intake, digest and utilize greater amounts of energy than their conspecifics (Buehler *et*
454 *al.*, 2010; Halsey *et al.*, 2019). Therefore, ontogenetic variations like sexual maturation and
455 reproduction, might influence other physiological, i.e., INE systems such as the immune system
456 or the very HPA axis leading to variations in INPs throughout life (Johnston *et al.*, 2012; Segner,
457 Verburg-van Kemenade & Chadzinska, 2017) and acting as endogenous disruptors that
458 influence at the individual level without having a notorious impact on the population global
459 arrangement.

460 INP changes may synthesize a shift in INE responses and potential. This may then imply a
461 trade-off in energy allocation (Garland *et al.*, 2022) and would be limited in its possibilities and
462 scope by how much energy individuals can efficiently use at any given moment (Buehler *et al.*,
463 2010; Hasselquist & Nilsson, 2012; Yang *et al.*, 2021). Therefore, considering that we do not
464 know when individuals will pay the cost of an energetic demand, possibly some individuals
465 would express the effect of chronic stress immediately after the experiment is finished and
466 others would show carry-over effects by responding later to the challenge (Senner *et al.*,
467 2015). Even so, since all the probabilities of transition seem to increase after the stress
468 protocol, this may indicate that individuals would be more flexible after the stress protocol, or
469 at least changing their INP would be less expensive in terms of energy budget, which would
470 represent an extra advantage to deal with environmental complexity (Pirotta *et al.*, 2022). In
471 the recent years, questionings have arisen about how individuals will cope with, for instance,

472 weather variations related to climate change and its consequences at different scales
473 (Burggren, 2018; Cunningham, Gardner & Martin, 2021). Possibly, an increased plasticity in the
474 INE interplay response might represent an advantage to cope with environmental variability
475 and more flexible individuals might increase their probability of survival due to their greater
476 adaptability to different and challenging situations (Cockrem, 2013). Moreover, as variations in
477 temperature may also imply changes in pathogens richness and burden the higher flexibility
478 underlying a change in an INP would lead to an increased ability to cope with them (Sergio,
479 Blas & Hiraldo, 2018). The previous interpretation of our results in the context of global
480 warming would also be valid for a variety of scenarios to come: pollution, alteration of
481 husbandry procedures due to new regulations, and even selective processes could be analyzed
482 in this frame, if we can dissect how each challenge is integrated at the individual level. The
483 consecutive integration of the responses of the group of individuals defining the population,
484 will complement the information with a new perspective as previously shown. Additionally,
485 assessing INPs would allow to identify potential responses to environmental challenges in
486 poultry species allowing to develop management strategies associated to them (Nazar &
487 Estevez, 2022) and this may possibly be applied in other domestic species of economic
488 interest.

489 In summary, our research provides new insights into understanding how species respond to
490 the passage of time and face environmental challenges across different scales of analysis. Even
491 in highly selected species like quail, it appears that individuals retain their vital ability to adapt
492 their physiological capabilities over lifespan due to internal physiological changes, such as
493 sexual maturation or aging. However, when integrated, these changes seem to be less relevant
494 at the population scale, as seen in the case of INPs. On the contrary, external challenges would
495 act as disruptive events causing changes at the population scale but having a slight effect on
496 the individuals.

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Experimental design: F.N.N, R.H.M.,S.G.C. Laboratory Analysis: M.F.P, V.I.C; R.P; FNN. Statistical Analysis and figures preparation: A.M; Original draft writing: A.M., F.N.N. Manuscript revision: F.N.N, R.H.M.,S.G.C; R.P. V.I.C,M.F.P. All authors have reviewed and approved the final manuscript.

Data Availability: Data is available at https://data.mendeley.com/datasets/vs48x3x9f2/1. Contact e-mail: franconicolas.nazar@ehu.eus

References

Ashley, N.T. & Demas, G.E. (2017). Neuroendocrine-immune circuits, phenotypes, and interactions. *Hormones and Behavior* **87**, 25–34.

Auer, K.E., Kußmaul, M., Möstl, E., Hohlbaum, K., Rülicke, T. & Palme, R. (2020). Measurement of Fecal Testosterone Metabolites in Mice: Replacement of Invasive Techniques. *Animals (Basel)* **10**, 165.

Bishop, Y.M.M., Fienberg, S.E. & Holland, P.W. (1974). Models for Measuaring Change. In *Discrete Multivariate Analysis. Theory and practice*: 257–279.

Blas, J. & Fairhurst, G.D. (2021). Stress ecophysiology. In *Sturkie's Avian Physiology*: 1029–1078.

Bókony, V., Lendvai, Á.Z., Likér, A., Angelier, F., Wingfield, J.C. & Chastel, O. (2009). Stress Response and the value of reproduction: Are birds prudent parents? *American Naturalist* **173**, 589–598.

Buehler, D.M., Tieleman, B.I. & Piersma, T. (2010). How Do Migratory Species Stay Healthy Over the Annual Cycle? A Conceptual Model for Immune Function and For Resistance to Disease. *Integrative and Comparative Biology* **50**, 346–357.

Burggren, W. (2018). Developmental phenotypic plasticity helps bridge stochastic weather events associated with climate change. *Journal of Experimental Biology* **221**, jeb161984.

Caliva, J.M., Kembro, J.M., Pellegrini, S., Guzman, D.A. & Marin, R.H. (2017). Unexpected results when assessing underlying aggressiveness in Japanese quail using photocastrated stimulus birds. *Poultry Science* **96**, 4140–4150.

Cockrem, J.F. (2013). Corticosterone responses and personality in birds: Individual variation and the ability to cope with environmental changes due to climate change. *General and Comparative Endocrinology* **190**, 156–163.

Cohen, A.A., Martin, L.B., Wingfield, J.C., McWilliams, S.R. & Dunne, J.A. (2012). Physiological regulatory networks: Ecological roles and evolutionary constraints. *Trends in Ecology and Evolution* **27**, 428–435.

Colditz, I.G., Hine, B.C., Colditz, I.G. & Hine, B.C. (2016). Resilience in farm animals: biology, management, breeding and implications for animal welfare. *Animal Production Science* **56**, 1961–1983.

Crawley, M.J. (2007). *The R Book*. *The R Book*. Firt. Chichester, UK: John Wiley & Sons.

541 Cunningham, S., j, Gardner, J., L. & Martin, R., O. (2021). Opportunity costs and the response of
542 birds and mammals to climate warming. *Frontiers in Ecology and the Environment* **19**,
543 300–307.

544 Dhabhar, F.S. (2009). Enhancing versus Suppressive Effects of Stress on Immune Function:
545 Implications for Immunoprotection and Immunopathology. *Neuroimmunomodulation*
546 **16**, 300.

547 Elenkov, I.J., Kvetnansky, R., Hashiramoto, A., Bakalov, V.K., Link, A.A., Zachman, K., Crane, M.,
548 Jezova, D., Rovensky, J., Dimitrov, M.A., Gold, P.W., Bonini, S., Fleisher, T., Chrousos,
549 G.P. & Wilder, R.L. (2008). Low- versus high-baseline epinephrine output shapes
550 opposite innate cytokine profiles: presence of Lewis- and Fischer-like neurohormonal
551 immune phenotypes in humans? *Journal of immunology (Baltimore, Md.: 1950)* **181**,
552 1737–1745.

553 Forsman, A. (2015). Rethinking phenotypic plasticity and its consequences for individuals,
554 populations and species. *Heredity* **115**, 276–284.

555 Garcia-Reyero, N. (2018). The clandestine organs of the endocrine system. *General and*
556 *Comparative Endocrinology* **257**, 264–271.

557 Garland, T., Downs, C.J. & Ives, A.R. (2022). Trade-offs (And constraints) in organismal biology.
558 *Physiological and Biochemical Zoology* **95**, 82–112.

559 Giayetto, O., Videla, E.A., Chacana, P., Jaime, C., Marín, R.H. & Nazar, F.N. (2020). Modulating
560 offspring responses: Concerted effects of stress and immunogenic challenge in the
561 parental generation. *Journal of Experimental Biology* **223**.

562 Del Giudice, M., Buck, C.L., Chaby, L.E., Gormally, B.M., Taff, C.C., Thawley, C.J., Vitousek, M.N.
563 & Wada, H. (2018). What Is Stress? A Systems Perspective. *Integrative and*
564 *comparative biology* **58**, 1019–1032.

565 Goerlich, V.C., Nätt, D., Elfwing, M., Macdonald, B. & Jensen, P. (2012). Transgenerational
566 effects of early experience on behavioral, hormonal and gene expression responses to
567 acute stress in the precocial chicken. *Hormones and Behavior* **61**, 711–718.

568 Guibert, F., Lumineau, S., Kotrschal, K., Möstl, E., Richard-Yris, M.-A. & Houdelier, C. (2013).
569 Trans-generational effects of prenatal stress in quail. *Proceedings of the Royal Society*
570 *B: Biological Sciences* **280**, 20122368.

571 Halsey, L.G., Green, J.A., Twiss, S.D., Arnold, W., Burthe, S.J., Butler, P.J., Cooke, S.J., Grémillet,
572 D., Ruf, T., Hicks, O., Minta, K.J., Prystay, T.S., Wascher, C.A.F. & Careau, V. (2019).
573 Flexibility, variability and constraint in energy management patterns across vertebrate
574 taxa revealed by long-term heart rate measurements. *Functional Ecology* **33**, 260–272.

575 Hasselquist, D. & Nilsson, J.Å. (2012). Physiological mechanisms mediating costs of immune
576 responses: What can we learn from studies of birds? *Animal Behaviour* **83**, 1303–1312.

577 Huss, D., Poynter, G. & Lansford, R. (2008). Japanese quail (Coturnix japonica) as a laboratory
578 animal model. *Lab Anim (NY)* **37**, 513–519.

579 Jacobs, J.D. & Wingfield, J.C. (2000). Endocrine Control of Life-Cycle Stages: A Constraint on
580 Response to the Environment? *The Condor* **102**, 35–51.

581 Jimeno, B., Briga, M., Hau, M. & Verhulst, S. (2018). Male but not female zebra finches with
582 high plasma corticosterone have lower survival. *Functional Ecology* **32**, 713–721.

583 Johnston, C.E., Hartley, C., Salisbury, A.-M. & Wigley, P. (2012). Immunological Changes at
584 Point-of-Lay Increase Susceptibility to Salmonella enterica Serovar Enteritidis Infection
585 in Vaccinated Chickens. *PLOS ONE* **7**, e48195.

586 Klein, S.L. & Flanagan, K.L. (2016). Sex differences in immune responses. *Nat Rev Immunol* **16**,
587 626–638.

588 Kogut, M.H. (2022). Immunophysiology of the avian immune system. In *Sturkie's Avian*
589 *Physiology*: 571–590.

590 Koutsos, E.A. & Klasing, K.C. (2014). *Factors Modulating the Avian Immune System. Avian*
591 *Immunology*. Elsevier Ltd.

592 Lattin, C.R., Waldron-Francis, K. & Romero, L.M. (2013). Intracellular glucocorticoid receptors
593 in spleen, but not skin, vary seasonally in wild house sparrows (Passer domesticus).
594 *Proceedings. Biological sciences* **280**.

595 Lee, K.A. (2006). Linking immune defenses and life history at the levels of the individual and
596 the species. *Integrative and Comparative Biology* **46**, 1000–1015.

597 Lipshutz, S.E., George, E.M., Bentz, A.B. & Rosvall, K.A. (2019). Evaluating testosterone as a
598 phenotypic integrator: From tissues to individuals to species. *Molecular and Cellular*
599 *Endocrinology* **496**, 110531.

600 Loonstra, A.H.J., Verhoeven, M.A., Senner, N.R., Hooijmeijer, J.C.E.W., Piersma, T. & Kentie, R.
601 (2019). Natal habitat and sex-specific survival rates result in a male-biased adult sex
602 ratio. *Behavioral Ecology* **30**, 843–851.

603 Marin, R.H., Benavidez, E., Garcia, D.A. & Satterlee, D.G. (2002). Sex Differences in Central
604 Benzodiazepine Receptor Densities and Circulating Corticosterone Release After Acute
605 Stress in Broiler Chicks1. *Poultry Science* **81**, 261–264.

606 Mcewen, B.S. & Wingfield, J.C. (2003). The concept of allostasis in biology and biomedicine.
607 *Hormones and behavior* **43**, 2–15.

608 Møller, A.P., Sorci, G. & Erritzøe, J. (1998). Sexual Dimorphism in Immune Defense. *The*
609 *American Naturalist* **152**, 605–619.

610 Moore, M.P. & Martin, R.A. (2019). On the evolution of carry-over effects. *Journal of Animal*
611 *Ecology* **88**, 1832–1844.

612 Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., And, ~ & Coffman, R.L. (1986).
613 Definition According to Profiles of Lymphokine Activities and Secreted Proteins **136**.

614 Nazar, F.N. (2015). Determinación de fenotipos inmunoneuroendócrinos y su modulación por
615 situaciones de estrés crónico en 2 modelos de aves que difieren en el grado de
616 domesticación: Coturnix coturnix y Gallus gallus. *Universidad Nacional de Córdoba*
617 **Córdoba**, 140.

618 Nazar, F.N., Barrios, B.E., Kaiser, P., Marin, R.H. & Correa, S.G. (2015). Immune Neuroendocrine
619 Phenotypes in Coturnix coturnix: Do Avian Species Show LEWIS/FISCHER-Like Profiles?
620 *PLOS ONE* **10**, e0120712.

621 Nazar, F.N. & Estevez, I. (2022). The immune-neuroendocrine system, a key aspect of poultry
622 welfare and resilience. *Poultry Science* **101**, 101919–101919.

623 Nazar, F.N., Magnoli, A.P., Dalcero, A.M. & Marin, R.H. (2012). Effect of feed contamination
624 with aflatoxin B1 and administration of exogenous corticosterone on Japanese quail
625 biochemical and immunological parameters. *Poultry Science* **91**, 47–54.

626 Nazar, F.N. & Marin, R.H. (2011). Chronic stress and environmental enrichment as opposite
627 factors affecting the immune response in Japanese quail (Coturnix coturnix japonica).
628 *Stress (Amsterdam, Netherlands)* **14**, 166–173.

629 Nazar, F.N., Videla, E.A., Fernandez, M.E., Labaque, M.C. & Marin, R.H. (2018). Insights into
630 thermal stress in Japanese quail (Coturnix coturnix): dynamics of immunoendocrine
631 and biochemical responses during and after chronic exposure. *Stress* **21**, 257–266.

632 Nazar, N.F., Estevez, I., Correa, S.G. & Marin, R.H. (2017). Stress Induced Polarization of
633 Immune-Neuroendocrine Phenotypes in Gallus gallus. *Scientific Reports* **7**, 1–11.

634 Nystrand, M. & Dowling, D.K. (2020). Effects of immune challenge on expression of life-history
635 and immune trait expression in sexually reproducing metazoans - A meta-analysis.
636 *BMC Biology* **18**, 1–17.

637 O'Connor, C.M., Norris, D.R., Crossin, G.T. & Cooke, S.J. (2014). Biological carryover effects:
638 linking common concepts and mechanisms in ecology and evolution. *Ecosphere* **5**,
639 art28.

640 Padgett, D.A. & Glaser, R. (2003). How stress influences the immune response. *Trends in*
641 *Immunology* **24**, 444–448.

642 Palme, R. & Möstl, E. (1997). Measurement of cortisol metabolites in faeces of sheep as a
643 parameter of cortisol concentration in blood. *Int. J. Mammal. Biol.* **62**, 192–197.

644 Piersma, T. & Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design.
645 *Trends in Ecology and Evolution* **18**, 228–233.

646 Pirotta, E., Thomas, L., Costa, D.P., Hall, A.J., Harris, C.M., Harwood, J., Kraus, S.D., Miller,
647 P.J.O., Moore, M.J., Photopoulou, T., Rolland, R.M., Schwacke, L., Simmons, S.E.,
648 Southall, B.L. & Tyack, P.L. (2022). Understanding the combined effects of multiple
649 stressors: A new perspective on a longstanding challenge. *Science of the Total*
650 *Environment* **821**, 153322.

651 Roberts, M.L., Buchanan, K.L., Evans, M.R., Marin, R.H. & Satterlee, D.G. (2009). The effects of
652 testosterone on immune function in quail selected for divergent plasma corticosterone
653 response. *Journal of Experimental Biology* **212**, 3125–3131.

654 Romero, L.M., Dickens, M.J. & Cyr, N.E. (2009). The reactive scope model - A new model
655 integrating homeostasis, allostasis, and stress. *Hormones and Behavior* **55**, 375–389.

656 Saino, N., Ambrosini, R., Caprioli, M., Romano, A., Romano, M., Rubolini, D., Scandolara, C. &
657 Liechti, F. (2017). Sex-dependent carry-over effects on timing of reproduction and
658 fecundity of a migratory bird. *Journal of Animal Ecology* **86**, 239–249.

659 Schoenle, L.A., Zimmer, C., Miller, E.T. & Vitousek, M.N. (2021). Does variation in
660 glucocorticoid concentrations predict fitness? A phylogenetic meta-analysis. *General*
661 *and Comparative Endocrinology* **300**, 113611.

662 Schöffmann, G., Winter, P., Palme, R., Pollak, A., Trittenwein, G. & Golej, J. (2009).
663 Haemodynamic changes and stress responses of piglets to surgery during total
664 intravenous anaesthesia with propofol and fentanyl. *Lab Anim* **43**, 243–248.

665 Segner, H., Verburg-van Kemenade, B.M.L. & Chadzinska, M. (2017). The immunomodulatory
666 role of the hypothalamus-pituitary-gonad axis: Proximate mechanism for

667 reproduction-immune trade offs? *Developmental & Comparative Immunology*,
668 Neuroendocrine-immune system interactions: An evolutionary perspective **66**, 43–60.

669 Senner, N.R., Conklin, J.R. & Piersma, T. (2015). An ontogenetic perspective on individual
670 differences. *Proceedings of the Royal Society B: Biological Sciences* **282**.

671 Sergio, F., Blas, J. & Hiraldo, F. (2018). Animal responses to natural disturbance and climate
672 extremes: a review. *Global and Planetary Change* **161**, 28–40.

673 Shankar, A., Graham, C.H., Canepa, J.R., Wethington, S.M. & Powers, D.R. (2019).
674 Hummingbirds budget energy flexibly in response to changing resources. *Functional*
675 *Ecology* **33**, 1904–1916.

676 Shini, S. & Kaiser, P. (2009). Effects of stress, mimicked by administration of corticosterone in
677 drinking water, on the expression of chicken cytokine and chemokine genes in
678 lymphocytes. *http://dx.doi.org/10.1080/10253890802526894* **12**, 388–399.

679 Spedicato, G.A. & Signorelli, M. (2014). The markovchain Package: A Package for Easily
680 Handling Discrete Markov Chains in R. *Cran*.

681 Stearns, S.C. (1989). The Evolutionary Significance of Phenotypic Plasticity: Phenotypic sources
682 of variation among organisms can be described by developmental switches and
683 reaction norms. *BioScience* **39**, 436–445.

684 Sternberg, E.M., Hill, J.M., Chrousos, G.P., Kamilaris, T., Listwak, S.J., Gold, P.W. & Wilder, R.L.
685 (1989a). Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis
686 activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. *Proc*
687 *Natl Acad Sci U S A* **86**, 2374–2378.

688 Sternberg, E.M., Young, W.S., Bernardini, R., Calogero, A.E., Chrousos, G.P., Gold, P.W. &
689 Wilder, R.L. (1989b). A central nervous system defect in biosynthesis of corticotropin-
690 releasing hormone is associated with susceptibility to streptococcal cell wall-induced
691 arthritis in Lewis rats. *Proceedings of the National Academy of Sciences of the United*
692 *States of America* **86**, 4771–4775.

693 Superina, M., Carreño, N. & Jahn, G.A. (2009). Characterization of seasonal reproduction
694 patterns in female pichis Zaedyus pichiy (Xenarthra: Dasypodidae) estimated by fecal
695 sex steroid metabolites and ovarian histology. *Animal Reproduction Science* **116**, 358–
696 369.

697 Taborsky, B., English, S., Fawcett, T.W., Kuijper, B., Leimar, O., McNamara, J.M., Ruuskanen, S.
698 & Sandi, C. (2021). Towards an Evolutionary Theory of Stress Responses. *Trends in*
699 *Ecology & Evolution* **36**, 39–48.

700 Uchiyama, R., Moritomo, T., Kai, O., Uwatoko, K., Inoue, Y. & Nakanishi, T. (2005). Counting
701 absolute number of lymphocytes in quail whole blood by flow cytometry. *The Journal*
702 *of veterinary medical science* **67**, 441–444.

703 Valdebenito, J.O., Halimubieke, N., Lendvai, Á.Z., Figuerola, J., Eichhorn, G. & Székely, T. (2021).
704 Seasonal variation in sex-specific immunity in wild birds. *Sci Rep* **11**, 1349.

705 Vincze, O., Vágási, C.I., Pénzes, J., Szabó, K., Magonyi, N.M., Czirják, G.Á. & Pap, P.L. (2022).
706 Sexual dimorphism in immune function and oxidative physiology across birds: The role
707 of sexual selection. *Ecology Letters* **25**, 958–970.

708 Walsberg, G.E. (2003). How useful is energy balance as a overall index of stress in animals?
709 *Hormones and Behavior* **43**, 16–17.

710 Wei, R., Listwak, S.J. & Sternberg, E.M. (2003). Lewis hypothalamic cells constitutively and
711 upon stimulation express higher levels of mRNA for pro-inflammatory cytokines and
712 related molecules: comparison with inflammatory resistant Fischer rat hypothalamic
713 cells. *Journal of Neuroimmunology* **135**, 10–28.

714 Wingfield, J.C., Pérez, J.H., Krause, J.S., Word, K.R., González-Gómez, P.L., Lisovski, S. &
715 Chmura, H.E. (2017). How birds cope physiologically and behaviourally with extreme
716 climatic events. *Philosophical Transactions of the Royal Society B: Biological Sciences*
717 **372**, 20160140.

718 Yang, X., Heinemann, M., Howard, J., Huber, G., Iyer-Biswas, S., Le Treut, G., Lynch, M.,
719 Montooth, K.L., Needleman, D.J., Pigolotti, S., Rodenfels, J., Ronceray, P., Shankar, S.,
720 Tavassoly, I., Thutupalli, S., Titov, D. V., Wang, J. & Foster, P.J. (2021). Physical
721 bioenergetics: Energy fluxes, budgets, and constraints in cells. *Proceedings of the*
722 *National Academy of Sciences* **118**, e2026786118.

723 Zelazowski, P., Smith, M.A., Gold, P.W., Chrousos, G.P., Wilder, R.L. & Stemberg, E.M. (1992).
724 In vitro Regulation of Pituitary ACTH Secretion in Inflammatory Disease Susceptible
725 Lewis (LEW/N) and Inflammatory Disease Resistant Fischer (F344/N) Rats. *NEN* **56**,
726 474–482.

727 Zimmer, C., Larriva, M., Boogert, N.J. & Spencer, K.A. (2017). Transgenerational transmission of
728 a stress-coping phenotype programmed by early-life stress in the Japanese quail.
729 *Scientific Reports* **7**, 1–19.

730 **Figure legends**

731 Fig. 1: Schematic conceptual representation of INPs in Coturnix coturnix (Nazar et al. 2015).
732 The variables set to determine the existence of avian INPs are represented at the side of each
733 bird. The size of the variable indicates if the animals show high or low responses in each of the

734 parameters in the INPs. LYMPH: lymphoproliferative response to PHA-P; Ab SRBC: antibody
735 response against SRBC; FLD: frequency of leukocyte distribution; level of expression of
736 mediators: IFN-γ and IL-1β (pro-inflammatory); and IL-4 and 13 (anti-inflammatory). "Fischer-
737 like" quail with high CORT levels also manifest high FLD and IL- 13, but low Lymph, Ab SRBC,
738 IFN-γ and IL-1β levels. "Lewis-like" counterparts have low CORT as well as low FLD and IL-13
739 responses, but high Lymph, Ab SRBC, IFN-γ and IL-1β responses. These two extreme groups of
740 birds do not differ in their IL-4 levels.

741 Fig. 2: Summary of the experimental protocol followed in this research. INPs were assessed in
742 a group of 96 quail during four experimental stages: 1) juveniles, 2) adults before an
743 unpredictable chronic stress protocol, 3) adults after an unpredictable chronic stress protocol,
744 4) senescent. Abrreviations: wk. (weeks), UCSE (Unpredictable Chronic Stress Exposure).

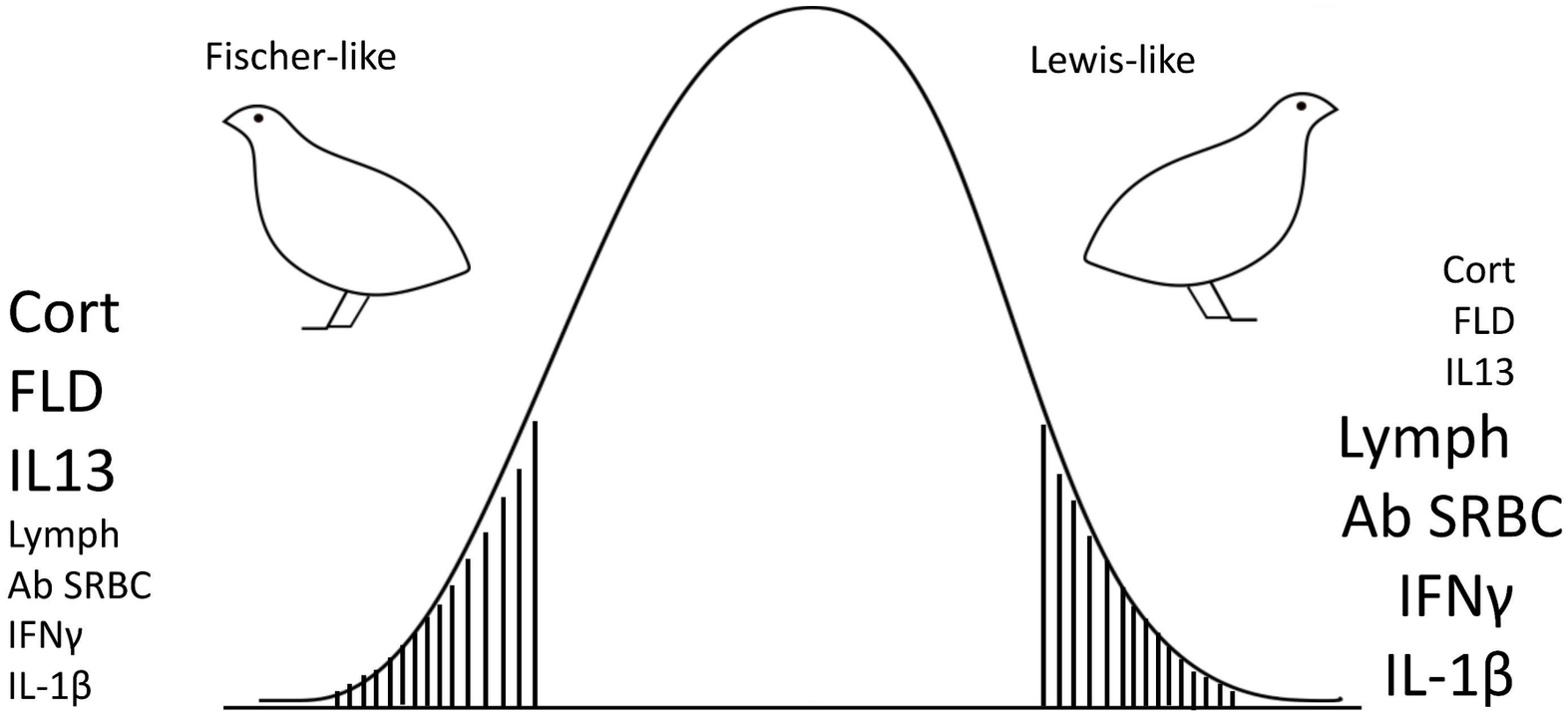
745 Fig. 3: Principal component analysis plots exploring data variability and clustering procedure.
746 Each dot represents one bird and each variable is represented by an arrow. Each plot
747 represents the INP during one stage of the experiment.

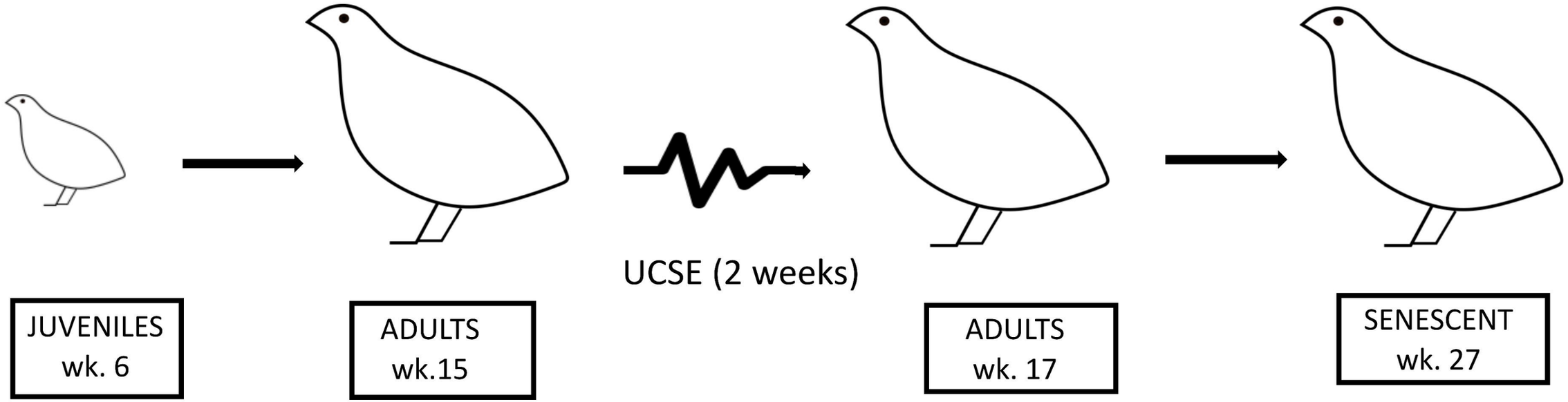
748 Fig. 4: Linear discriminant analysis plots. Each plot represents the INP during one stage of the
749 experiment. The variables Limph, Testosterone, AbSRBC, IFN-G and IL.4 showed similar
750 canonical axis 1 and canonical axis 2 values and therefore appear overlapped in the center of
751 the graph. In the adults post stress graph, also corticosterone had a similar canonical axis
752 displacement.

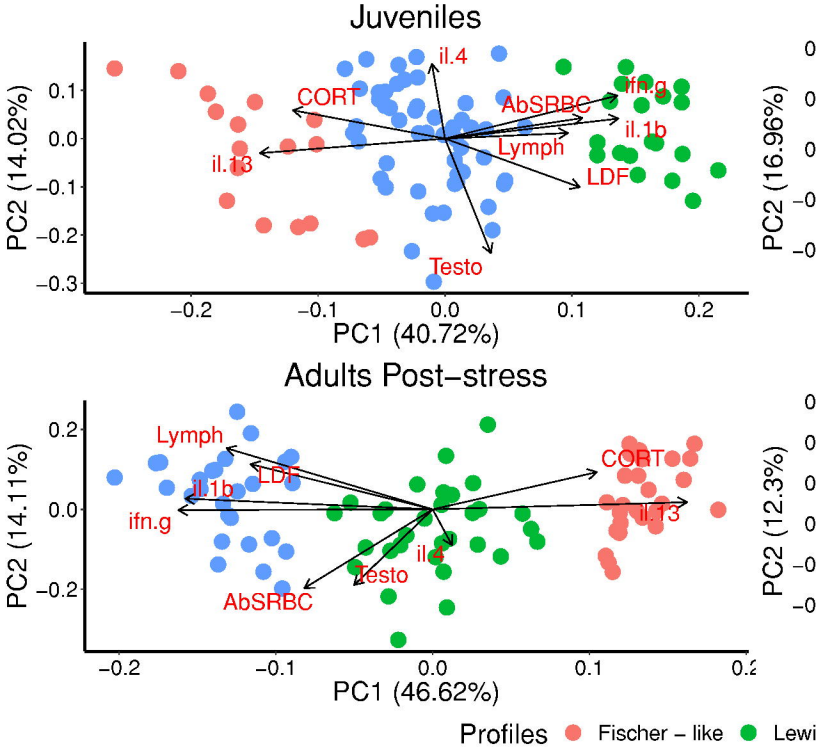
753 Fig. 5: Phenotype frequency distribution during the four stages of the experiment. Each plot
754 represents the INPs frequency during one stage of the experiment.

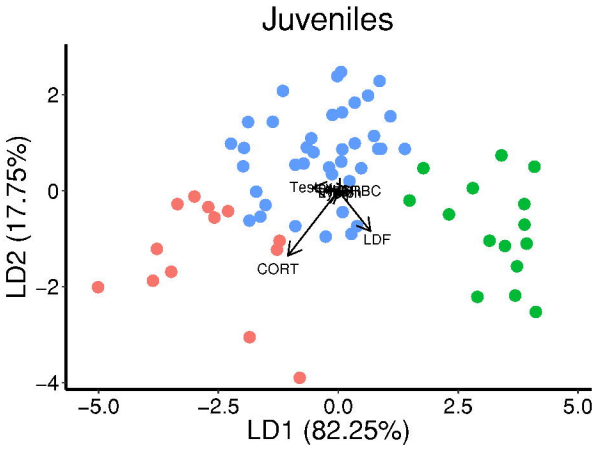
755 Fig. 6: Plot of transition probabilities. Arrows indicate changes from one phenotype to the
756 other or the permanence in that INP. Transition probabilities are indicated on the lines.

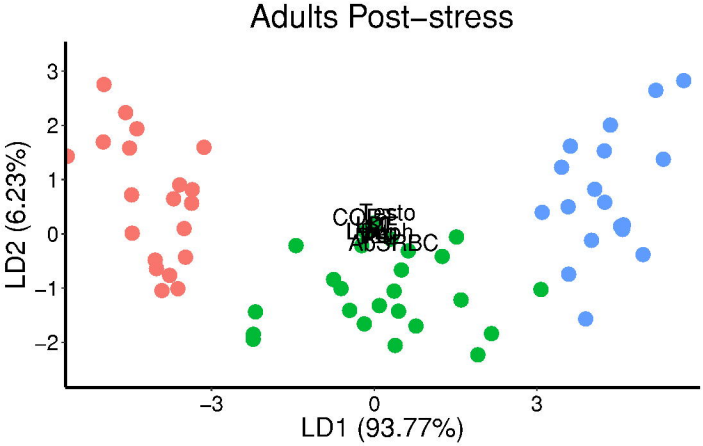
757


Fischer-like
Lewis-like
Cort
FLD
IL13
Lymph
Ab SRBC
IFNγ
IL-1β
Cort
FLD
IL13
Lymph
Ab SRBC
IFNγ
IL-1β

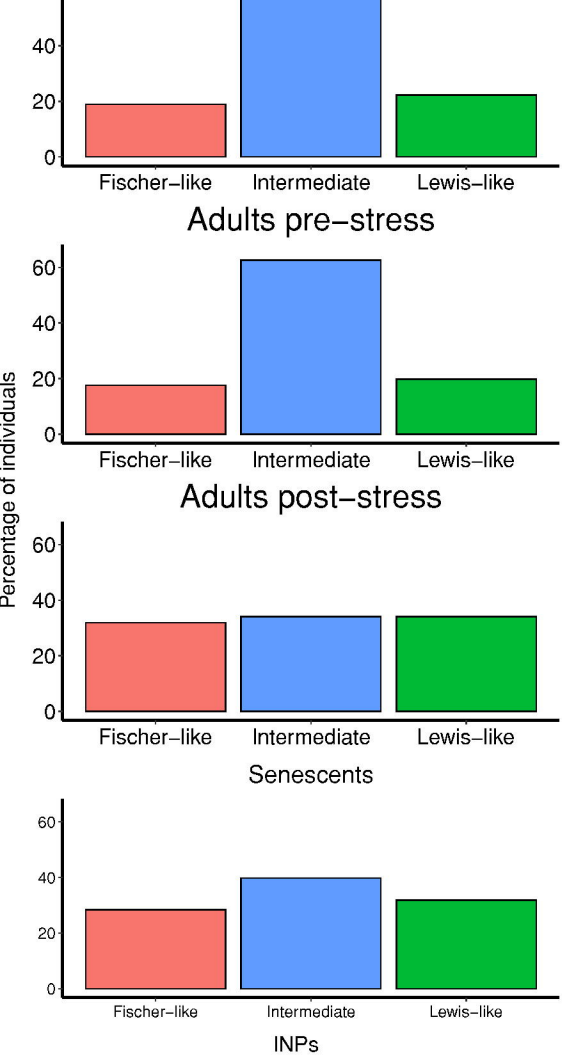

UCSE (2 weeks)
JUVENILES
wk. 6
ADULTS
wk.15
ADULTS
wk. 17
SENESCENT
wk. 27


Juveniles
il.4
CORT
AbSRBC
ifn.g
il.1b
Lymph
il.13
LDF
Testo
PC2 (14.02%)
PC1 (40.72%)
PC2 (16.96%)
Adults Post−stress
Lymph
LDF
il.1b
ifn.g
CORT
il.13
il.4
Testo
AbSRBC
PC2 (14.11%)
PC1 (46.62%)
PC2 (12.3%)
Profiles
Fischer − like
Lewi

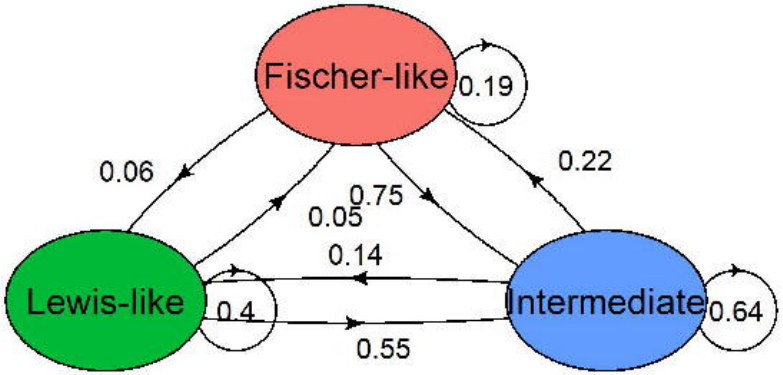




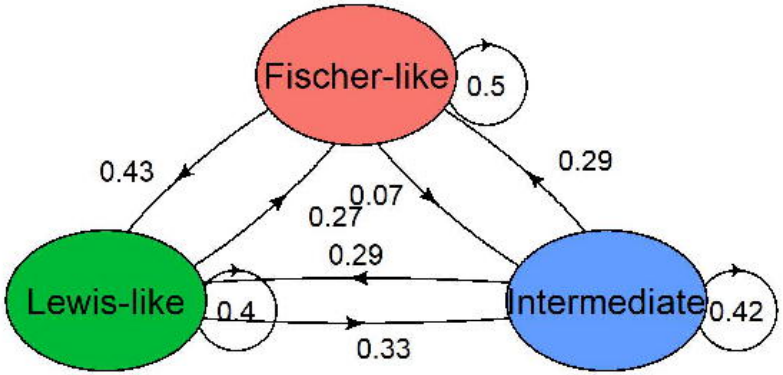
Profiles Fischer−like Le


Percentage of individuals
0
20
40
Fischer−like
Intermediate
Lewis−like
Adults pre−stress
0
20
40
60
Fischer−like
Intermediate
Lewis−like
Adults post−stress
0
20
40
60
Fischer−like
Intermediate
Lewis−like
Senescents
0
20
40
60
Fischer−like
Intermediate
Lewis−like
INPs

Transition from juveniles to adults pre-stress



Transition from adults pre-stress to adults post-stress



Transition from adults porst stress to senescence

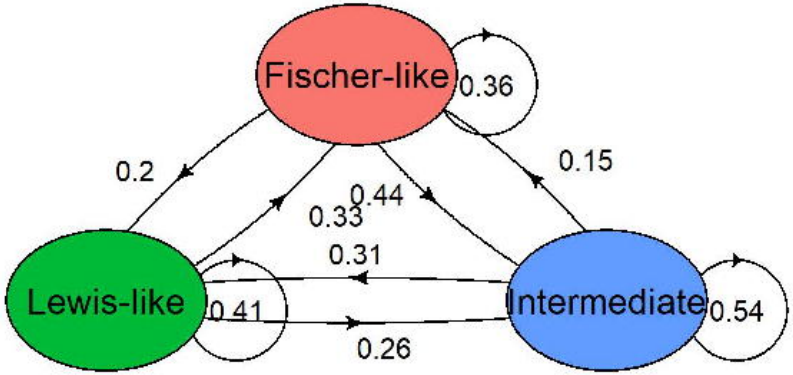


Table 1: Proportion analysis to assess differences among phenotypes in the three ontogenetic transitions studied.

	Juveniles	pre-stress adults	p-value
Fischer-like	0.19	0.18	0.5
Intermediate	0.55	0.63	0.67
Lewis-like	0.22	0.20	0.43
	pre-stress adults	post-stress adults	p-value
Fischer-like	**0.18**	**0.31**	**0.03**
Intermediate	**0.63**	**0.32**	**0.00005**
Lewis-like	**0.2**	**0.37**	**0.0093**
	post-stress adults	Senescent	p-value
Fischer-like	0.31	0.28	0.5
Intermediate	0.32	0.43	0.5
Lewis-like	0.37	0.32	0.67

Table 2: Proportion analysis comparing differences among sexes during the four experimental stages: juveniles, adults pre-stress, adults post-stress and senescent. Significative differences are indicated in bold.

Experimental Stage	Phenotype	Males	Females	p-value
Juveniles	Lewis-like	0.65	0.35	0.05692
	Intermediate	0.45	0.55	0.2186
	Fischer-like	0.41	0.59	0.25
Adults pre-stress	**Lewis-like**	**0.78**	**0.22**	**0.00135**
	Intermediate	0.54	0.46	0.2268
	Fischer-like	**0.13**	**0.86**	**0.00005**
Adults post-stress	Lewis-like	0.48	0.52	0.5
	Intermediate	**0.78**	**0.22**	**0.00006**
	Fischer-like	**0.27**	**0.73**	**0.001141**
Senescent	**Lewis-like**	**0.89**	**0.11**	**0.000000009**
	Intermediate	0.51	0.51	0.5
	Fischer-like	**0.16**	**0.84**	**0.000003**

Table 3: Transition matrices estimating the probability of an individual changing its phenotype from one experimental stage to the other or the probability of remaining in their original phenotype. On the left are represented the number (in absolute values) of individuals that belong to a determined INP and remained there in the next stage or had a different INP during the next stage. On the right is represented the transitions probabilities matrix created with data on the left. Transitions represented: from juveniles to adults pre-stress, from adults pre-stress to adults post-stress, and adults post-stress to senescence. Note that for DTMC analysis probabilities were calculated considering the partial totals for each phenotype in each stage.

		Absolute values				Probabilities			
		Adults pre-stress							
		Fischer-like	Lewis-like	Intermediate	Total	Fischer-like	Lewis-like	Intermediate	Total probabilities
Juveniles	Fischer-like	3	1	12	16	0.19	0.06	0.75	1.00
	Lewis-like	1	8	11	20	0.05	0.40	0.55	1.00
	Intermediate	11	7	33	51	0.22	0.14	0.64	1.00
		Adults post-stress							
Adults pre-stress	Fischer-like	7	6	1	15	0.50	0.43	0.07	1.00
	Lewis-like	4	6	5	15	0.27	0.40	0.33	1.00
	Intermediate	15	15	21	51	0.29	0.29	0.42	1.00
		Senescent							
Adults post-stress	Fischer	9	5	11	25	0.36	0.20	0.44	1.00
	Lewis	9	11	7	27	0.33	0.41	0.26	1.00
	Intermediate	4	8	14	26	0.15	0.31	0.54	1.00