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

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Abstract

In avian models (quail, *Coturnix coturnix*; 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), 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,

30 displaying greater homogeneity among INPs, provides the population with a greater advantage
31 to confronting potential environmental challenges to come.

32 **Introduction**

33 During an organism's lifetime, there are compromises in resource allocation among competing
34 traits known as trade-offs [1,2]. Such trade-offs may lead to individual differences within a
35 population causing immediate or delayed effects on fitness that can lead to changes in
36 individuals' phenotype [1,3]. Coping with a dynamic environment requires the mobilization of the
37 so called allostatic mediators, i.e., glucocorticoids and cytokines, that regulate a variety of
38 processes to ensure the individual's survival by operating at different concentrations in
39 accordance with organism´s demands [4]. Allostatic mediators work coordinating responses
40 among metabolic pathways which trigger physiological changes in several systems in response
41 to both internal and environmental keys [3,5]. Certain alterations are linked to natural processes
42 in an animal's life cycle such as sexual maturation, reproduction, or growth which are
43 energetically demanding mechanisms that require changes in resource allocation [6,7]. There are
44 other stimuli known as stressors that can disrupt homeostasis and trigger emergency
45 adjustments in animals' physiology and behavior, such as loss of social rank, severe weather,
46 habitat destruction, extreme climatic events and even human disturbance [8]. Therefore, stress
47 coping induces an emergency life history state that directly impacts energy allocation, thereby
48 reducing the energy available for other essential physiological functions, such as reproduction
49 or growth [9,10].

50 When the exposure to stressors is prolonged, animals develop a particular and potentially
51 persistent state of physiological imbalance, known as chronic stress (CS). In this state, the
52 physiological response either persists long after the stressor has ceased, or is repeatedly
53 activated [8,11]. CS implies heightened exposure of the organism to glucocorticoids and other
54 allostatic mediators, each with the potentiality to impact several physiological variables such

55 as white blood cell proliferation, cytokine secretion, antibody production, and cytolytic
56 activity, among others [12–15]. Consequently, CS can deplete an organism's available resources,
57 reducing its ability to cope with future stressors. Alternatively, CS may provide information to
58 deal with subsequent challenges influencing fitness critical variables like survival or
59 reproductive success [16] that can extend beyond the individual, with transgenerational effects
60 that impact subsequent generations [17–20]. Even so, coping with challenges will be conditioned
61 by the individual's specific phenotype and its capacity to adaptively shape its response to the
62 environment [3,21].

63 Differences among sexes can also influence immune and neuroendocrine responses due to the
64 effect of estrogens and testosterone and even some aspects of life history can be related [22,23].
65 It is widely accepted that females tend to allocate more resources to reproduction than males,
66 given the demands of egg development and parental care [24]. As a result, females exhibit a Th2-
67 biased immune response, with lower costs in terms of energy budget [24,25]. On the contrary, the
68 highest reproduction investment in males is located on mate attraction thus, they have more
69 energy available for an Th1 biased immunological response, i.e., cell mediated response [24,26].
70 Also, in birds, an anatomical difference is in accordance with this physiological observations,
71 males tend to have a smaller spleen than females [27]. This organ is the main leucocytes
72 producer in avians, therefore, differences in size between sexes can be related to variation in
73 parasites sensitivity [27]. Additionally, males tend to have a higher corticosterone response than
74 females, which can influence their survival rate [28].

75 Ontogeny is another relevant factor to be considered when studying coping mechanisms and
76 its effect will be logically influenced according to the individual's life stage. In general, when
77 juveniles face some kind of challenge this can cause changes in the reaction norm of certain
78 physiological responses causing permanent changes in the phenotype configuration [21,29]. On
79 the contrary, facing challenges during adulthood may lead to less permanent responses, for

80 that reason is considered that adults acquire a "reversible state effect" in which they will be
81 able to switch among phenotypes acquiring new configurations in response to the
82 environment [1,30]. To differentiate permanent transformation in the reaction norm that happen
83 when challenges occur in juvenile stages from those reversible in response to the environment
84 during adulthood, Piersma and Drent (2003) proposed the name "phenotypic flexibility" to the
85 second ones. This name refers to changes that occur when environmental conditions vary
86 rapidly and over shorter timescales than a lifetime. The ability of changing and acquiring a
87 "reversible state effect" may turn into an evolutionary advantage because the bigger the
88 diversity of traits in a population, the more prepared it will be to face future unknown
89 challenges [31].

90 Modifying phenotype configuration may lead to a reorganization in resource allocation that
91 can have an influence on fitness [32,33]. In some cases, these effects will be immediate and in
92 others will not, driving to carry-over effects that can influence on individual's performance to
93 future situations [34] and impact on processes at the population level such as survival rate [35,36]. In
94 some cases, carry-over effects are not seen until the next generation and the price that
95 individuals pay for facing a challenge are evident in their descendance [1,20]. Therefore,
96 understanding how ontogeny and potential carry-over effects influence alterations in their
97 immune and neuroendocrine configuration is crucial. This information is valuable for assessing
98 the impact of challenges in both the short and long term, both at the individual and population
99 levels.

100 In the last decades, immune neuroendocrine phenotypes (INPs) have been described in
101 mammalians including humans [37], and related species like rodents [38]. Later on, in
102 representatives of different evolutionary histories such as avian species like hens (*Gallus*
103 *gallus*; Nazar *et al.*, 2017), or quail (*Coturnix coturnix*; Nazar, 2015). INPs are a set of
104 characteristics that identify individuals in their neuro-hormonal and immunological responses,

similar among them but different from another group within the same animal population (Sternberg *et al.*, 1989; Elenkov *et al.*, 2008; Ashley and Demas, 2017). As a consequence, INPs provide a valuable framework for assessing population responses to chronic and stressful environmental challenges (Sternberg *et al.*, 1989; Nazar *et al.*, 2017). INPs are characterized by neuroendocrine mediator concentrations, activity and expression of hormonal receptors, and cytokine belonging to pro or anti-inflammatory profiles. In quail and hens three INPs have been described (i) the Lewis-like phenotype characterized by low corticosterone (CORT) levels, high proinflammatory mediators' levels (IFN-γ and IL-1β), high lymphoproliferative response to Phytohemagglutinin-p (PHA-P), high antibody response against to sheep red blood cells (SRBC), low innate : acquired immunity relationship evaluated by the frequency of leukocyte distribution (FLD) and anti-inflammatory mediators' response (IL-13, IL-4); (ii) the Fischer-like phenotype, characterized by high CORT levels, high levels of anti-inflammatory mediators (IL-4 and IL-13), high FLD, and low lymphoproliferative response to PHA-P, Ab SRBC, and proinflammatory response [42]; and (iii) the intermediate phenotype, in between these two extreme phenotypes (Fig. 1).

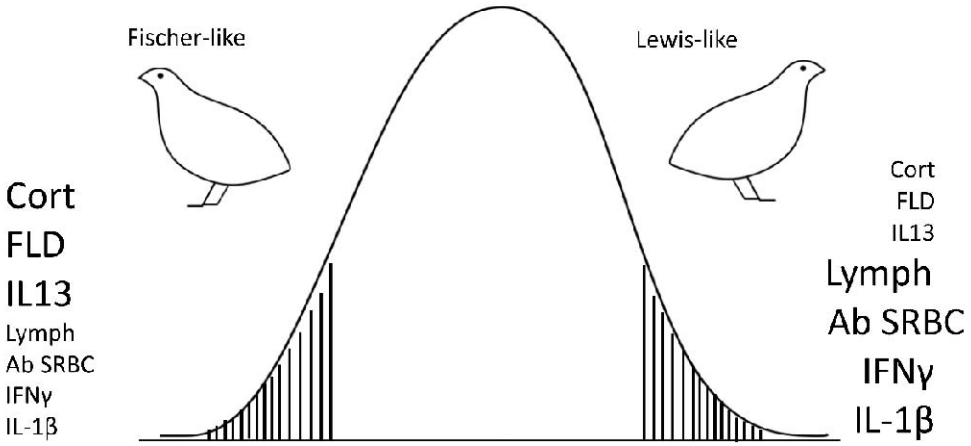


Fig. 1: Schematic conceptual representation of INPs in Coturnix coturnix (Nazar et al. 2015). The variables set to determine the existence of avian INPs are represented at the side of each bird. The size of the variable indicates if the animals show high or low responses in each of the parameters in the INPs. LYMPH: lymphoproliferative response to PHA-P; Ab SRBC: antibody response against SRBC; FLD: frequency of leukocyte distribution; level of expression of

mediators: IFN-γ and IL-1β (pro-inflammatory); and IL-4 and 13 (anti-inflammatory). "Fischer-like" quail with high CORT levels also manifest high FLD and IL- 13, but low Lymph, Ab SRBC, IFN-γ and IL-1β levels. "Lewis-like" counterparts have low CORT as well as low FLD and IL-13 responses, but high Lymph, Ab SRBC, IFN-γ and IL-1β responses. These two extreme groups of birds do not differ in their IL-4 levels

The ability of individuals to deal with a disease will be related to the INP presented (Sternberg *et al*., 1989; Wei *et al*., 2003; Nazar *et al.*, 2015, 2017). As a consequence INPs frequencies in a population can affect its ability to cope with immune neuroendocrine challenges because a new and differential response will be given to the presence of parasites, and/or other pathogens affecting individuals within the population [15,43]. Also, INPs imply an underlying differential INE response, i.e., of the hypothalamus pituitary adrenal axis (HPA), Fischer-like individuals have a higher corticosterone response than Lewis-like individuals [15,42]. This differential response to corticosterone will impact various other life-history traits, such as metabolic rate (Jimeno et al., 2018b), and the behaviors of the animals [44]. Consequently, INE configuration (either synthesized or not in the concept of an INP) could be an indicator of the population's substrate to cope with environmental challenges.

As stated before, in stressful situations, the frequency of INPs changes [15]. However, the contribution of each phenotype to this new configuration is still not clear. We are not certain whether the majority of individuals altering their phenotype belong to the intermediate INP category, or if those from one extreme phenotype transition to the opposite extreme, or any other potential combination. Understanding the dynamics of change at the individual level can illuminate phenomena even at the population level. It offers complementary information that is currently lacking regarding individuals' physiological integration of environmental and ontogenetic challenges. In this context, we studied INPs configuration during different ontogenetic stages in Japanese quail as an avian model (juveniles and adults). As adults, we conducted further studies to address whether exposure to a disruptive challenging event, characterized by unpredictable and diverse chronic stressor exposures (UCSE), could modify the configuration of INPs at both the individual and population levels, and whether these changes could persist until senescence. We hypothesized that at a population level, INPs will

153 be modulated by UDCS acting as a disruptive force that alters the frequency of the phenotypes
154 within the population. We expect that CS will have effects on the variables that integrate the
155 INE interface, modifying INPs' expression at the population level due to individual's quail
156 adjustments.

157 **Results**

158 The three groups identified by the cluster analysis are represented in the PCA plots (Fig.3),
159 using biplots as a graphical strategy to represent the phenomena as previously mentioned.
160 Cluster analyses was performed as previously described [15]. Briefly K-means analysis distributed
161 the birds in 3 main dissimilar groups during the four experimental stages studied. In the first
162 two experimental stages (juveniles and adults pre-stress) the number of individuals belonging
163 to the intermediate phenotype is higher than the number of individuals belonging to extreme
164 phenotypes, i.e., Fischer-like and Lewis-like, as it can be seen in the PCA plots (Fig. 2a and b).
165 After the stress protocol the number of individuals with Fischer and Lewis like phenotypes
166 increased and this change in the representation of individuals with extreme phenotypes
167 remained until senescence (Fig. 2b and c). The three groups, as separate corpus of birds,
168 remained differentiated along the four stages of this experiment.

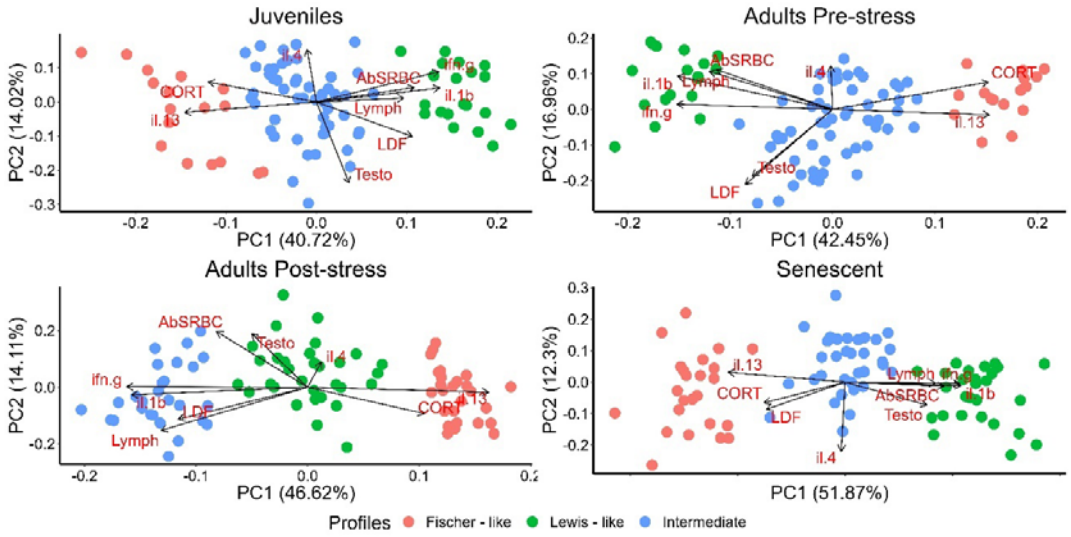


Fig. 2: Principal component analysis plots exploring data variability and clustering procedure. Each dot represents one bird and each variable is represented by an arrow. Each plot represents the INP during one stage of the experiment.

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

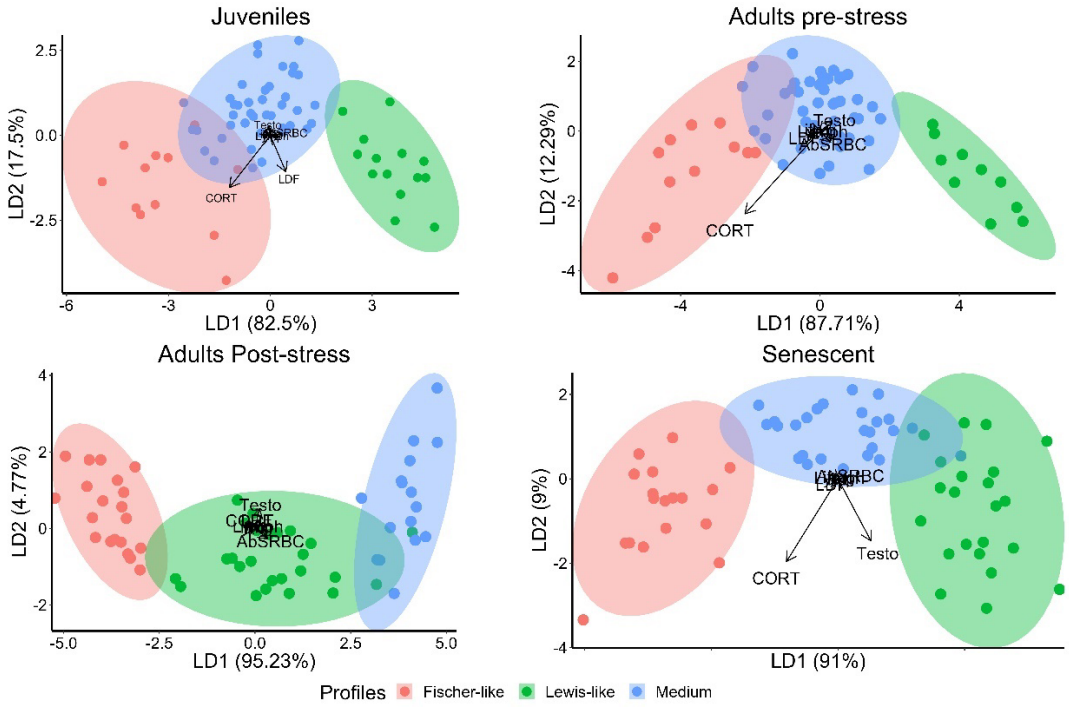


Fig. 3: Linear discriminant analysis plots. Each plot represents the INP during one stage of the experiment. The variables Limph, Testosterone, AbSRBC, IFN-G and IL.4 showed similar canonical axis 1 and canonical axis 2 values and therefore appear overlapped in the center of the graph. In the adults post stress graph, also corticosterone had a similar canonical axis displacement.

The frequency of the INPs indicated that, in the population studied, the prevailing phenotype during the two first experimental stages (juveniles and adults pre-stress), was the intermediate one, characterized by immune-neuroendocrine responses of intermediate magnitude. After stress, the frequency of the extreme phenotypes Lewis-like and Fischer-like increased significantly (Fig. 3, Table 3; p-value=0.0093 for Lewis-like and p-value = 0.03 for Fischer-like). Also, a reduction in the proportion of individuals with intermediate phenotype was observed in this experimental stage (p-value=0.00005). During senescence, frequencies of the extreme phenotypes seemed to sustain a uniform distribution without significant changes compared to the previous stage (adults post-stress) (Fig. 3). Table 1 shows the results of the Proportion test comparing transitions among phenotypes. A significant increase in the proportion of extreme phenotypes and a reduction of the intermediate phenotype was observed only in the transition from adults' pre-stress to adults' post-stress (Indicated in bold). In all the other transitions, no significant differences were found.

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

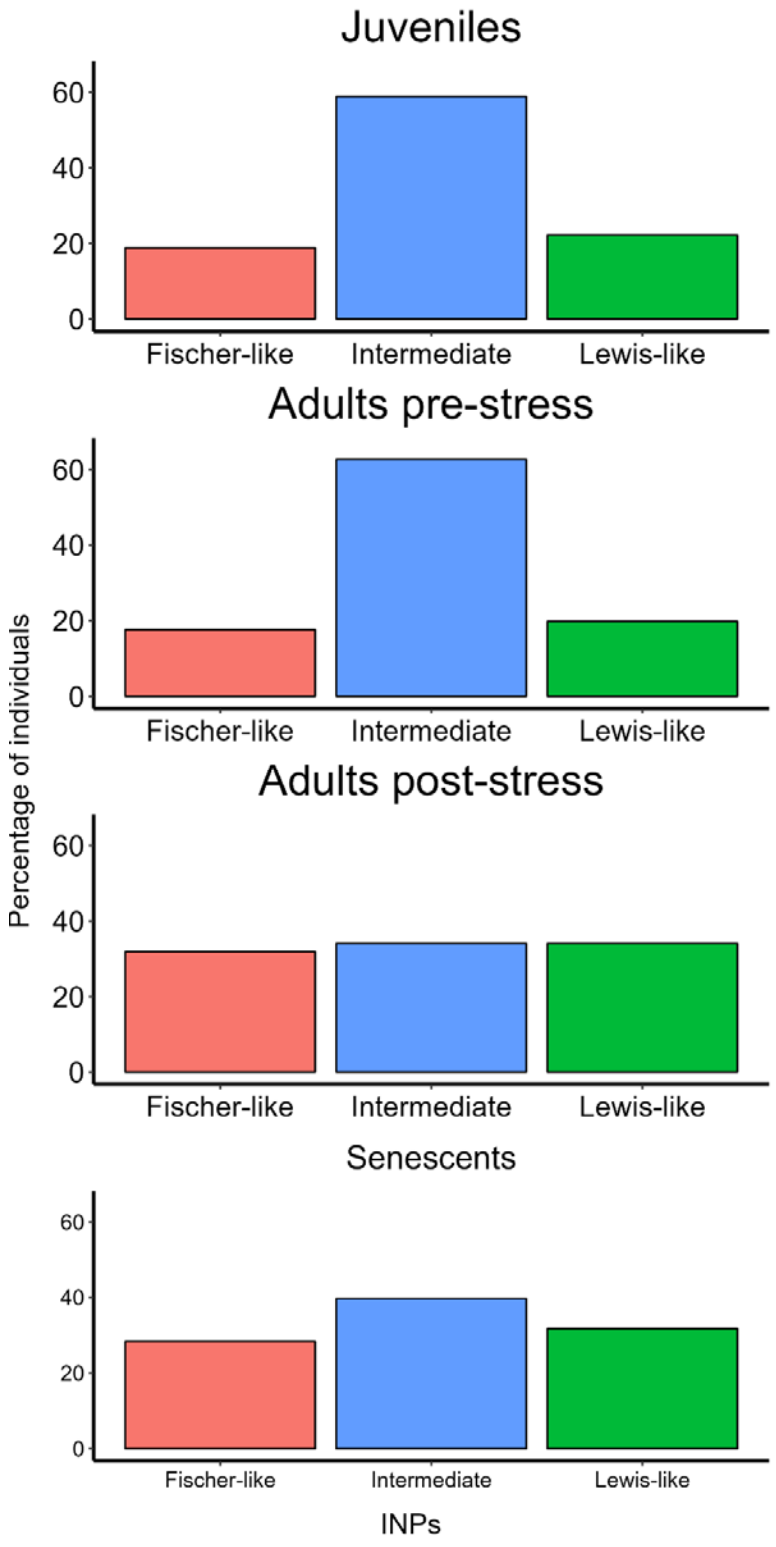


Fig. 4: Phenotype frequency distribution during the four stages of the experiment. Each plot represents the INPs frequency during one stage of the experiment.

208 The proportion analysis comparing the phenotypes among sexes within the same experimental
209 stage (Table 2) indicated that the proportion of females showing a Fischer-like phenotype was
210 significatively higher than males with this INP in adults pre-stress (p-value= 0.00005), in adults
211 post-stress (p-value = 0.001141), and in senescent quail (p-value=0.000003). On the contrary,
212 the proportion of males presenting a Lewis-like INP was significantly higher than the
213 proportion of females in the adults' pre-stress (p-value=0.00135), and in the senescent
214 experimental stages (p-value=0.000000009). In addition, the frequency of males with an
215 intermediate phenotype was significantly higher than the proportion of females in the adults'
216 post-stress experimental stage (p-value=0.00006). Only a trend for females showing a reduced
217 proportion of Lewis-like phenotype compared than males was found during the juvenile
218 assessment (Table 2).

219 **Table 2:** Proportion analysis comparing differences among sexes during the four experimental stages: juveniles,
220 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**

221

222 The probability of an individual changing its phenotype from one experimental stage to the
223 next one or the probability of remaining in their original phenotype was evaluated using a
224 DTMC analysis (Table 3). As shown, during the transition of juveniles to adults, the probability
225 of changing from Lewis-like or Fischer-like phenotypes to the intermediate was the highest in

both cases (0.55 and 0.75 respectively). In addition, most of the individuals belonging to the intermediate type remained in that state (0.64; Table 3; Fig. 5). After the stress protocol, the probability of individuals changing from Lewis-like (0.33) or Fischer-like (0.07) phenotypes to the intermediate phenotypes decreased (Table 3; Fig. 5), and the probability of individuals to change from intermediate phenotype to a Lewis-like (0.29) or a Fischer-like (0.29) increased. Moreover, the probability of Lewis-like individuals changing their phenotype to Fischer-like and vice versa (0.43 and 0.27 respectively) also increased in this transition. Finally, during the transition from post-stress adults to senescence (Table 3; Fig.5), the probability of individuals changing from an intermediate phenotype to a Lewis-like (0.31) remained high. The probability of an individual changing from a Lewis-like to Fischer-like or inversely (0.33 and 0.20 respectively) decreased compared to the transition from pre-stress adults and post-stress adults.

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-	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-	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

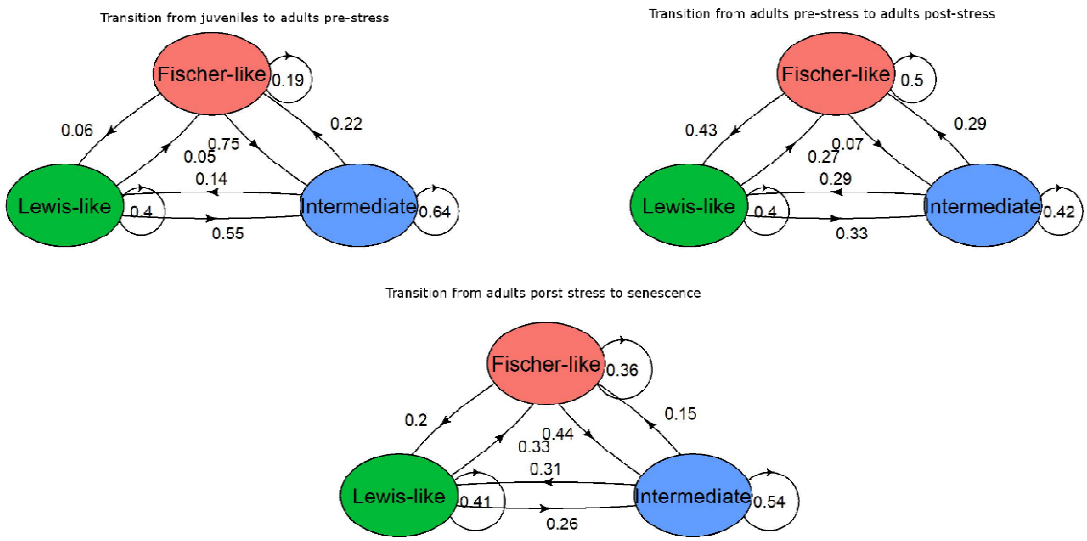


Fig. 5: Plot of transition probabilities. Arrows indicate changes from one phenotype to the other or the permanence in that INP. Transition probabilities are indicated on the lines.

Discussion

This study assesses changes in INE variables that lead to the configuration of INPs throughout ontogeny (juvenile and adult stages) evaluating also whether a significant disruptive event can modify previous adult configuration and whether this alteration persists when aging into senescence. In particular, we analyzed whether a chronic unpredictable stress disturbance at the INE level influences INPs proportions, their distributions at the population level and the probability of change (INP assessments in adults pre- and post-stress). It is of outmost importance to highlight that INPs are not considered here a fixed entity, but rather the array of INE responses that a particular individual can deploy in a certain moment of its ontogeny. Thus, each of the evaluated stages provide differential and complementary information. The two first stages i.e., juveniles and adults (Fig. 2), are related to physiological changes associated with regular life processes such as sexual maturation and growth [3]. Lastly, and using the second stage as an adult (non-disturbed) baseline, the third and fourth stages i.e., adults after a stress protocol and senescent individuals (Fig. 2), are related to the ability of individuals to cope with external challenges (post-stress sampling) and how challenged individuals reach senescence (last sampling).

264 Findings showed that it is possible to identify three different INPs in the population during the
265 whole study and the stages evaluated (Fig. 3). Corticosterone was determined as one of the
266 most important predictors of INPs during the four stages studied. Nevertheless, LDAs showed
267 that the relationship among the variables also changed along the stages (Fig. 3) which may be,
268 at least in part, related to the reorganization in resource allocation due to ontogenetic
269 variations [1,3]. Indeed, findings are consistent with the idea that, throughout their lives,
270 individuals undergo processes influenced by the costs of living, resulting in changes in the
271 relationships between immune and neuroendocrine factors and their respective significance
272 [6,7].

273 Some authors proposed that the stress response is influenced by both the life history traits of
274 the species [45] and the response potential of different tissues [46]. Furthermore the INPs as a
275 partially new factor to be considered and included in these equations has been suggested [37–
276 39,41,47]. Fischer-like individuals might be more related to anti-inflammatory and antibody-
277 mediated immune responses than Lewis-like individuals [15,42,48]. Therefore, individuals
278 expressing either one of or other of these two extreme phenotypes would have different
279 abilities to cope with diverse environmental conditions, and, as a consequence, they may show
280 distinct environmental sensitivity to stressors understanding them as challenges [49]. Changes in
281 INP configuration from one life stage to the other would imply a variation at the very individual
282 level in the ability to cope with a variety of challenges [15,38,42,47]. For example, and from a
283 theoretical point of view, if one individual with an intermediate INP before stress switches to a
284 Lewis-like after a chronic stress disturbance, its ability to develop robust inflammatory
285 responses will be increased, and therefore also their potential to deal with certain types of
286 pathogens [15,37,38]. On the contrary, if an individual acquires a Fischer-like configuration, the
287 outcome of its response to infections would be opposite to that of a Lewis-like configuration
288 [15,37,38]. These examples are just based on some of the shifts observed and, it is worth

289 highlighting, that these and any other shift in INP needs to be understood as a synthesis of a
290 change in the components of the INE system being integrated for analysis.

291 In juvenile and adult pre-stress, the frequency of extreme phenotypes was low, and the
292 frequency of the intermediate phenotype was notoriously higher (Fig. 4), which might
293 resemble a Gaussian distribution. Even so, no significant increase in the frequencies of the
294 transition from juveniles to adults was observed (Table 1). On the contrary, after the chronic
295 stress protocol, the population acquired a new distribution, more uniform, with a significant
296 increase in the proportion of the two extremes phenotypes and reduction of the intermediate
297 representatives during the transition from pre-stress to post- stress adults (Table 1). It is
298 relevant to recall that the stress protocol included not just a variety of different stressors but
299 also a strong unpredictable nature. Hence, as individuals confront pathogens by developing
300 the most effective response allowed by their immunological potential [33], shifting the
301 population to a better-balanced representation of INPs can be viewed as a highly efficient
302 strategy to deal with a broader range of immune challenges. This new distribution may imply
303 different possibilities to deal with environmental challenges, whereas a population "over-
304 expressing" only one INP may find this possibility limited [31,42]. Remarkably, this new
305 distribution remained until senescence (Fig. 4) without significant changes in the proportion of
306 individuals belonging to one or other INP (Table 1). These results suggest that the INP induced
307 changes, and therefore the population ability to deal with future unpredictable challenges,
308 have long lasting carry-over effects [1,15,50].

309 Additionally, the proportion of females expressing Fischer-like phenotype was significantly
310 higher than the proportion of males during adults pre-stress, adults post-stress and senescent
311 stages. On the contrary, males expressed a higher frequency of Lewis-like phenotype during
312 the same stages. These results may provide us with information on how both sexes cope with
313 challenges. Our results seem to be clear in indicating that some kind of polarization in the

314 expression of INPs occurs in the population, with males expressing more frequently a Th1
315 response than females, being more prepared for intracellular infections [51]. Possibly, this
316 polarization would be related to different energetic request in males and female in
317 reproduction investment: males tend to invest more energy in matte attraction reducing their
318 investment in immunological response to parasites [24,26]. Additionally, studies done in zebra
319 finches and broilers, indicated that males have a higher corticosterone response to stress [52]
320 and a lower survival rate after a stress induction than females [28]. Considering that Lewis-like
321 individuals will have lower basal corticosterone than Fischer-like or intermediate individuals,
322 increasing the number of males with this phenotype might represent an advantage, because
323 these individuals might be less responsive to future challenges increasing their probability of
324 survival. This proposal requires further study, and the relation between INPs and sex is a factor
325 that should be considered in future research.

326 From the individual's perspective, during the four stages studied, most quail acquired different
327 INPs configuration during the experiment, even without environmental challenges (Fig. 5).
328 Changes in INPs might be related to phenotypic flexibility meaning that some individuals would
329 display a variable expression of INPs within their lifespan [21]. Changes in the phenotype along
330 an individual's life can progress linearly, affecting the outcome of the next developmental step
331 mainly during juveniles stages, or can occur in a more flexible manner during adulthood when
332 individuals may change adaptively and repeatedly in response to environmental conditions [1,6].
333 The ability to vary INP in response to the environment will represent an immunological
334 advantage in some cases but, would this change worst the pain? Possibly it would depend on
335 the crosstalk of ontogenetic demands, individuals' energy budget and environmental
336 challenges [3,53,54]. Even if in our experiment, individuals were fed all in the same manner and
337 had water *ad libitum*, this does not mean that the use of energy is identical by all of them but
338 at least the access was similar. Some individuals will have a greater capacity to distribute
339 energy to all aspects of their life than others, and they will be able to intake, digest and utilize

340 greater amounts of energy than their conspecifics [33,55]. Therefore, ontogenetic variations like
341 sexual maturation and reproduction, might influence other physiological, i.e., INE systems such
342 as the immune system or the very HPA axis leading to variations in INPs throughout life [7,56] and
343 acting as endogenous disruptors that influence at the individual level without having a
344 notorious impact on the population global arrangement.

345 INP changes may synthesize a shift in INE responses and potential. This may then imply a
346 trade-off in energy allocation [3] and would be limited in its possibilities and scope by how much
347 energy individuals can efficiently use at any given moment [9,33,57]. Therefore, considering that
348 we do not know when individuals will pay the cost of an energetic demand, possibly some
349 individuals would express the effect of chronic stress immediately after the experiment is
350 finished and others would show carry-over effects by responding later to the challenge [1]. Even
351 so, since all the probabilities of transition seem to increase after the stress protocol, this may
352 indicate that individuals would be more flexible after the stress protocol, or at least changing
353 their INP would be less expensive in terms of energy budget, which would represent an extra
354 advantage to deal with environmental complexity[58]. In the recent years, questionings have
355 arisen about how individuals will cope with, for instance, weather variations related to climate
356 change and its consequences at different scales[30,59]. Possibly, an increased plasticity in the
357 immune neuroendocrine interplay response might represent an advantage to cope with
358 environmental variability and more flexible individuals might increase their probability of
359 survival due to their greater adaptability to different and challenging situations[60]. Moreover, as
360 variations in temperature may also imply changes in pathogens richness and burden the higher
361 flexibility underlying a change in an INP would lead to an increased ability to cope with them
362 [61]. The previous interpretation of our results in the context of global warming would also be
363 valid for a variety of scenarios to come: pollution, alteration of husbandry procedures due to
364 new regulations, and even selective processes could be analyzed in this frame, if we can
365 dissect how each challenge is integrated at the individual level. The consecutive integration of

366 the responses of the group of individuals defining the population, will complement the
367 information with a new perspective as previously shown.

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

376 **Materials and Methods**

377 *Animals and husbandry*

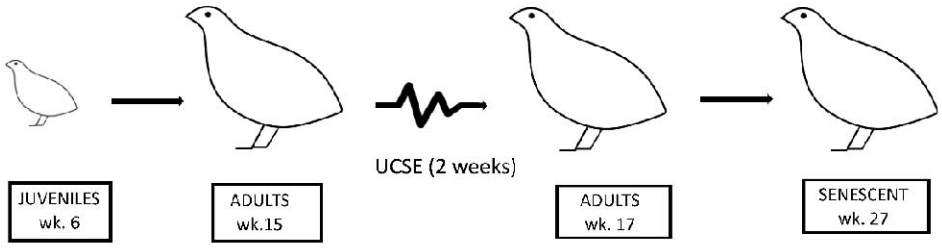
378 The present study used adult Japanese quail because they are recognized as an useful
379 laboratory model because of their physiology, small size, short life cycle, and low maintenance
380 cost [62]. The quail were raised as described elsewhere [63,64]. Animal care was provided in
381 adherence to Institutional Animal Care and Use Committee guidelines. The experiment
382 procedures were approved by the ethical committee at Instituto de Investigaciones Biológicas
383 y Tecnológicas (IIByT) in compliance with the legislation regarding the use of animals for
384 experimental and other scientific purposes (ACTA CICUAL N° 27, 09/04/2015) and are in
385 accordance with ARRIVE guidelines.

386 Briefly, from the first day of age (DA), quail were housed in enriched rearing boxes measuring
387 90x90x60 cm (length x width x height) in mixed groups of 40 individuals. A wire-mesh floor (1
388 cm grid) was raised 5 cm to allow excreta passage, and 50% of the total surface was covered
389 with corrugated cardboard. Each box had a feeder arranged in the front, 16 automatic drinkers
390 ("nipple" type), and a cover lid to prevent the escape of the birds and the loss of heat. The

391 breeding temperature was lowered gradually and weekly from hatching to 28 DA, starting
392 from an initial temperature of 37ºC until reaching 24ºC [65]. The ambient relative humidity
393 recorded ranged between 50 and 60%. The quail were kept in a daily cycle of 14 h of light (330-
394 350 lx) and 10 h of dark (14:10 L:D) during the study, turning on the lights at 06:00 h. At 28 DA,
395 quail were sexed using breast plumage coloration and wing banded for later identification. At
396 35 DA, 48 male-female pairs were randomly re-housed in 39x19x25 cm (length x width x
397 height) pedigree enclosures. The environmental enrichment program included corrugated
398 cardboard and plastic grass on the floor, and rough plastic sheets and wooden boards as
399 enrichment elements that were alternated weekly to minimize the habituation and to avoid
400 the disturbance of a more frequent change [66]. Quail starter diet (24% crude protein; 2900 kcal
401 metabolizable energy kg−1) was used for the first 4 weeks of life. A laying diet was provided
402 afterward (21% protein, 2750 kcal ME kg−1). The supply of water and food was *ad libitum*.

403 *Experimental design:*

404 The experiment consisted in evaluating selected INE representative variables (previously
405 associated with INPs, see below) in four target stages of analysis (Fig. 6). 1) juvenile quail (6
406 wks of age); 2) adult quail (15 wks of age), when birds evidenced full maturity (fully active
407 foam gland in males and females peaking egg production (~one egg per day) and prior to birds'
408 exposure to a chronic disruptive event (UCSE protocol, supplementary table S1); 3) After the
409 UCSE ended (17 wks of age) and 4) during senescence (27 wks of age).



410
411 *Fig. 6: Summary of the experimental protocol followed in this research. INPs were assessed in a group of 96 quail*

412 *during four experimental stages: 1) juveniles, 2) adults before an unpredictable chronic stress protocol, 3) adults*
413 *after an unpredictable chronic stress protocol, 4) senescent. Abrreviations: wk. (weeks), UCSE (Unpredictable Chronic*
414 *Stress Exposure)*

415 *Variables tested:* The set of variables evaluated for characterizing INPs in every individual are
416 briefly described as fully descriptions are provided elsewhere [14,15,42].

417 *Response to PHA-P injection:* this is a measure pro-inflammatory potential of the birds. Briefly,
418 after measuring with a caliper the wing web thickness, a 0.05 ml. intradermal injection of a 1
419 mg/ml solution of PHA-P in phosphate-buffered saline (PBS) was administered in the wing web
420 area. On the following day (24 h post-PHA-P), the dermal swelling response was measured as
421 the percentage of increase in wing web thickness at the injection site. Measurements were
422 recorded to the nearest 0.01 mm using a mechanical micrometer [67].

423 *Antibody response to SRBC*: this parameter was used to evaluate the induced humoral immune
424 response. The antibody titer was assessed with a micro agglutination assay [66]. 20 µl of
425 complement-inactivated plasma (through heating to 56°C) were serially diluted in 20 µl of PBS
426 (1:2, 1:4, 1:8, up to 1:512). Next, 20 µl of a 2% suspension of SRBC in PBS were added to all
427 wells. Microplates were incubated at 40°C for 1 h and hemagglutination of the test plasma
428 samples was compared to the blank (PBS only) and negative controls (wells with no SRBC
429 suspension). Antibody titers were reported as $\log_2$ of the highest dilution yielding significant
430 agglutination. The results represent the average of two duplicates for each animal.

431 *Corticosterone and testosterone analyses*: Plasma samples were extracted with diethylether
432 (10 times the volume). Afterwards, the ether phase was transferred into a new vial,
433 evaporated and dissolved in assay buffer [68]. Aliquots (in duplicate) were quantified in a
434 corticosterone and testosterone enzyme immunoassay (EIA). Details of the EIAs, including
435 cross-reactivities of the antibody, are given in Palme and Möstl (1997)[69] and Auer et al.
436 (2020)[70]. Steroid concentrations are expressed as nanograms of hormone per ml of plasma
437 (ng/ml).

438 *Frequency of leukocyte subpopulation distribution (FLD)*: First, cell count was done by flow
439 cytometry. To do this, 40 µl of total blood were stained with a fluorescent lipophilic dye (3, 3`-
440 dyhexiloxacarbocyanine iodide; DiOC6, Molecular Probes) to obtain absolute counts of
441 erythrocytes, lymphocytes, monocytes, thrombocytes and granulocytes as described
442 elsewhere [71]. Then, the FLD number was calculated using the following formula: FLD = number
443 of granulocytes/(number of lymphocytes + number of monocytes) and is an indicator of the
444 innate: acquired immunity ratio.

445 *Cytokines:* To determine cytokine mRNA expression levels, peripheral lymphocytes were
446 isolated and total RNA was extracted from cells according to the manufacturer's instructions
447 and as previously described [72]. Then, total RNA was reverse-transcribed. The resulting cDNA
448 was stored at -80°C until used for real-time PCR. For quantitative real-time PCR assays, specific
449 primers for quail IL-1β, IL- 4, IL-13, and IFN-γ genes were used as previously reported [73]. β-actin
450 was used as a reference housekeeping gene. Real-time PCR was performed with a Step One
451 Plus Detection System (Real-Time PCR System- Thermo Fisher Scientific). The results represent
452 the average from three technical replicates for each analyzed animal. The level of expression
453 of each target gene was calculated using the formula: Gene Level = 2-(Target Gene Ct – β-Actin
454 Ct). The value obtained was used to compare the level of expression of each molecule [42].

455 *Statistical analysis:*

456 INPs were formerly described using cluster analysis in order to explore the degree of similarity
457 among the birds in the study on the bases of the analyzed variables. Particularly, with the aim
458 of identifying the best partition of the birds, described by the best orthogonal linear
459 combinations of the factors according to the least-squares criterion, a K-means analysis was
460 performed. The following 9 standardized variables were used in the analysis: plasma
461 corticosterone, testosterone, IFN-γ and IL-4, IL-1β, IL-13, skin-swelling response to PHA-P,
462 antibody response against SRBC and FLD ratio. Based on the theoretical framework previously

463 exposed, the clustering was performed with a K value of 3 (2 subgroups with representatives
464 of potential extreme INPs and 1 intermediate subgroup). Euclidean metrics were used for
465 computing the distance between points and cluster centers. To graphically represent the
466 results of the clustering in a multivariate manner, a Principal Component Analysis (PCA) was
467 used with the clustering variables as explanatory variables [15,74]. Also, on the data from each
468 stage of the experiment, we performed a linear discriminant analysis (LDA) to characterize
469 variation in the population [74]. LDA was used to recognize the parameters that are more
470 important to identify INPs during each stage of this study. Due to autocorrelation, two of the
471 four cytokines were removed to do LDA analysis. We chose IL-4 as representative of the anti-
472 inflammatory response and IFN-γ as representative of the pro-inflammatory response. To
473 adjust the model, we randomly selected 75% of the data of each experimental stage, and then
474 we tested its predictive power with the remaining 25% of the data. To compare the variation in
475 the frequency of INPs from one experimental stage to the other, we did a proportion analysis.
476 We also compared the proportion of males and females belonging to each phenotype.

477 We evaluated the transition probability from one INP to the others during the four
478 experimental stages using discrete-time Markov chains (DTMC; Spedicato and Signorelli, 2014).
479 This kind of analysis is adequate to evaluate changes in individuals at successive points in time.
480 To do this, we built transition matrices among the four stages of the experiment (Table 3): first
481 a matrix between juveniles and adults, second, a matrix between adults pre-stress and post-
482 stress, and third a matrix between adult post-stress and senescent individuals [76]. Transition
483 matrices estimate the probability of an individual changing its phenotype. For example, if at
484 the juvenile stage, 16 individuals show a Fischer-like phenotype, we intended to know how
485 many of those individuals remained Fischer-like when they reach adulthood and how many
486 changed to Lewis-like or the intermediate phenotype. We also evaluated the number of
487 individuals that remained in the same phenotype. To estimate probabilities, we divided the
488 number of individuals that had changed by the total of individuals of each phenotype in the

489 previous state (see Table 3 of the results section) [75]. Since in some cases, the INP could not be
490 established during the four stages, the total number of individuals in each transition may be
491 variable but in the three transitions studied the sample was always more than 70 individuals,
492 thus the results can be considered representative of INPs configuration in the species and of
493 changes during the experiment.

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499 **Authors Contributions:**

500 Experimental design: F.N.N, R.H.M.,S.G.C. Laboratory Analysis: M.F.P, V.I.C; R.P; FNN.
501 Statistical Analysis and figures preparation: A.M; Original draft writing: A.M., F.N.N. Manuscript
502 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
503 final manuscript.

504 **Data Availability:** Data is available on reasonable request. Contact e-mail:
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700 **Figure legends**

701 Fig. 2: Schematic conceptual representation of INPs in Coturnix coturnix (Nazar et al. 2015).
702 The variables set to determine the existence of avian INPs are represented at the side of each
703 bird. The size of the variable indicates if the animals show high or low responses in each of the
704 parameters in the INPs. LYMPH: lymphoproliferative response to PHA-P; Ab SRBC: antibody
705 response against SRBC; FLD: frequency of leukocyte distribution; level of expression of
706 mediators: IFN-γ and IL-1β (pro-inflammatory); and IL-4 and 13 (anti-inflammatory). "Fischer-
707 like" quail with high CORT levels also manifest high FLD and IL- 13, but low Lymph, Ab SRBC,
708 IFN-γ and IL-1β levels. "Lewis-like" counterparts have low CORT as well as low FLD and IL-13
709 responses, but high Lymph, Ab SRBC, IFN-γ and IL-1β responses. These two extreme groups of
710 birds do not differ in their IL-4 levels.

711 Fig. 2: Principal component analysis plots exploring data variability and clustering procedure.
712 Each dot represents one bird and each variable is represented by an arrow. Each plot
713 represents the INP during one stage of the experiment.

714 Fig. 3: Linear discriminant analysis plots. Each plot represents the INP during one stage of the
715 experiment. The variables Limph, Testosterone, AbSRBC, IFN-G and IL.4 showed similar
716 canonical axis 1 and canonical axis 2 values and therefore appear overlapped in the center of
717 the graph. In the adults post stress graph, also corticosterone had a similar canonical axis
718 displacement.

719 Fig. 4: Phenotype frequency distribution during the four stages of the experiment. Each plot
720 represents the INPs frequency during one stage of the experiment.

721 Fig. 5: Plot of transition probabilities. Arrows indicate changes from one phenotype to the
722 other or the permanence in that INP. Transition probabilities are indicated on the lines.

723 Fig. 6: Summary of the experimental protocol followed in this research. INPs were assessed in
724 a group of 96 quail during four experimental stages: 1) juveniles, 2) adults before an
725 unpredictable chronic stress protocol, 3) adults after an unpredictable chronic stress protocol,
726 4) senescent. Abrreviations: wk. (weeks), UCSE (Unpredictable Chronic Stress Exposure)

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