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1 **Coping with extremes: remarkably blunt adrenocortical responses to acute stress in two**
2 **sympatric snow finches on the Qinghai-Tibet Plateau during winter relative to other**
3 **seasons.**

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22 **ABSTRACT**

23 The extreme climatic conditions (ECCs) of the Qinghai-Tibet Plateau impose strong selective
24 pressures on the evolution of phenotypic traits in free-living animals. It is not well understood
25 how animals on the Qinghai-Tibet Plateau modify their adrenocortical functions in response
26 to both predictable and unpredictable events of ECCs, especially when the available resources
27 are lowest during the wintering life-history stage. To uncover potential physiological
28 mechanisms, we studied the life history stage dependent features of morphology, the plasma
29 corticosterone response to acute stress and brain glucocorticoid receptor (GR) and
30 mineralocorticoid receptor (MR) mRNA expression in two sympatric snow finches: the
31 white-rumped snow finch (*Onychostruthus taczanowskii*, WRSF); and the rufous-necked
32 snow finch, *Montifringilla ruficollis*, RNSF) in Qinghai Province, China. Our results showed
33 that (a) baseline corticosterone and stressor-induced corticosterone levels significantly varied
34 with life history stage, but not between the species; (b) in WRSF, GR mRNA expression in the
35 paraventricular nucleus was higher in the wintering stage compared to the pre-basic molt
36 stage. There were no differences in hippocampus MR mRNA expression between stages in
37 either species; (c) in the wintering stage, the suppression of corticosterone secretion in both
38 species was an unexpected strategy in free-living animals. Both convergent and divergent
39 phenotypic traits of adrenocortical responses to acute stress in two sympatric snow finches
40 contribute to our understanding of the coping mechanisms of closely related species in the
41 severe winter on the Qinghai-Tibet Plateau.

42

43 **Keywords:** corticosterone, corticosterone receptor, extreme climatic conditions,

44 Qinghai-Tibet Plateau, snow finch, stress response

45 **1. Introduction**

46 Extreme climatic conditions (ECCs) are characterized by dramatic variations such as
47 high frequencies and long durations of environmental climatic events. These can occur on a
48 predictable schedule or as unpredictable perturbations (Van de Pol et al., 2017; Wingfield et
49 al., 2017). For free-living animals, ECCs function as unique and critical driving forces on the
50 evolution of both genotypic and phenotypic traits (Møller, 2011; Bailey and Pol, 2016;
51 Natarajan et al., 2016). The relationships among life histories, physiology, and phylogeny are
52 critical for understanding how genotypes and phenotypes evolve with ECCs (Roff, 1992;
53 Laiolo et al., 2015). Currently little is known about the mechanisms by which free-living
54 animals modify their phenotypic traits in response to both predictable life-history cycles and
55 unpredictable, life-threatening, perturbations (Richardson et al., 2003; Li et al., 2008, 2011;
56 Wingfield et al., 2017). Furthermore, there is little or no information on whether physiological
57 traits necessary to cope with ECCs are highly conserved in closely related species
58 (Richardson et al., 2003; Li et al., 2008, 2012; Wingfield et al., 2017). As such it is important
59 to uncover the physiological coping mechanisms in free-living animals responding to ECCs
60 (Bailey and Pol, 2016; Wingfield et al., 2017).

61 In vertebrates, the hypothalamic-pituitary-adrenal (HPA) axis, has been shown to be one
62 of the fundamental mediators of coping mechanisms as hormones from the HPA orchestrate a
63 suite of behavioral and physiological traits in response to both predictable and unpredictable
64 environmental stimuli (Romero, 2002; Landys et al., 2006; Wingfield et al., 2017). Generally,
65 baseline circulating glucocorticoid levels regulate, for example, metabolism and

66 osmoregulation *via* the mineralocorticoid receptor (MR) in response to predictable
67 perturbations (Sapolsky et al., 2000; Romero, 2002; Landys et al., 2006). In free-living birds,
68 baseline corticosterone levels vary with life-history stages, with peaks occurring during the
69 breeding and nadirs in the pre-basic molt life history stage (Romero, 2002). Such fine-tuned
70 variations in baseline corticosterone levels are associated with life history stage dependent
71 daily life processes and physiological activities (reviewed by Romero, 2002; Romero et al.,
72 2005; Romero and Wingfield, 2016).

73 In contrast, stressor-induced glucocorticoid levels are often involved in redirecting
74 physiological and behavioral responses by activation of the emergency life history stage
75 through binding the low-affinity glucocorticoid receptor (GR) in response to unpredictable
76 life-threatening perturbations (Romero and Wingfield, 2016; Wingfield et al., 2017).
77 Short-term increases in glucocorticoid levels can produce “leave it” or “take it” behavioral
78 strategies through suppression of “unnecessary” functions so that all available energy
79 resources can be devoted to self-preservation (Sapolsky et al., 2000; Wingfield and Kitaysky,
80 2002). Previous evidence had shown that the lowest amplitude of stressor-induced
81 corticosterone levels tends to occur in the pre-basic molt life history stage, but the greatest
82 amplitude of stress response occurs in other LHSs such as breeding and winter (Romero, 2002;
83 Lattin et al., 2016; Romero and Wingfield, 2016). Furthermore, stressor-induced
84 corticosterone levels can also vary with breeding sub-stages, reflecting substantial trade-offs
85 between reproduction and immediate survival (Li et al., 2016). Therefore, both life history
86 stage dependent baseline and stressor-induced corticosterone variations in free-living birds

87 provide quantifiable ways to identify coping mechanisms of physiological and behavioral
88 responses to extreme environmental conditions (Wingfield et al., 2017).

89 In recent years an increasing number of studies have focused on life history stage
90 dependent strategies of adrenocortical responses to acute stress (Romero, 2002; Lattin et al.,
91 2016; Romero and Wingfield, 2016). To date, there are no consistent patterns in
92 stressor-induced corticosterone levels for those species breeding in ECCs (Wingfield et al.,
93 1994, 1995, 2017). For example, several avian species nesting in the Arctic or desert areas
94 suppress stressor-induced corticosterone levels relative to other LHSs but others do not
95 (Wingfield et al., 1992, 1995; Walker et al., 2015; Krause et al., 2016; Romero and Wingfield,
96 2016). Indeed, similar patterns of stress response modulation determined in closely related
97 species during specific life history stages regardless of environment, indicate that
98 corticosterone responses may also be highly conserved and affected by phylogeny (Breuner et
99 al., 2003; Li et al., 2008, 2011, 2012). To date no studies have investigated whether life
100 history stage dependent corticosterone stress responses are similar in closely related
101 co-occurring species that live year round in extreme environments, especially during the
102 wintering stage when environmental conditions can be severe.

103 Actions of glucocorticoids mediated by binding to intra-cellular receptors (MR and GR)
104 in target tissues (Proszkowiec-Weglarz and Porter, 2010; Krause et al., 2015). Previous
105 studies in the arctic-breeding white-crowned sparrows (*Zonotrichia leucophrys gambelii*)
106 during the pre-parental sub-stage of breeding showed higher brain MR mRNA expression, but
107 not GR mRNA, compared to the parental sub-stage (Breuner et al., 2003; Krause et al., 2015).

108 To the contrary, temperate-zone breeding house sparrows (*Passer domesticus*) had the highest
109 brain GR binding capacity for corticosterone but not MR binding capacity in the pre-breeding
110 sub-stage compared to the breeding sub-stages (Lattin and Romero, 2013). How brain GR and
111 MR levels vary with life history stage, and between closely related species in ECCs remains
112 largely unknown.

113 The Qinghai-Tibet Plateau is one of the largest and harshest environments for life in the
114 world (Wang et al., 2008), characterized by highly predictable and unpredictable climatic
115 conditions. Unlike lowland temperate-zone areas, the alpine meadow ecosystem on the
116 Qinghai-Tibet Plateau has a dramatic seasonal fluctuation of gross primary productivity (Kato
117 et al., 2004). Environmental resources available to free-living birds are lowest during winter,
118 and highest during summer. From the perspectives of energetic cost and perturbation
119 resistance potential, determined by body condition, food resource availability, location etc.
120 (Wingfield et al., 2011, 2017), increased corticosterone stress responses may come at a cost of
121 consumption of energetic resources for self-preservation rather than for a LHS such as
122 breeding (Sapolsky et al., 2000; Wingfield and Kitaysky, 2002). Under extreme conditions, a
123 stronger corticosterone stress response may not be an optimal strategy and even may result in
124 high mortality (Wingfield and Ramenofsky, 2011). On the contrary, a blunted corticosterone
125 response may be a better strategy when the energetic costs of emergency life history stage
126 exceed the current cumulative energetic resources available (McEwen and Wingfield, 2003;
127 Wingfield et al., 2017). Therefore, it is important to explore how free-living animals on the
128 Qinghai-Tibet Plateau adjust their adrenocortical responses to acute stress when the external

129 environment is at its most severe in winter, *i.e.*, reduced food resources, extreme cold, and
130 increased energy-demands of interspecific and intraspecific competition. Here, we
131 hypothesized that free-living birds on the Qinghai-Tibet Plateau in winter have lower
132 stressor-induced corticosterone responses, when energetic costs are increasing, relative to
133 those in summer. Furthermore, we predict that such a life history stage dependent coping style
134 would be highly conserved among closely related species.

135 The white-rumped snow finch (*Onychostruthus taczanowskii*, WRSF) and the
136 Rufous-necked snow finch (*Montifringilla ruficollis*, RNSF) are both native to the
137 Qinghai-Tibet Plateau, and are distributed almost sympatrically year-round as typical resident
138 species (Lu et al., 2009; the phylogenetic relationships in the snow finch complex are shown
139 in Fig. S1, supplementary material). To evaluate our above hypothesis, we determined the life
140 history stage dependent features of body mass, body condition, and adrenocortical response to
141 capture-restraint stress in WRSF and RNSF across the early breeding, late breeding, pre-basic
142 molt, and wintering life history stages. We further examined brain GR and MR mRNA
143 expression in the two species during the breeding and wintering stages. We predicted that (1)
144 both WRSF and RSNF would exhibit conserved features in stress physiology (baseline and
145 stressor-induced corticosterone levels, expression of MR and GR) across life history stages
146 because they share similar climatic and habitat conditions; (2) In the wintering life history
147 stage, both WRSF and RSNF would also express suppressed corticosterone response-related
148 features relative to other life history stages due to strong selective pressure of extreme
149 environments on the coping mechanisms of physiology and behavior.

150

151 **2. Materials and methods**

152 **2.1. Animals and study locations**

153 Free-living adult WRSFs and RNSFs were studied at Qinghai Lake (37°02.216' N
154 99°44.293' E, Elevation, 3215 m), Wenquan (35°24.258' N 99°25.876' E, Elevation, 3946 m),
155 Huashixia (35°06.345' N 98°51.390' E, Elevation, 4106 m), Maduo (34°54.821' N 98°12.598'
156 E, Elevation, 4268m), and Nangqian (32°20.92' N, 96°45.761' E, Elevation, 4100m) Qinghai
157 Province, China (Fig. S2, supplementary material). Samples were collected in the spring
158 (April 17 to May 25; when birds were establishing territories, mating and egg-laying, i.e. the
159 early breeding sub-stage), summer (July 2 to 17; the nestling period, i.e. the late breeding
160 sub-stage), Autumn (August 1 to 26; the pre-basic molt stage), and winter (January 23 to
161 February 10; also called the non-breeding stage) of 2006-2008. Sample sizes from each life
162 history stage for the measurements of morphology, corticosterone response, and
163 corticosterone receptors are summarized in Table S1 (supplementary material).

164 **2.2. Capture-restraint stress protocol and sampling**

165 All birds were captured opportunistically using mist nets followed by a standardized
166 capture stress protocol to assess sensitivity of the HPA axis in response to acute unpredictable
167 stressors (Wingfield et al., 1992; Li et al., 2008). To reduce variation in circulating
168 corticosterone levels due to daily biological rhythms, all the birds were sampled from 0800 h
169 to 1300 h during winter and spring (short day-length) and from 0700 h to 1200 h during

170 summer and autumn (long day-length). Within 3 min of capture, approximately 40 μ l of blood
171 was collected aseptically by piercing the alar vein using a 26-gauge needle. The blood was
172 transferred into heparinized microhematocrit capillary tubes and plasma assayed for baseline
173 corticosterone levels. After initial blood sampling, the birds were then placed in opaque cloth
174 bags, and subsequent blood samples were collected at 10 min, 30 min, and 60 min intervals
175 after capture to create a plasma profile of acute corticosterone secretion during continued
176 handling, and restraint. Blood samples were stored on ice in the field for 3–4 h before they
177 were centrifuged at 855g for 10 min. Plasma samples were separated and stored at -20°C until
178 assay.

179 After blood sampling, each bird was weighed to the nearest 0.1g, scored for furcular fat
180 on a semi-quantitative scale of 0 (no fat visible) to 5 (bulging fat deposit; Wingfield and
181 Farner, 1978). Wing, tarsus, and beak lengths were also measured. To estimate the absolute
182 size of energy reserves, we calculated a body condition index for each species by using
183 mass/size residuals (*i.e.*, the principal component of wing, beak and tarsus lengths). Since
184 both WRSF and RNSF are monomorphic species, sex identification was unavailable for those
185 individuals during the wintering and the early breeding stages. However, sex identification for
186 both species in the late breeding and pre-basic molt life history stages was determined by the
187 presence or absence of a brood patch (a female-specific trait). Finally, all birds captured were
188 individually marked for field identification with a numbered metal leg band and then released.

189 Birds sampled for tissues were euthanized within 3 min of capture using isoflurane
190 inhalation followed by decapitation. Whole brains were wrapped in aluminum foil and frozen

191 in liquid nitrogen until they could be moved to a -80 °C storage freezer. All protocols were
192 approved by the Institutional Animal Care and Use Committees of the Institute of Zoology,
193 Chinese Academy of Sciences, China, the University of Washington, Seattle and the
194 University of California, Davis, USA, and were carried out under the auspices of scientific
195 collecting permits issued by the Departments of Wildlife Conservation (Forestry Bureau) of
196 Qinghai Province, China.

197 **2.3. Corticosterone assays**

198 The corticosterone assay followed the protocols of Wingfield et al. (1992) and Li et al.
199 (2008). Briefly, 10-15µl of plasma was equilibrated overnight with 2000 cpm of
200 ³H-corticosterone to determine individual recoveries. Samples were extracted in 4 ml of
201 freshly redistilled dichloromethane, dried under nitrogen and re-suspended in 550 µl
202 phosphate-buffered saline with 1% gelatin. All samples were run in duplicate, and assay
203 values were corrected for plasma volume and individual recoveries (ranged from 87.3% to
204 99.4%). Inter-assay and intra-assay coefficients of variation were 12.6% and 9.1%,
205 respectively.

206 **2.4. In situ hybridization histochemistry for MR and GR mRNA**

207 The methodology for MR and GR *in-situ* hybridization procedures have been previously
208 described in detail (Dickens et al., 2009). Whole brains were sectioned coronally at 15 µm on
209 a cryostat and sections were thaw-mounted onto polysine, RNAase free, and pre-treated glass
210 microscope slides. Marker slides were created by collecting every sixth section for staining
211 using cresyl violet. Slides were stored at -80 °C with silica pellets as desiccant until *in-situ*

212 hybridization was performed. Slides were selected for *in-situ* hybridization after investigating
213 marker slides in conjunction with the canary, *Serinus canaria*, stereotaxic atlas (Stokes et al.,
214 1974) to identify regions of interest. Briefly, 500-bp fragments of the zebra finch GR
215 (Genbank: XM_002186722) or MR (Genbank: DQ539433) were sub-cloned into PGEM-7.
216 GR sense and antisense riboprobes in the presence of 35S-UTP, with SP6- and T7-RNA
217 polymerase were generated by *in-vitro* transcription, after plasmid linearization with EcoRI or
218 HindIII, respectively. Similarly, the MR sense and antisense riboprobes in the presence of
219 35S-UTP, with T7- and SP6-RNA polymerase were developed by *in-vitro* transcription after
220 plasmid linearization with HindIII or ApaI, respectively. Both the MR and GR genes are
221 highly conserved, with identities between chicken (galliform, *Gallus gallus*) and zebra finch
222 (passerine, *Taeniopygia guttata*) of 90% and 88%, respectively (Dickens et al., 2009). Slides
223 were dipped in the autoradiographic emulsion, air-dried, and left to expose in sealed boxes for
224 5 weeks. The slides were then developed and counterstained with hematoxylin-eosin,
225 dehydrated, and cover slipped with DPX mountant (Sigma, St Louis, MO, USA).
226 Hybridization of sections with GR or MR sense riboprobes, or pre-treatment with RNase-A
227 prior to hybridization with the GR or MR antisense riboprobes, did not result in any
228 detectable hybridization signal.

229 **2.5. Quantification of relative silver grain density for GR mRNA and MR mRNA**

230 Slides were examined under bright field microscopy. Sections containing the
231 paraventricular nucleus and hippocampus were determined in combination with the canary
232 stereotaxic atlas (Stokes et al., 1974), and the marker slides containing hematoxylin-eosin
233 counterstaining to locate brains regions and reveal neuroanatomical landmarks. Images were

234 captured at $\times 20$ magnification using a Nikon E600 microscope, Zeiss Axiocam 105 color
235 camera, and Zen Capture Software.

236 Expression of GR or MR mRNA was quantified by determining the density of silver
237 grains using ImageJ analysis software (NIH, Bethesda, MD, USA) over an area of known size
238 (0.01260 or 0.01234 mm²). The proportion of the set area overlaid with silver grain was
239 recorded as an average of 12 measurements per bird (taken over two consecutive sections) for
240 both paraventricular nucleus (GR) and hippocampus (MR). The percentage of background
241 silver grain was also noted over the same given area for each section and subtracted to correct
242 for any inconsistencies. All slides were coded so that the identity of the groups was unknown
243 during image analysis.

244 **2.6. Statistical analysis**

245 The maximal corticosterone level was calculated as the capture-restraint stressor-induced
246 maximum level each individual achieved over the 60 minute restraint period. This level is
247 generally much higher than baseline corticosterone level (i.e. the plasma level of
248 corticosterone in the first sample collected – within 3 minutes of capture). The total integrated
249 corticosterone values were determined as the area under curve calculated by trapezoid rule
250 using Graph Pad Prism, *version 5.01* (Graph Pad Software Inc., San Diego, CA, USA; Li et
251 al., 2008). Fold change was determined by using the ratio of maximal corticosterone and
252 baseline corticosterone levels. To determine the potential effects of sex differences during the
253 late breeding and pre-basic molt stages, we compared body mass, fat score, body condition
254 and corticosterone stress response using independent sample *t*-tests between sexes in the

255 WRSF and the RNSF. Given that there were no significant differences in those measurements
256 (Table S2, supplementary material), we then combined data from both sexes of each species
257 during a specific stage in following analysis.

258 For those variables that met normality (body condition, maximal corticosterone, fold
259 change, total integrated corticosterone, GR mRNA expression), we examined potential
260 differences using a linear mixed model (LMM) fitted with the restricted maximum likelihood
261 (REML) method in SPSS 21.0. Specifically, the fixed effects of species, life history stage and
262 sub-stage, and the interaction between species and life history stage, with year and site as
263 random factors were tested. For those variables that did not meet normality (body mass, fat
264 score, MR mRNA expression), we examined life history stage differences in each species
265 using Kruskal-Wallis tests with the exception of MR mRNA expression where we used a
266 Mann-Whitney *U* test for RNSF. Species differences of these variables within a life history
267 stage were also compared using a Mann-Whitney *U* tests. Differences between pairs of means
268 were identified by Bonferroni-adjusted *post hoc* tests based on model-predicted estimated
269 marginal means in either LMMs or Kruskal-Wallis tests. Effect sizes were estimated using
270 *Cohen's d* for *U* tests and partial omega-squared (ω^2) for LMM or ANOVA to measure the
271 strength of statistically significant differences between groups (Elis, 2010). Differences were
272 considered significant at $p < 0.05$. All data are presented as means \pm SEM.

273

274 **3. Results**

275 **3.1. Variations of morphological traits across life history stages and between species**

276 Body mass and body condition of both WRSF and RNSF varied with life history stages
277 (Tables 1 and 2). WRSFs were lighter and in worse body condition while RNSFs were heavier
278 and in better condition during the wintering relative to the pre-basic molt life history stages
279 (Fig. 1A,B; Table S3, supplementary material). WRSFs were heavier than RNSFs during the
280 early breeding, late breeding and pre-basic molt life history stages, in better condition during
281 the pre-basic molt, but in worse condition during the winter than RNSFs (Table 1, Fig. 1A-B).

282 The fat score of both species varied across life history stages, with significantly more fat
283 in winter and early breeding relative to late breeding and pre-basic molt (Table 2, Fig. 1C;
284 Table S3, supplementary material). WRSF had significantly greater fat stores than RNSF
285 during late breeding, but less fat in pre-basic molt (Fig. 1C; Table S3, supplementary
286 material).

287 **3.2. Variations of baseline and stressor-induced corticosterone levels across life history** 288 **stages and between species**

289 Baseline corticosterone levels of both species varied across life history stages (Table 1).
290 *Post hoc* tests showed that WRSF had significantly higher baseline corticosterone levels
291 during early breeding and winter relative to pre-basic molt, whereas RNSF exhibited higher
292 baseline corticosterone levels during the winter compared to both late breeding and pre-basic
293 molt (Fig. 2A; Table S3, supplementary material). However, baseline corticosterone levels did
294 not vary between species at different life history stages (Table 1; Fig. 2A).

295 Stressor-induced maximal corticosterone levels, total integrated corticosterone levels and
296 fold change also did not vary with species but were different across life history stages (Table 2;

297 Fig. 2B-D). *Post hoc* tests showed that both species had significantly lowered maximal
298 corticosterone levels, total integrated corticosterone levels, and fold change during winter
299 than those from other life history stages (Fig. 2B-D, F; Table S3, supplementary material).
300 Maximal corticosterone and total integrated corticosterone levels of WRSF were significantly
301 lower during pre-basic molt relative to both early and late breeding. Maximal corticosterone
302 and total integrated corticosterone levels of RNSF did not vary across early breeding, late
303 breeding, and pre-basic molt. Fold change also showed no differences with these life history
304 stages in both species (Fig. 2B-D; Table S3, supplementary material).

305 **3.3. Variations of corticosterone receptors across life history stages and between species**

306 GR mRNA varied with life history stage, but MR mRNA did not (Tables 1 and 2). *Post*
307 *hoc* results revealed no differences in GR mRNA between early breeding and winter in both
308 species. However, WRSF had significantly higher levels of GR mRNA in the wintering life
309 history stage compared with those during the pre-basic molt life history stage (n.a. for RNSF
310 because we had no comparable data during the pre-basic molt life history stage; Figs. 3-4;
311 Table S3, supplementary material). Both GR mRNA and MR mRNA did not vary with species
312 (Tables 1 and 2).

313

314 **4. Discussion**

315 We determined life history stage dependent variations in baseline and stressor-induced
316 corticosterone levels, and brain MR and GR mRNA expression of both WRSF and RSNF. As

317 predicted, we identified a highly conserved adrenocortical response to acute stress in the two
318 closely related species. Notably, both WRSF and RSNF during the wintering life history stage
319 exhibited markedly increased baseline, blunted stressor-induced corticosterone levels (a
320 significantly lowered fold change) relative to other life history stages (Fig. 2). As far as we are
321 aware, this is a unique coping strategy in response to one of the most extreme environmental
322 exposures.

323 **4.1. Annual changes of morphological traits in response to predictable perturbations**

324 In birds, increased fat deposition in the non-breeding life history stage such as wintering
325 is a physiological strategy for maintaining higher energetic reserves to cope with both the
326 predictable and unpredictable events at that time (Witter and Cuthill, 1993; Rogers and Reed,
327 2003). Consistently, we found that both WRSF and RSNF exhibited similar patterns in annual
328 changes of fat deposition as observed for other passerines (Pravosudov and Grubb, 1997;
329 Cresswell, 1998). However, we found markedly lowered body mass in WRSF, whereas mass
330 increased in RSNF during the wintering life history stage relative to others. Such
331 species-dependent differences may result from the divergences in both behavioral and
332 ecological traits in relation to life history stages. For example, in the wintering life history
333 stage, WRSFs outcompeted RSNFs in rural areas (away from the buildings of human beings)
334 ate more seeds. By contrast, RSNFs moved to urban areas where resources and shelters were
335 less intensely competitive and fed on starchy materials from human food waste (Li et al.,
336 under review). Overall, the underlying physiological mechanism of such distinct differences
337 in morphology warrants for further investigation.

338 **4.2. Annual changes of corticosterone response traits in relation to predictable and**
339 **unpredictable perturbations**

340 The nadirs of baseline circulating corticosterone levels in both species occurred in the
341 pre-basic molt life history stage, which is in line with previous findings in several other avian
342 species (Romero, 2002; Cornelius et al., 2011; Romero and Wingfield, 2016). The reduced
343 baseline corticosterone levels at this period can prevent protein catabolic activity, resulting in
344 higher quality of feathers (Romero, 2002; Romero et al., 2005; Cornelius et al., 2011).
345 Surprisingly, there were no significant differences in baseline corticosterone levels between
346 the late breeding and pre-basic molt life history stages, which is in line with an invasive
347 species - Eurasian tree sparrows (*Passer montanus*) on the Qinghai-Tibet Plateau (Li et al.,
348 2008; Qu et al., 2019). Corticosterone can promote energy catabolism through several
349 metabolic pathways to support energy demand for physiological and behavioral processes
350 (Romero, 2002; Romero et al., 2005). Whether such a strategy of lowered baseline
351 corticosterone levels after raising young successfully can divert resources for energy
352 accumulation when the environmental resources are in the most abundant periods remains to
353 be clarified. On the other hand, peak baseline corticosterone levels occurred both in the
354 wintering and early breeding life history stages of WRSF, and in the wintering life history
355 stage for RNSF. Our results differ from those findings showing upregulated baseline
356 corticosterone levels are necessary for providing the extra energy needed for mating and
357 territory defense during breeding (Romero, 2002; Goymann et al., 2006; Wada et al., 2006;
358 Love et al., 2014). Generally, increased baseline corticosterone levels are associated with

359 poorer body condition, contributing to resource allocation towards immediate survival when
360 the environment becomes more challenging (Romero, 2002; Bonier et al., 2009; Wingfield
361 and Ramenofsky, 2011). Up-regulation of baseline corticosterone in WRSFs and RNSFs
362 during the wintering and early breeding life history stages might be explained as a unique
363 strategy for coping with the most challenging period of their environment on the
364 Qinghai-Tibet Plateau.

365 The nadirs of all the stressor-induced corticosterone levels (maximal corticosterone, total
366 integrated corticosterone, and fold change) in both WRSF and RNSF occurred in the
367 wintering life history stage but not in the pre-basic molt stage (Fig. 2). This differs from
368 previous findings in both migratory and non-migratory passerines (Romero, 2002; Li et al.,
369 2008, 2012; Romero and Wingfield, 2016). Earlier studies have shown that those migratory
370 species wintering in much less severe environments exhibited significantly stronger
371 corticosterone response. This may be because environmental resources available in those
372 environments can maximize perturbation resistance potential in the face of life-threatening
373 and unpredictable perturbations (Wingfield et al., 1994, 1995, 2017; Romero, 2002).
374 Increased stressor-induced corticosterone levels could contribute to optimizing individual
375 survival by triggering the emergency life history stage and mobilize energy reserves to cope
376 with the perturbation (see Landys et al., 2006; Wingfield et al., 2017). In contrast, suppressed
377 stressor-induced corticosterone responses in non-migratory, wintering, snow finches in the
378 severe environment of the Qinghai-Tibet Plateau may be an energy-saving strategy to resist
379 predictable extreme winter conditions. To what extent this may increase the vulnerability of

380 snow finches to additional unpredictable events superimposed on winter conditions remains to
381 be determined but such a scenario could quickly reduce perturbation resistance potential to
382 zero or even become negative (allostatic overload type 1; see Wingfield et al., 2017). This is a
383 concern especially in the light of global climate change. Considering that higher energetic
384 requirements are necessary for immediate survival in such low ambient temperature during
385 winter, whether an increase in baseline corticosterone levels and suppression in corticosterone
386 response to a standardized stressor is a unique physiological and ecological strategy of
387 avoiding extra costs in relation to a severe environment also remains to be determined.

388 To explore changes of downstream glucocorticoid receptors, we found that brain MR
389 mRNA did not vary across life history stages, and may have a permissive effect for changing
390 baseline corticosterone titers (Sapolsky et al., 2000; Lattin et al., 2012; Lattin and Romero,
391 2015). Our results are in line with those findings in other passerines indicating no significant
392 differences in brain MR mRNA between the breeding and pre-basic molt life history stages
393 (Breuner and Orchinik, 2001; Lattin and Romero, 2013). However, WRSFs had significantly
394 higher levels of GR mRNA in the paraventricular nucleus of the hypothalamus during the
395 wintering life history stage relative to the pre-basic molt stage. GR mRNA density varied
396 seasonally (Lattin and Romero, 2015) although the exact mechanism remains to be delineated
397 (Herman and Tasker, 2016). Therefore, wintering WRSF exhibited a remarkable suppression
398 of the corticosterone response at the adrenal level, but a significantly increased sensitivity of
399 negative feedback for corticosterone at the hypothalamus level. Future investigations on the

400 regulatory mechanisms of MR and GR gene expressions are needed for understanding the
401 coping strategy of WRSF and RNSF in the harsh environments of the Qinghai-Tibet Plateau.

402 **4.3. Variations of morphological and hormonal traits between species**

403 WRSFs and RNSFs had different patterns of body mass change and body condition, *i.e.*,
404 RNSFs were heavier and had better body condition during the winter relative to other stages,
405 but WRSF did not. The underlying explanation may be because WRSF remained in the
406 plateau areas often distant from human-occupied sites, while RNSF shifted their winter range
407 to human-occupied sites so as to take advantage of human resources and shelters (Li et al.,
408 under review). However, there were no significant differences in baseline and stress-induced
409 corticosterone levels, brain GR and MR mRNA between WRSF and RNSF at any point
410 indicating that coping strategies of stress physiology may be highly conserved traits in the
411 harsh environment of the Qinghai-Tibet Plateau. Such a conserved strategy of stress responses
412 in relation to phylogeny also occurs in other passerines, e.g., the genus *Passer* (Li et al., 2008)
413 and *Carduelis* (Li et al., 2012).

414 **5. Conclusions**

415 Our studies revealed that peak baseline corticosterone levels occurred in the wintering
416 and early breeding life history stages for WRSF and in the wintering life history stage for
417 RNSF. The two closely related species also showed similar changes in stressor-induced
418 corticosterone levels, fold change, and seasonal changes in GR and MR mRNA expressions.
419 In the wintering life history stage, the increase of baseline corticosterone levels and the
420 suppression of corticosterone secretion in response to acute stress in both species may be a

421 unique coping mechanism and specific energy-saving life history stage-dependent strategy
422 because they have limited perturbation resistance potential at this time. Therefore, the
423 convergent pattern of the life history stage-dependent adrenocortical response to acute stress
424 in these two closely related species may reflect highly conserved mechanisms of hormonal
425 regulation in response to both predictable environmental conditions and potential additional
426 unpredictable environmental perturbations. This unique strategy may be derived from strong
427 selective pressure of extreme environments of the Qinghai-Tibet Plateau. Our work in snow
428 finches native to the Qinghai-Tibet Plateau contributes to our understanding of the coping
429 mechanisms in severe environments, especially in the most challenging season when there are
430 limited cumulative energy resources. Species differences, even when closely related, reveal
431 how adjustments in morphology physiology and behavior may change according to local
432 conditions such as favoring human-occupied sites versus remaining in rural habitats.
433 Furthermore, measuring changes in down-stream components of the adrenocortical response
434 to acute stress such as MR and GR also reveal novel potential mechanisms of coping with
435 environmental change.

436

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447

448 **Author contributions**

449 F.L. and J.C.W. conceived the ideas and design methodology, and D.L., J.E.D., G.W., V.R.B.,
450 and S.L.M. contributed to data collection and laboratory assays; D.L. analysed the data and
451 led the writing of the manuscript with the help of G.N. and Y.S. All authors contributed
452 critically to the drafts and gave final approval for publication.

453

454 **Additional information**

455 **Competing Interests:** The authors declare that they have no competing interests.

456

457 **Appendix A. Supplementary data**

458 Supplementary data may be found online in the Appendix A section at the end of the article.

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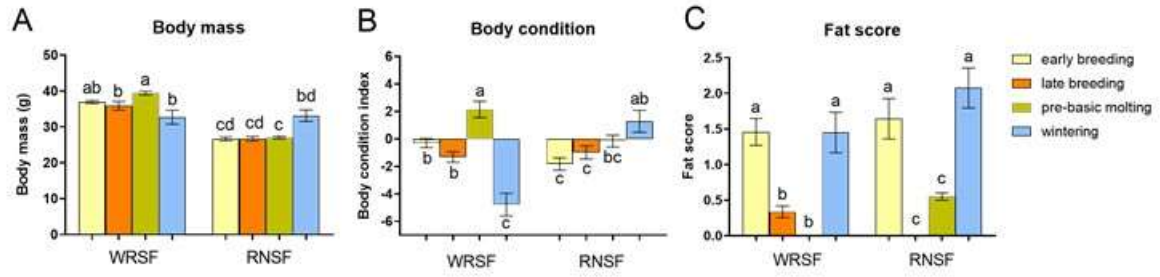
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- 608

609 **Figures**



610

611 **Fig. 1.** Comparisons of morphological variables during the early breeding, late breeding, pre-basic
 612 molt, and wintering life history stages in the white-rumped snow finch (*Onychostruthus taczanowskii*,
 613 WRSF) and the rufous-necked snow finch (*Montifringilla ruficollis*, RNSF). A) Body mass, B) body
 614 condition, C) fat score. Groups with different letters are significantly different from one another by life
 615 history stages or by species ($p < 0.05$).

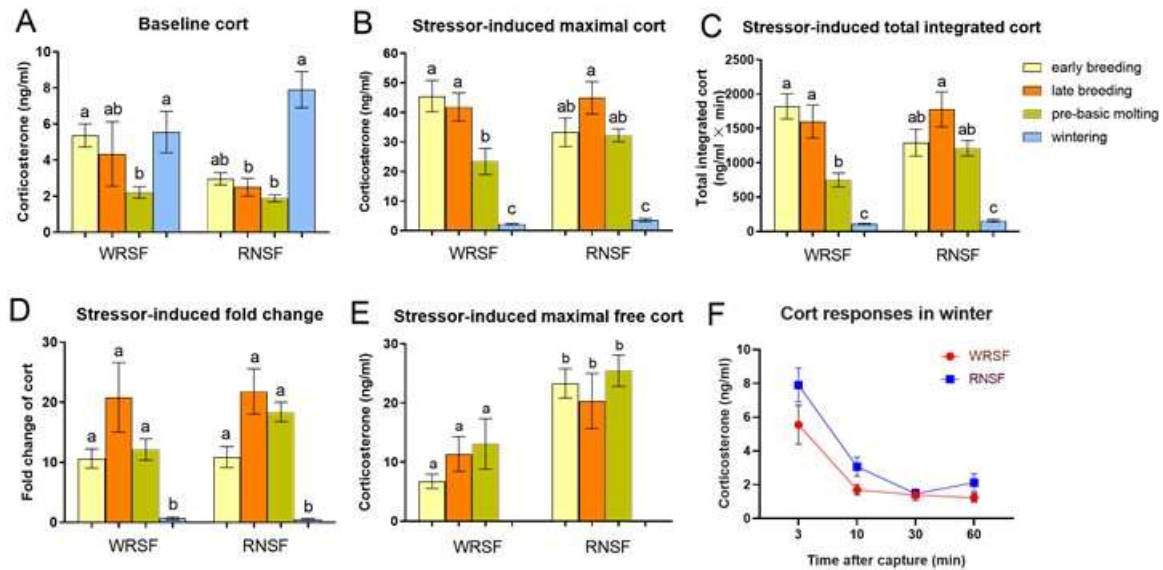
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622 **Fig. 2.** Comparisons of corticosterone (cort) responses during the early breeding, late breeding,

623 pre-basic molt, and wintering life history stages in the white-rumped snow finch (*Onychostruthus*

624 *taczanowskii*, WRSF) and the rufous-necked snow finch (*Montifringilla ruficollis*, RNSF). A) Baseline

625 corticosterone levels, B) stressor-induced maximal corticosterone levels, C) stressor-induced total

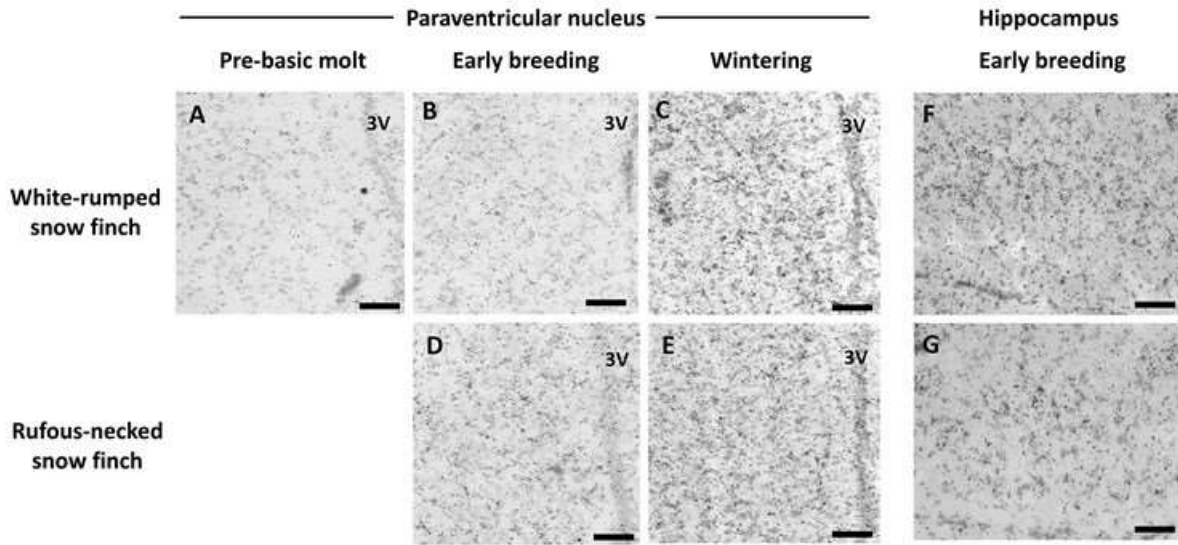
626 integrated corticosterone levels, D) stressor-induced fold change of corticosterone, E) stressor-induced

627 maximal free corticosterone levels (n.a. in the wintering life history stage because samples were

628 unavailable), F) adrenocortical responses to capture stress in WRSF and RNSF during the wintering

629 life history stages. Groups with different letters are significantly different from one another by

630 life-history stages or by species ($p < 0.05$).



631

632 **Fig. 3.** Representative photomicrographs of autoradiographs in bright field showing cells expressing
 633 glucocorticoid receptor mRNA in the paraventricular nucleus (PVN; A-E) of the hypothalamus and
 634 mineralocorticoid receptor mRNA in the hippocampus (F & G) of the white-rumped snow finch
 635 (*Onychostruthus taczanowskii*; A-C & F) and rufous-necked snow finch (*Montifringilla ruficollis*; D,
 636 E & G) during the pre-basic molt (A), early breeding (B, D, F & G), and wintering life history stages
 637 (C & E). Hybridisation is visible as silver grains (black dots) over cell bodies. 3V = Third ventricle.

638 Scale bars = 100 µm.

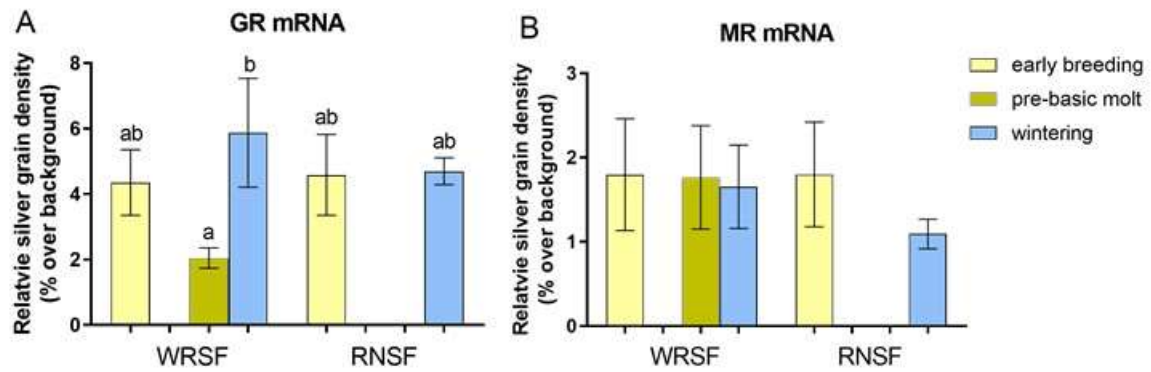
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645 **Fig. 4.** (A-B) Comparisons of glucocorticoid receptor (GR, A) mRNA expression in paraventricular
 646 nucleus (PVN) of the hypothalamus and mineralocorticoid receptor (MR,B) mRNA expression in
 647 hippocampus (Hp) in the white-rumped snow finch (*Onychostruthus taczanowskii*, WRSF) and the
 648 rufous-necked snow finch (*Montifringilla ruficollis*, RNSF) during the early breeding, pre-basic molt
 649 (n.a. for RNSF), and wintering life history stages. Groups with different letters are significantly
 650 different from one another by life-history stages ($p < 0.05$).

651

652

653 **Table 1**

654 Comparisons of fat score, body mass, plasma baseline corticosterone (cort) levels, and
 655 hippocampus mineralocorticoid receptor (MR) mRNA expression in the white-rumped snow
 656 finch (*Onychostruthus taczanowskii*, WRSF) and the rufous-necked snow finch
 657 (*Montifringilla ruficollis*, RNSF) among different life-history stages (the early breeding, late
 658 breeding, pre-basic molt, and wintering life history stages) by Kruskal–Wallis tests, and
 659 comparisons of those variables between species at certain life history stages in Mann–
 660 Whitney *U* test.

661

Response	Species	df	χ^2 value	<i>n</i>	ω^2	Life-history	df	<i>Z</i>	<i>n</i>	Cohen's <i>d</i>
Body mass	WRSF	3.55	20.949	<0.0	0.30	Early breeding	1.2	3.999	<0.00	5.613
	RNSF	3.50	10.009	0.01	0.27	Late breeding	1.1	3.776	<0.00	4.360
						Pre-basic molt	1.2	4.751	<0.00	6.510

Fat score						Wintering	1.2	-0.021	0.984	0.065
	WRSF	3.55	39.727	<0.0	0.42	Early breeding	1.2	-0.743	0.474	0.284
	RNSF	3.51	43.748	<0.0	0.56	Late breeding	1.1	3.373	0.007	1.886
						Pre-basic molt	1.2	-2.39	0.017	0.901
Baseline cort						Wintering	1.2	-1.384	0.166	0.575
	WRSF	3.48	16.074	0.00	0.12	Early breeding	1.2	1.919	0.057	1.041
	RNSF	3.40	32.722	<0.0	0.63	Late breeding	1.1	0.001	1.000	0.680

Variable	Body mass, fat score, and body condition		Corticosterone response		Mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA					
	WRSF	RNSF	WRSF	RNSF	WRSF	RNSF				
Early breeding	25	7	25	6	5	5				
Late breeding	9	13	8	13	n.a.	n.a.				
Pre-basic molt	15	15	15	16	6	n.a.				
Wintering	11	20	6	14	5	10				
MR mRNA					Pre-basic molt	1.2	0.519	0.624	0.581	
					Wintering	1.1	-1.228	0.219	0.725	
	WRSF	2.13	0.781	0.67	-0.0	Early breeding	1.6	-0.447	0.786	0.627
	RNSF	1.13	18.000	0.44	0.05	Late breeding	n.a.	n.a.	n.a.	n.a.
						Pre-basic molt	n.a.	n.a.	n.a.	n.a.
					Wintering	1.1	1.269	0.230	0.800	

Partial omega-squared (ω^2) and Cohen's *d* are measures of effect size for Kruskal–Wallis tests and Mann–Whitney *U* test, respectively. Significant factors ($p < 0.05$) and medium ($\omega^2 > 0.06$; Cohen's *d* > 0.5) or large size effects ($\omega^2 > 0.14$;

662

663 **Table S1** Sample size of body mass, fat score, body condition, corticosterone (cort) response, brain
664 mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA in the white-rumped snow finch
665 (*Onychostruthus taczanowskii*, WRSF) and the Rufous-necked snow finch (*Montifringilla ruficollis*, RNSF)
666 during the early breeding, late breeding, pre-basic molt, and wintering life history stages.

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668

669 **Table S2** Comparisons of body mass, fat score, body condition, and corticosterone (cort) response in
 670 independent sample *t*-tests between sexes in the white-rumped snow finch (*Onychostruthus taczanowskii*,
 671 WRSF) and the rufous-necked snow finch (*Montifringilla ruficollis*, RNSF) during the late breeding and
 672 pre-basic molt life history stages.

Variable	WRSF			RNSF								
	Late breeding			Pre-basic molt			Late breeding			Pre-basic molt		
	<i>t</i> value	df	<i>p</i> value	<i>t</i> value	df	<i>p</i> value	<i>t</i> value	df	<i>p</i> value	<i>t</i> value	df	<i>p</i> value
Body mass	-0.067	6	0.949	1.165	14	0.264	0.697	11	0.500	-0.280	13	0.784
Fat score	-0.509	7	0.626	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.902	9	0.018
Body condition index	-0.216	6	0.836	0.813	13	0.431	-0.417	11	0.685	-0.064	13	0.950
Baseline cort	-1.215	5.211	0.276	-0.725	13	0.481	0.841	8.743	0.423	0.128	9.299	0.901
Maximal cort	-0.943	6	0.382	0.876	14	0.396	0.013	10	0.990	-0.210	14	0.836
Fold increase	-0.832	6	0.437	0.848	12	0.413	-0.373	10	0.717	0.718	14	0.485
Total integrated cort	n.a.	n.a.	n.a.	0.081	9.822	0.937	-0.173	10	0.866	-0.792	14	0.441
There were not enough sample sizes for male WRSFs in the late breeding stage for total integrated cort levels.												

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Variable	Species	Life-history stage	Late breeding	Pre-basic molt	Wintering
Body mass*	WRSF	Early breeding	0.341	0.067	0.292
		Late breeding		0.001	1.000
		Pre-basic molt			0.001
	RNSF	Early breeding	1.000	1.000	0.208
		Late breeding		1.000	0.041
		Pre-basic molt			0.141
Body condition	WRSF	Early breeding	1.000	0.008	<0.001
		Late breeding		0.004	0.010
		Pre-basic molt			<0.001
	RNSF	Early breeding	1.000	0.662	0.013
		Late breeding		1.000	0.035
		Pre-basic molt			0.384
Fat score*	WRSF	Early breeding	0.029	<0.001	1.000
		Late breeding		0.535	0.046
		Pre-basic molt			<0.001
	RNSF	Early breeding	0.001	0.005	1.000
		Late breeding		1.000	<0.001
		Pre-basic molt			<0.001
Baseline cort*	WRSF	Early breeding	0.307	0.002	1.000
		Late breeding		1.000	0.419
		Pre-basic molt			0.030
	RNSF	Early breeding	0.285	0.030	1.000
		Late breeding		1.000	<0.001
		Pre-basic molt			<0.001
Maximal cort	WRSF	Early breeding	1.000	<0.001	<0.001
		Late breeding		0.008	<0.001
		Pre-basic molt			<0.001
	RNSF	Early breeding	0.849	1.000	<0.001
		Late breeding		0.341	<0.001
		Pre-basic molt			<0.001
Total integrated cort	WRSF	Early breeding	1.000	<0.001	<0.001

		Late breeding		0.006	<0.001
		Pre-basic molt			0.001
	RNSF	Early breeding	1.000	1.000	<0.001
		Late breeding		0.227	<0.001
		Pre-basic molt			0.001
Fold increase	WRSF	Early breeding	0.059	1.000	<0.001
		Late breeding		0.323	<0.001
		Pre-basic molt			0.001
	RNSF	Early breeding	0.131	0.618	<0.001
		Late breeding		1.000	<0.001
		Pre-basic molt			0.037
PVN GR mRNA	WRSF	Early breeding	n.a.	0.256	0.874
		Late breeding		n.a.	n.a.
		Pre-basic molt			0.020
	RNSF	Early breeding	n.a.	n.a.	0.920
		Late breeding		n.a.	n.a.
		Pre-basic molt			n.a.
Significant factors ($p < 0.05$) are shown in bold type.					

675 **Table S3** Multiple comparison results of significant variables (see Tables 1 and 2) across different
676 life-history stages (the early breeding, late breeding, pre-basic molt, and wintering stages) from Kruskal–
677 Wallis tests (variable with asterisk) or linear mixed model (LMM) in the white-rumped snow finch
678 (*Onychostruthus taczanowskii*, WRSF) or the rufous-necked snow finch (*Montifringilla ruficollis*, RNSF).

679

680 **Table 2** The comparisons of body condition index, plasma corticosterone (cort) response
681 (maximal corticosterone levels, fold increase, total integrated corticosterone levels, baseline
682 and maximal free corticosterone levels), and paraventricular nucleus glucocorticoid receptor
683 (GR) mRNA expression in the white-rumped snow finch (*Onychostruthus taczanowskii*,
684 WRSF) and the rufous-necked snow finch (*Montifringilla ruficollis*, RNSF) during the early
685 breeding, late breeding, pre-basic molt, and wintering life history stages in a linear mixed
686 model (LMM) by considering species, life-history stage (stage), and interaction of species and
687 life history stage as fixed factors, and sampling site and year as random factors.

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Response variable	Mixed model	df	F value	p value	ω^2	Response variable	Mixed model	df	F value	p value	ω^2
Body condition index	Intercept	1, 104	10.321	0.002		Maximal cort	Intercept	1, 92	642.926	<0.001	
	Species	1, 104	1.929	0.168	0.004		Species	1, 92	2.038	0.157	0.001
	Stage	3, 104	7.923	<0.001	0.067		Stage	3, 92	40.606	<0.001	0.479
	Species × stage	3, 104	18.700	<0.001	0.299		Species × stage	3, 92	1.271	0.289	0.007
Fold increase	Intercept	1, 98	1314.721	<0.001		Total integrated cort	Intercept	1, 91	1069.402	<0.001	
	Species	1, 98	0.772	0.382	-0.002		Species	1, 91	0.201	0.655	0.023
	Stage	3, 98	63.240	<0.001	0.584		Stage	3, 91	45.737	<0.001	0.604
	Species × stage	3, 98	1.922	0.131	0.003		Species × stage	3, 91	2.617	0.056	0.017
GR mRNA	Intercept	1, 23	113.738	<0.001							
	Species	1, 23	0.278	0.603	0.002						
	Stage	2, 23	4.491	0.023	0.244						
	Species × stage	1, 23	0.616	0.44	0.025						

	stage			0						
Partial omega-squared (ω^2) is a measure of effect size for LMMs. Significant factors ($p < 0.05$) and medium ($\omega^2 > 0.06$) or large size effects ($\omega^2 > 0.14$) are shown in bold type.										

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