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noraceh@ceh.ac.uk

1 **Rapid stress-induced inhibition of plasma testosterone in free-**
2 **ranging male rufous-winged sparrows, *Peucaea carpalis*:**
3 **characterization, time course, and recovery**

4
5 Pierre Deviche^{a*}, Sisi Gao^a, Scott Davies^a, Peter J. Sharp^b, and Alistair Dawson^c

6
7 ^a School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA

8 ^b The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian EH25 9RG,
9 Scotland, United Kingdom

10 ^c Centre for Ecology & Hydrology, Bush Estate, Penicuik, Midlothian EH26 0QB,
11 Scotland, United Kingdom

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17
18
19 * Corresponding author:

20 Email: deviche@asu.edu; phone: 480 965 0726; fax: 480 965 6899.

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23 Figures: 5; Tables: 1.

25 ABSTRACT

26 Chronic stress generally inhibits the activity of the reproductive system. Acute stress also is
27 often inhibitory, but the mechanism involved and its persistence of action once animals are no
28 longer exposed to the stressor are poorly understood. We investigated the effect of capture and
29 restraint stress on plasma testosterone (T), luteinizing hormone (LH), and corticosterone
30 (CORT) in free-ranging male rufous-winged sparrows, *Peucaea carpalis*. Stress decreased
31 plasma T between 10 and 30 min after capture and restraint but did not influence plasma LH,
32 the main hormone that controls T secretion, suggesting that stress did not decrease plasma T
33 by inhibiting LH secretion. The stress-induced decrease in plasma T was associated with
34 elevated plasma CORT, but there was no evidence that these effects were functionally related.
35 Plasma stress-induced T was positively related to plasma initial T measured within 2 min of
36 capture. This relationship was, however, complex as plasma T decreased proportionally more in
37 response to stress in sparrows with high than low plasma initial T. The relative sensitivity to a
38 same stressor was, therefore, individually variable and this variation was related to initial plasma
39 T. Birds caught and restrained for 30 min, and then released on their breeding territory before
40 recapture up to 6 hours later, maintained depressed plasma T, indicating that the effect of acute
41 stress on this hormone persists after the stressor removal. These studies provide new
42 information on the effects of acute stress on plasma T in free-ranging birds. In particular, they
43 are among the first to characterize the time course and to describe the persistence of these
44 effects. The findings also contribute to identifying factors that are associated with individual
45 differences in plasma hormone levels.

46

47

48 **Keywords:** Androgen - Bird - Corticosterone - Field endocrinology - Luteinizing Hormone –
49 Reproduction.

50

51 **1. Introduction**

52

53 Vertebrates generally respond to adverse conditions (“stressors”) by activation of the
54 hypothalamo-pituitary gland-adrenal (HPA) axis, resulting in elevated secretion and plasma
55 concentrations of glucocorticoids such as cortisol or corticosterone (CORT). Chronic (long-term)
56 and acute (i.e., within minutes to hours) activation of the HPA axis is often associated with
57 complex biochemical, behavioral, and physiological responses including changes in the activity
58 of the reproductive system [60,63]. Chronic stress often suppresses reproductive function
59 [7,53,19]. In males, acute stress likewise suppresses plasma testosterone (T). Examples
60 include fish (brown trout, *Salmo trutta*: [47], amphibians (Ocoee salamander, *Desmognathus*
61 *ocoee*): [70], and several bird species (*Zonotrichia* spp.: [69,43]; zebra finch, *Taeniopygia*
62 *guttata*: [44]; house sparrows, *Passer domesticus*, Abert’s towhees, *Melozone aberti*, and
63 Cassin’s sparrows, *Peucaea cassinii*; Deviche, Gao, and Davies, personal observations). These
64 observations indicate that the phenomenon is widespread across vertebrate taxa. However,
65 other studies on birds found either an increase in plasma T (cockerel: [25]; European starling,
66 *Sturnus vulgaris*: [61]) in response to stress or a decrease or increase depending on plasma T
67 levels before stress (semipalmated sandpiper, *Calidris pusilla*: [21]). The factors (e.g., type of
68 stress: [25]) that account for interspecies differences in the plasma T response to acute stress
69 are largely unknown. Identifying these factors and elucidating their role should benefit from a
70 detailed characterization of the time course, magnitude, and duration of stress effects on
71 plasma T.

72 Previous studies imply that stress may directly affect testicular function. Acute stress in
73 the white-crowned sparrow, *Zonotrichia leucophrys* decreases plasma T but not luteinizing
74 hormone (LH; [69]). The same was observed in turkeys, *Meleagris gallopavo* [15] and in the
75 male rufous-winged sparrow, *Peucaea carpalis*, in which acute stress also did not affect the
76 plasma T response to a LH injection [13]. These results suggest that acute stress does not
77 decrease plasma T by impairing LH secretion or attenuating the testicular sensitivity to this
78 hormone [13]. Acute stress in birds typically increases plasma CORT in 5-10 min [44,33,69,13]
79 and in the rufous-winged sparrow, plasma T decreases by 30% - 50% after capture and
80 confinement for 15-30 min [13]. Avian testes contain glucocorticoid receptors [32] and in
81 mammals, glucocorticoids influence T production through direct actions on interstitial (Leydig)
82 testicular cells [14,24,27]. Direct actions of CORT on the avian testes may, therefore, also

83 mediate rapid effects of acute stress on plasma T, but this hypothesis has not been
84 investigated. One objective of the present investigation was to address these issues by
85 determining and comparing the time course of changes in plasma CORT, LH, and T in response
86 to acute stress. We hypothesized that plasma T begins to decline after less than 15 min of
87 stress and that the magnitude of the decrease increases as a function of the stress duration.
88 This hypothesis was tested by determining the effects of capture and restraint for 5, 10, or 20
89 min.

90 No study has, to our knowledge, investigated the time course of endocrine recovery from
91 mild acute stress in wild, free-ranging birds. The *second objective* of the present work was to
92 address this question by exposing sparrows to acute stress to decrease their plasma T,
93 releasing them on their breeding territory, and then recapturing them at various times and
94 sampling them again.

95

96

97 **2. Material and methods**

98

99 *2.1. Study species and location*

100

101 The studies used a sedentary, year-round territorial and socially monogamous Sonoran
102 Desert songbird, the adult male rufous-winged sparrow, and were performed in and in the
103 vicinity of the Santa Rita Experimental Range, Pima County, Arizona, USA, where the bird
104 commonly breeds [36]. Sparrows were sampled during 27th – 31st July 2010 ($n = 40$; Studies 1a
105 and 2a, see below) and 4th – 8th August 2011 ($n = 55$; Studies 1b and 2b). Seasonal
106 reproduction in Rufous-winged Sparrows is associated with the summer monsoon and birds
107 during the study period were in breeding condition [36,12,57].

108

109 *2.2. Capture and blood sample collection*

110

111 Sparrows were captured in response to simulated territorial intrusion (STI: conspecific
112 song playback), while they were on their breeding territory and using a Japanese mist net. As
113 shown in a previous study on this species, plasma T in males in breeding condition is not
114 influenced by exposure to STI for durations similar to those in the present investigation [12].

115 Captures took place between 5:30 AM and 17:15 PM. Within two min of capture, a blood
116 sample was collected from a jugular vein of each bird into a heparinized plastic syringe to
117 determine *plasma initial* (= baseline) hormone concentrations. In other bird species, plasma
118 CORT does not increase markedly until birds are exposed to the stress of capture and restraint
119 for at least 2 min ([50,52], but see [8]). Birds were then confined to individual breathable cloth
120 bags for 5, 10, 20 (Studies 1a,b) or 30 min (Studies 2a,b; details below) after which a second
121 blood sample was obtained (except Study 2a, see below) to determine plasma *stress-induced*
122 hormone concentrations. Capture followed with mild restraint is commonly used in wild birds to
123 acutely and non-invasively stimulate the HPA axis and elevate plasma CORT [5,3,13]. This
124 method does not induce maximum secretory activity of the adrenal glands, as shown by the
125 observation in acutely stressed birds that administration of adrenocorticotrophic hormone further
126 elevates plasma CORT ([17] and references therein). Individuals were randomly assigned to
127 experimental groups. At the end of the 30 minute period of confinement, sparrows in Studies 2a
128 and b were released and, if possible, subsequently recaptured (details below), and a blood
129 sample was collected from each bird within two min of recapture. The volume of individual blood
130 samples approximated 120 μ l (2 blood samples collected from a bird: Studies 1a,b and 2a) or
131 80 μ l (3 blood samples collected from a bird; Study 2b). Following collection, samples were
132 immediately placed on ice until processed later the same day in the laboratory. Plasma was
133 separated by centrifugation, collected, and frozen until assayed (see below).

134 Prior to release at the capture site, each sparrow received a uniquely numbered metal
135 leg band (US Geological Survey) and an intramuscular injection of 0.9 % NaCl in distilled water
136 (volume equal to that of blood taken). Standard measurements (wing chord, \pm 1 mm; weight, \pm
137 0.1 g) were taken from each individual and served to calculate individual body condition indices,
138 defined as the residuals of a reduced major axis linear regression of wing chord over body mass
139 [22].

140 All activities were pre-approved by the Arizona State University Institutional Animal Care
141 and Use Committee and conducted under appropriate permits issued by the Bird Banding
142 Laboratory (US Geological Survey), the US Fish and Wildlife Service, the Arizona Game and
143 Fish Department, and the Santa Rita Experimental Range.

144

145 *2.3. Sample sizes*

146 Rufous-winged sparrows weigh on average 15-16 g (Table 1) and the volume of blood
147 collected from one bird in one day could, therefore, not exceed ~250 μl [16], yielding
148 approximately 125 μl of plasma. As a result of the limited volume of plasma available, individual
149 plasma samples could be assayed only for two hormones (either LH and T or T and CORT; see
150 below). The individual sample volume (80 μl , yielding approximately 40 μl of plasma) of blood
151 collected from Study 2b birds (see below) did not make it possible to assay these samples for
152 more than one hormone (i.e., T).

153
154

155 *2.3.1. Studies 1a and 1b*

156 The objective of Studies 1a (2010) and 1b (2011) was to characterize the acute (within
157 20 min of capture) effects of capture and restraint on plasma LH (Study 1a), T (Studies 1a,b),
158 and CORT (Study 1b). Sparrows were caught as described above, bled, placed in a cloth bag
159 for 5 min (2010: $n = 10$; 2011: $n = 11$), 10 min (2010: $n = 10$; 2011: $n = 10$), or 20 min (2010: $n =$
160 8 ; 2011: $n = 8$), and then bled again.

161

162 *2.3.2. Studies 2a and 2b*

163 The main objective of Studies 2a and 2b was to determine the time course of recovery of
164 plasma T following release after birds had been restrained for 30 min as described above. In
165 Study 2a (2010), 12 sparrows were caught, bled, restrained for 30 min, and released on site.
166 Attempts to recapture these birds later the same day resulted in 8 recaptures (= 67% of initial
167 captures) between 1 hr 03 min and 1 hr 41 min (average: 1 hr 19 min) of release. The study was
168 repeated in 2011 (Study 2b) to include a broader range of durations between release and
169 recapture and to include collection of a blood sample after the 30 min period of restraint. In
170 Study 2b, 17 of 26 sparrows (= 65% of initial captures) were recaptured between 0 hr 38 min
171 and 6 hrs 41 min (average: 3 hrs 08 min) of release.

172

173

174 *2.4. Hormone assays*

175

176 *2.4.1. Luteinizing hormone*

177 Plasma LH concentrations were determined using a micromodification of a chicken LH
178 radioimmunoassay described previously [54]. The assay has been previously validated for use
179 in the Rufous-winged Sparrow [11]. Briefly, the reaction volume was 60 μ l, comprising 20 μ l of
180 plasma sample or standard, 20 μ l of primary rabbit LH antibody, and 20 μ l of I^{125} -labelled LH.
181 The primary antibody was precipitated to separate free and bound I^{125} label using 20 μ l of
182 donkey anti-rabbit precipitating serum and 20 μ l of non-immune rabbit serum. All samples were
183 measured in a single assay. The intra-assay coefficient of variation was 7.3 % and the minimum
184 detectable dose was 0.15 ng/ml.

185

186 *2.4.2. Testosterone and corticosterone*

187 Plasma T and CORT were measured as previously described [12,13], using commercial
188 enzyme-linked immunoassay kits (Enzo Life Sciences, Ann Arbor, MI) according to the
189 manufacturer's recommended procedure. Samples were assayed in duplicate and were
190 randomly assigned to assay plates except that for each hormone samples (2 or 3 depending on
191 the study) from a given sparrow were assayed on the same plate.

192 For the T assay, all samples collected during the same year were assayed together after
193 10x (2010) or 8x (2011) dilution in assay buffer. The primary antibody used in the T assay has
194 less than 5% crossreactivity with 17β -estradiol, dihydrotestosterone, CORT, and progesterone
195 (manufacturer's specifications). The mean interassay and intrassay coefficients of variation
196 were 12.3% (3 samples assayed on each plate) and 2.5% ($n = 126$ samples), respectively, and
197 the assay sensitivity was 15 pg/ml.

198 For the CORT assay (only samples collected in 2011) samples were assayed together
199 following 15x dilution in assay buffer. The primary antibody used in the CORT assay has less
200 than 2% crossreactivity with progesterone, T, aldosterone, and 17β -estradiol. The mean
201 interassay and intrassay coefficients of variation were 12.1% (3 samples assayed on each
202 plate) and 2.0% ($n = 58$ samples), respectively, and the assay sensitivity was 142 pg/ml.

203

204 *2.5. Statistical analyses*

205

206 Data were analyzed using Student's t-tests, simple and multiple linear regressions,
207 Spearman rank order correlations, and analyses of variance (ANOVA) followed, when
208 appropriate, with multiple pair-wise comparison tests (Student-Newman-Keuls tests (SNK) or, in

209 the case of one-way repeated factor ANOVAs, Bonferroni t-tests). Data sets to be analyzed by
210 ANOVA and that were not normally distributed were either normalized by transformation to Log
211 X or ranked before analysis [6]. Data that were not transformed or transformed to Log X before
212 analysis are presented as means \pm standard errors (s.e.'s). Data that were ranked before
213 analysis are presented as medians \pm 0.5 interquartile intervals. The statistical significance level
214 of all tests was set at $p = 0.05$. Data were analyzed using SigmaPlot Version 11.0 (Systat
215 Software Inc., San Jose, CA), Statistica Version 10 (StatSoft. Inc., Tulsa, OK), and GraphPad
216 Prism Version 5.04 (GraphPad Software, Inc., La Jolla, CA).

217

218

219 **3. Results**

220

221 *3.1. Endocrine effects of capture and restraint*

222

223 *3.1.1. Plasma corticosterone (Study 1b)*

224 We analyzed plasma CORT data using repeated measure ANOVA with stress (initial vs.
225 stress-induced) and restraint duration (5, 10, or 20 min) as independent factors. Plasma initial
226 CORT was similar in the three experimental groups of sparrows (p 's > 0.05 , SNK) and
227 increased within 5 min of capture (stress effect: $F_{1,57} = 87.243$, $p < 0.001$; Fig. 1). There was no
228 overall restraint duration effect ($p > 0.3$). Even though there was an interaction between this
229 factor and stress ($F_{2,57} = 5.738$, $p = 0.009$), plasma stress-induced CORT in the three groups of
230 birds did not differ (p 's > 0.05 , SNK).

231

232 *3.1.2. Plasma luteinizing hormone (Study 1a)*

233 We analyzed plasma LH data as described for plasma CORT. Neither stress nor
234 restraint duration influenced plasma LH (p 's > 0.25), and there was no interaction between
235 these factors ($p > 0.80$; Fig. 2).

236

237 *3.1.3. Plasma testosterone (Studies 1a, b)*

238 To determine whether capture and restraint influenced plasma T, we combined 2010
239 and 2011 data into a single data set that we analyzed by repeated measure ANOVA with year
240 (2010 vs. 2011), stress, and restraint duration as independent factors. Plasma T was, on

241 average, higher in 2010 than 2011 ($F_{1,51} = 20.60, p < 0.0001$). As samples collected in 2010 and
242 2011 were assayed independently, it is unknown whether this difference reflects genuine year
243 differences in plasma T or interassay differences. There was, however, no year x stress, year x
244 restraint duration, or year x stress x restraint duration interaction (p 's > 0.075) and data for the
245 two years were, therefore, combined in further analyses. Plasma T decreased in response to
246 stress ($F_{1,51} = 54.62, p < 0001$) and was influenced by the restraint duration ($F_{2,51} = 4.71, p =$
247 0.013), but there was a stress x restraint duration interaction ($F_{2,51} = 5.74, p = 0.006$; Fig. 3).
248 Plasma initial T was similar in the three experimental groups (SNK: p 's > 0.25). Plasma stress-
249 induced T was lower than corresponding plasma initial T after restraint for 10 or 20 min, but not
250 5 min.

251 To further characterize the time course of stress effects on plasma T, we used two-way
252 ANOVA for repeated measures to compare hormone levels in sparrows sampled in 2011 after
253 restraint for 20 min (Study 1b) or 30 min (Study 2b). Plasma stress-induced T was lower than
254 plasma initial T (stress effect: $F_{1,67} = 65.391, p < 0.001$), but restraint for 30 min did not
255 decrease plasma T more than restraint for 20 min (stress effect x restraint duration interaction: p
256 > 0.100).

257

258 *3.1.4. Further characterization of plasma initial and stress-induced testosterone (Studies 1a,b* 259 *and 2b)*

260 Plasma initial T in 2010 and 2011 was individually variable (2010: $n = 40$; range: 1.14 –
261 58.03 ng/ml; coefficient of variation (CV): 98 %; 2011: $n = 55$: range: 1.05 – 25.12 ng/ml; CV: 69
262 %). We used multiple linear regression with four independent factors (year, capture time, body
263 size (as estimated by wing chord), and body condition index) to investigate potential sources of
264 this variation. None of these factors contributed significantly to accounting for the observed
265 individual variation in plasma initial T (p 's ≥ 0.065).

266 We combined 2010 and corresponding 2011 data to determine the relationship between
267 plasma initial T and plasma T measured after restraint for 10 min ($n = 20$), 20 min ($n = 16$), or 30
268 min ($n = 26$) using linear regressions. Plasma T data in birds that we restrained for 5 min were
269 not analyzed as stress at this time did not affect the circulating concentration of this hormone
270 (Section 3.1.3). Plasma stress-induced T was in all cases positively associated with plasma
271 initial T (10 min: slope = 0.52 ± 0.06 , coefficient of determination, $r^2 = 0.81$; 20 min: slope = 0.21
272 $\pm 0.04, r^2 = 0.64$; 30 min: slope = $0.21 \pm 0.04, r^2 = 0.59, p$'s ≤ 0.0002 ; Fig. 4, left panels).

273 Extending other findings (Section 3.1.3), the decrease in plasma T associated with stress was
274 proportionally larger in sparrows that we restrained for 20 min or 30 min than for 10 min
275 (comparisons of linear regression line slopes: 10 min vs. 20 min: $F_{1,32} = 18.188$, $p < 0.001$; 10
276 min vs. 30 min: $F_{1,42} = 21.285$, $p < 0.0001$), but in this respect birds that we restrained for 20 or
277 30 min did not differ (id., $p > 0.90$).

278 Further analyses revealed complex relationships between plasma initial and stress-
279 induced T. The percentage decrease in plasma T relative to plasma initial T in response to
280 capture and restraint was a function of plasma initial T (Fig. 4, right panels). In birds that were
281 restrained for 10 min and especially 20 or 30 min, plasma T decreased proportionally more in
282 response to stress when plasma initial T was high than low. In all cases, experimental data fit
283 the equation of a three parameter exponential decay curve ($y = y_0 + ae^{-bx}$; r^2 's > 0.22 , p 's $<$
284 0.05).

285

286 *3.1.5. Correlation between plasma testosterone and corticosterone (Study 1b)*

287 We used the Spearman rank order correlation test to research associations between
288 plasma T and CORT in sparrows in which both hormones were measured immediately after
289 capture and then after restraint for 10 min or 20 min (Study 1b, $n = 18$). Samples from sparrows
290 that were sampled after restraint for 5 min were not included in these analyses as this
291 manipulation did not influence plasma T (Section 3.1.3). Plasma initial T was not correlated to
292 plasma initial CORT ($r = 0.267$, $p > 0.05$). There also was no correlation between plasma stress-
293 induced T and CORT ($r = -0.201$, $p > 0.05$). These data provide no evidence that the CORT
294 stress response accounted for the stress-induced reduction in plasma T.

295

296 *3.2. Plasma testosterone after on-site release (Study 2b)*

297

298 We compared plasma initial, stress-induced, and at recapture T to determine whether
299 the stress-induced decrease in plasma T dissipated after release. Birds at these three times had
300 different plasma T levels (repeated measure ANOVA: $F_{2,50} = 14.4$, $p < 0.001$; Fig. 5a).
301 Consistent with the results of Studies 1a, b (see above), restraint for 30 min decreased plasma
302 T (Bonferroni t-test: $p < 0.05$). Plasma T at recapture was still lower than at initial capture
303 (Bonferroni t-test: $p < 0.05$) and did not differ from plasma T at the time of release (id., $p > 0.05$).
304 The difference between plasma initial T and plasma T at the time at recapture was not related to

305 how long a bird had been released (Fig. 5b; linear regression: $p > 0.5$). The difference between
306 plasma stress-induced (i.e., at release) T and plasma T at recapture was likewise unrelated to
307 how long a bird had been released (Fig. 5c; linear regression: $p > 0.3$).

308 Collectively, the data offer no evidence that the stress-induced inhibition of plasma T
309 dissipated between release and recapture up to almost 7 hours later.

310

311 *3.3. Comparison of recaptured and not recaptured males (Studies 2a, b)*

312

313 Following initial capture and release, approximately one third of the birds sampled in
314 Studies 2a and 2b either were not re-sighted or were re-sighted in the vicinity of the capture site
315 but could not be recaptured. We analyzed whether recaptured males differed morphologically
316 and/or physiologically from those we did not recapture. For this, we compared data for five
317 parameters (capture time, plasma initial T, wing chord, body mass, and body condition index)
318 between the two male groups using two-way ANOVAs (independent factors: year (2010 vs.
319 2011) and recapture vs. no recapture). Males studied in 2010 were not bled at the end of the 30
320 min restraint period and before release (see Materials and Methods). Therefore, Student's t-test
321 was used to compare plasma stress-induced T in recaptured vs. not recaptured males sampled
322 during that year. In 2011 recaptured males were, on average, initially caught earlier in the day
323 than males that we did not recapture (year x recapture vs. no recapture interaction: $F_{1,37} = 6.43$,
324 $p = 0.016$; Table 1). Except for this difference, males that we recaptured did not differ in any
325 respect from males that we did not recapture (p 's > 0.1). These results suggest that individuals
326 that we did or not recapture were similar morphologically and physiologically.

327

328

329 **4. Discussion**

330

331 Consistent with previous studies on rufous-winged sparrows and some other species,
332 acute stress resulting from capture and handling decreased plasma T (see Introduction for
333 references). This decrease was observed within 10 min of capture and its magnitude increased
334 as a function of the duration of exposure to the stressor until birds were released 30 min after
335 capture. The stress-induced decrease in plasma T persisted for at least 6 hours after release
336 and was, therefore, relatively long-lasting. The present study is to our knowledge the first to

337 describe this persistence in any free-ranging avian species. A single previous study on free-
338 ranging birds other than rufous-winged sparrows investigated whether acute stress influences
339 plasma LH (white-crowned sparrow: [69]). As found here, the inhibitory effect of stress on
340 plasma T in these sparrows was not associated with a decline in plasma LH. The stress-induced
341 decline in plasma T was not related to the stress-induced increase in plasma CORT, indicating
342 that the decline in plasma T was not simply a function of increased CORT secretion.
343 Furthermore, the plasma T response to stress was relatively larger in males with high than low
344 plasma initial T, revealing a complex, plasma initial T-related T response to stress. These data
345 provide new insights on interactions between acute stress and reproductive hormones in free-
346 ranging birds and on the mechanisms that potentially mediate these interactions, and they
347 contribute to our understanding of the bases of individual differences in circulating androgen
348 levels in intact birds.

349

350 *4.1. Inhibitory effect of acute stress on plasma testosterone: characterization and mechanisms*

351

352 As previously reported for white-crowned [69] and rufous-winged sparrows [13], acute
353 stress in the present study decreased plasma T without affecting plasma LH. In a previous
354 investigation, acute stress also did not attenuate the LH response of male Rufous-winged
355 Sparrows to an injection of the gonadotropin-releasing hormone (GnRH) secretagogue N-
356 methyl-D,L-aspartate (NMA) or of GnRH itself, or the T response to LH administration [13].
357 These results suggest that acute stress in this species does not decrease plasma T by acting on
358 the hypothalamo-pituitary axis. Furthermore, the effect of stress was time-dependent: plasma T
359 decreased as little as 10 min after capture and restraint and then further decreased as the
360 restraint duration increased. The short latency for stress to affect plasma T and the time-
361 dependency of the T response suggest mediation of the response by one or several non-
362 genomic mechanisms. One such mechanism may consist of a rapid direct inhibition of testicular
363 function by glucocorticoids [14,24,27,39]. Alternatively, glucocorticoids may influence plasma T
364 by accelerating its clearance through interactions with plasma corticosterone-binding globulin,
365 which in birds binds CORT and T reversibly, competitively, and with high affinity [9]. To our
366 knowledge, no study has, however, investigated whether CORT influences T clearance in any
367 species.

368 Acute stress in rufous-winged sparrows had overall opposite effects on plasma CORT
369 (increase) and T (decrease; compare Figs. 1 and 3). However, and similar to the situation in the
370 black-legged kittiwake, *Rissa tridactyla* [19], sparrows showed no individual correlation between
371 plasma initial CORT and T. We also found no correlation between the plasma concentrations of
372 these hormones in stressed sparrows. These data do not refute the possibility that CORT
373 influences the plasma T response to stress, but they indicate that if present, this influence is
374 complex and not reflected simply in the plasma concentrations of these hormones.

375 Acute stress may, alternately, decrease plasma T through a glucocorticoid-independent
376 mechanism. One such mechanism may consist of a gonadotropin-inhibitory hormone- (GnIH)
377 mediated impairment of T production [41] and/or a suppression of testicular endocrine function
378 resulting from stress-mediated activation of a sympathetic nervous pathway terminating in the
379 gonads. This pathway and its inhibitory influence on T secretion during stress have been
380 defined in mammals [26,28]. Avian testes receive sympathetic innervation [65] but the function
381 of this innervation in birds has not been investigated.

382

383 *4.2. Persistence of the stress-induced plasma testosterone decrease: mechanism and* 384 *consequences*

385

386 Previous studies found that the adrenocortical CORT response to stress changes during
387 repeated stress exposure. For example, free-ranging female eastern bluebirds, *Sialis sialis*, had
388 similar plasma initial CORT but increased their plasma level of this hormone more when caught
389 and restrained for the first time than a second time weeks later [37]. Similarly, the effect of acute
390 stress on plasma CORT in captivity decreased during repeated exposure to stressors in rats
391 [18] and American kestrels, *Falco sparverius* [35]. These observations indicate that acute stress
392 can alter the HPA sensitivity to subsequent stress exposure, but do not indicate how long the
393 endocrine effects of acute stress persist following a single stressful event and once subjects are
394 no longer exposed to the stressor. To our knowledge the present investigation is the first to
395 address this issue in free-ranging birds. In rufous-winged sparrows that we caught and
396 restrained for 30 min and then released on site, plasma T decreased by approximately 50% and
397 then remained low for at least 6 hours. During this period, plasma T levels did, on average, not
398 differ from levels at the time of release. Thus, the post-release endocrine effect of acute stress
399 persisted for several hours during which birds exhibited no sign of recovery.

400 Sparrows were recaptured using conspecific song playbacks and while still on their
401 breeding territory. Acute stress did, therefore, not lead to territory abandonment or eliminate
402 aggressive responses. However, as we did not study the behavior of the experimental birds, it
403 cannot be excluded that post-release low plasma T was associated with a partial inhibition of
404 spontaneous or STI-induced song rate or aggressivity. This hypothesis would be consistent with
405 the mounting body of evidence demonstrating that T can exert rapid effects (min to hours;
406 review: [42]). For example, in the White-crowned Sparrow T withdrawal after chronic treatment
407 with this hormone reduced the size of and increased the density of neurons in one brain region
408 involved in song production, the HVC, within 12 hours [59]. In the castrated male Japanese
409 quail, T treatment for one day sufficed to increase the size of the preoptic nucleus, which
410 controls reproductive behavior, and the expression of aromatase in this region [4]. In fish, an
411 opportunity to increase social status was followed within 30 min by increased expression of
412 reproductive behavior and plasma androgen levels [40], and T administration stimulated males
413 to approach females within 45 min [34]. In light of these findings, low plasma T for several hours
414 after release, as seen in the present study, may have been associated with subtle behavioral
415 and/or physiological changes.

416 Whether this was the case requires further investigation because it is unknown whether
417 a decrease in plasma T such as found here (average ~50%), and irrespective of the duration of
418 this decrease, is behaviorally or physiologically consequential. Two commonly used
419 experimental approaches to investigate the effects of T consist of (a) castration and (b)
420 hormone replacement to castrates or administration to intact subjects with naturally low plasma
421 T. Castration eliminates the main source of T, resulting in the steroid circulating at negligible or
422 undetectable concentrations. This manipulation in birds negatively influences the brain
423 production of T-sensitive enzymes such as aromatase [64], the size and neuronal
424 characteristics of androgen-sensitive brain regions [58], and the expression of androgen-
425 dependent behaviors [1,23,48]. In the second approach, T is often administered chronically to
426 result in circulating levels of the hormone that are similar to naturally maximum levels [2,49].
427 However, we know little about the shape of the relationship between plasma T concentrations
428 varying within the physiological range and the expression of androgen-dependent behavioral,
429 morphological, and physiological traits. As is commonly the case in other endocrine systems
430 [45], this relationship appears to be non-linear [30,31] and this non-linearity may contribute to

431 the frequently reported absence of correlation in intact birds between plasma T and the
432 expression of T-dependent behavior (e.g., [56]).

433

434 *4.3. Plasma testosterone: individual variability and relationship between plasma initial and* 435 *stress-related hormone levels*

436

437 Plasma T in most high and middle latitude breeders undergoes large seasonal changes
438 and is generally highest during the breeding season [46,62,55]. This pattern can be modified by
439 social factors, which in some circumstances rapidly elevate plasma T above seasonal levels
440 [66,68,51]. As a result, plasma T at any given time reflects the combined influence of seasonal
441 and facultative regulatory factors [20]. The multiplicity and complexity of mechanisms that
442 regulate plasma T usually result in large inter-individual variability, as noted in previous studies
443 [29] as well as in the present investigation. Individual variation in plasma initial T in rufous-
444 winged sparrows was not accounted for by the capture time, the body size, the body condition
445 index, or exposure to STI [12]. We found plasma initial and stress-related T concentrations to be
446 positively related (Fig. 4, left panels). However, close examination of the data revealed this
447 relationship to be complex: when restrained for 10, 20, or 30 min, sparrows with initially low
448 plasma T decreased their hormone level proportionally less than birds with initially high plasma
449 T (Fig. 4, right panels). For example, after restraint for 20 or 30 min plasma T had, on average,
450 decreased by approximately 20% in birds with initially low (≤ 5 ng/ml) plasma T, but by
451 approximately 70% in birds with initially high (≥ 10 ng/ml) plasma T. Thus, individuals with low
452 plasma initial T were relatively more resistant to the effects of acute stress than those with high
453 plasma initial T. A somewhat similar situation was observed in the semipalmated sandpiper [21].
454 In this species, plasma T in response to capture and restraint stress increased and decreased in
455 birds with initially low and high plasma T, respectively. What is the potential significance of
456 these observations?

457 Seasonally (i.e., in many bird species, photoperiodically) regulated plasma T levels are
458 thought to be necessary and sufficient to maintain androgen-dependent physiological and
459 behavioral functions such as reproductive behavior, spermatogenesis, and secondary sexual
460 characters [10,20]. In previous avian and non-avian studies showing that acute stress inhibits
461 plasma T [43,47,69,70], this inhibition was only partial, resulting in stress-induced plasma T
462 remaining within 25% - 75% of plasma initial T. We found here that when exposed to a same

463 stressor, birds with high plasma initial T decreased their plasma T proportionally more than birds
464 with low plasma initial T. Furthermore, restraint for 10 min decreased plasma T less than
465 restraint for longer durations, but the effect of restraint for 20 min or 30 min on plasma T were
466 similar. These findings, along with data indicating that social interactions can in some situations
467 increase T secretion within minutes, confirm that plasma T levels are labile and prone to rapid
468 changes. The available results are consistent with the hypothesis that when faced with acute
469 stress, organisms decrease their plasma T, but not below the seasonally appropriate level
470 necessary to maintain essential androgen-dependent functions. Further research is warranted
471 to identify the putative mechanism that controls the balance between inhibition and maintenance
472 of plasma T above physiologically and behaviorally necessary levels.

473

474

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476

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478

479

480 **References**

481

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- 689

690 **Figure legends**

691

692 **Fig. 1.** Plasma corticosterone (CORT; medians + 0.5 interquartile intervals) of adult male
693 rufous-winged sparrows, *Peucaea carpalis*, within 2 min of capture (Initial) and after 5, 10, or 20
694 min of restraint (Stress-induced). Sample sizes are indicated within columns. An asterisk
695 denotes a statistically significant increase relative to plasma initial T ($p \leq 0.05$; Student-
696 Newman-Keuls test).

697

698 **Fig. 2.** Plasma luteinizing hormone (LH; means + s.e.'s) of adult male rufous-winged sparrows,
699 *Peucaea carpalis* measured within 2 min of capture (= Initial) and after restraint for 5, 10, or 20
700 min (= Stress-induced). Sample sizes are indicated within columns.

701

702 **Fig. 3.** Plasma testosterone (T; means + s.e.'s) of adult male rufous-winged sparrows, *Peucaea*
703 *carpalis* measured within 2 min of capture (= Initial) and after restraint for 5, 10, or 20 min (=
704 Stress-induced). An asterisk denotes a statistically significant decrease relative to plasma initial
705 T ($p < 0.05$; Student-Newman-Keuls test); n.s. = $p > 0.05$. Sample sizes are indicated within
706 columns.

707

708 **Fig. 4.** Relationships in adult male rufous-winged sparrows, *Peucaea carpalis*, between plasma
709 initial testosterone (T) and plasma T after capture and restraint for 10, 20, or 30 min (left
710 panels); and plasma initial T and the percentage decrease in plasma T associated with capture
711 and restraint for 10, 20, or 30 min (right panels). On each panel, each point represents one
712 different individual.

713

714 **Fig. 5.** (a) Plasma testosterone (means \pm s.e.'s; $n = 17$) of adult male rufous-winged sparrows,
715 *Peucaea carpalis*, at capture (Initial), after 30 min of restraint (30 min stress), and at recapture
716 0.5 – 7 hrs after release (Recapture). Means with the same letter do not differ significantly ($P >$
717 0.05 ; Bonferroni t-test). (b) Difference between plasma T at recapture and plasma initial T of the
718 same males as in panel (a). (c) Difference between plasma T at recapture and at release of the
719 same males as in panel (a). Each point on panels b and c represents one individual.

Figure 1.

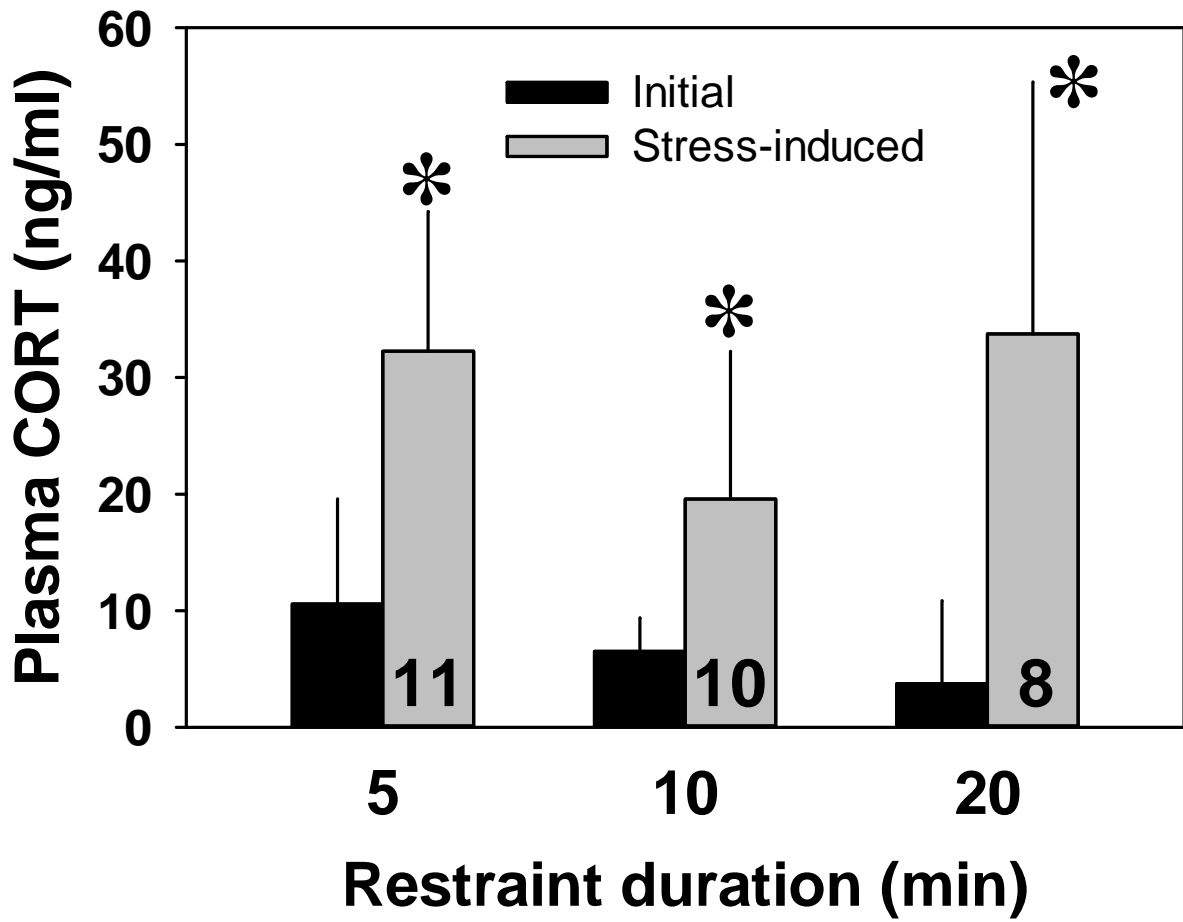


Figure 2.

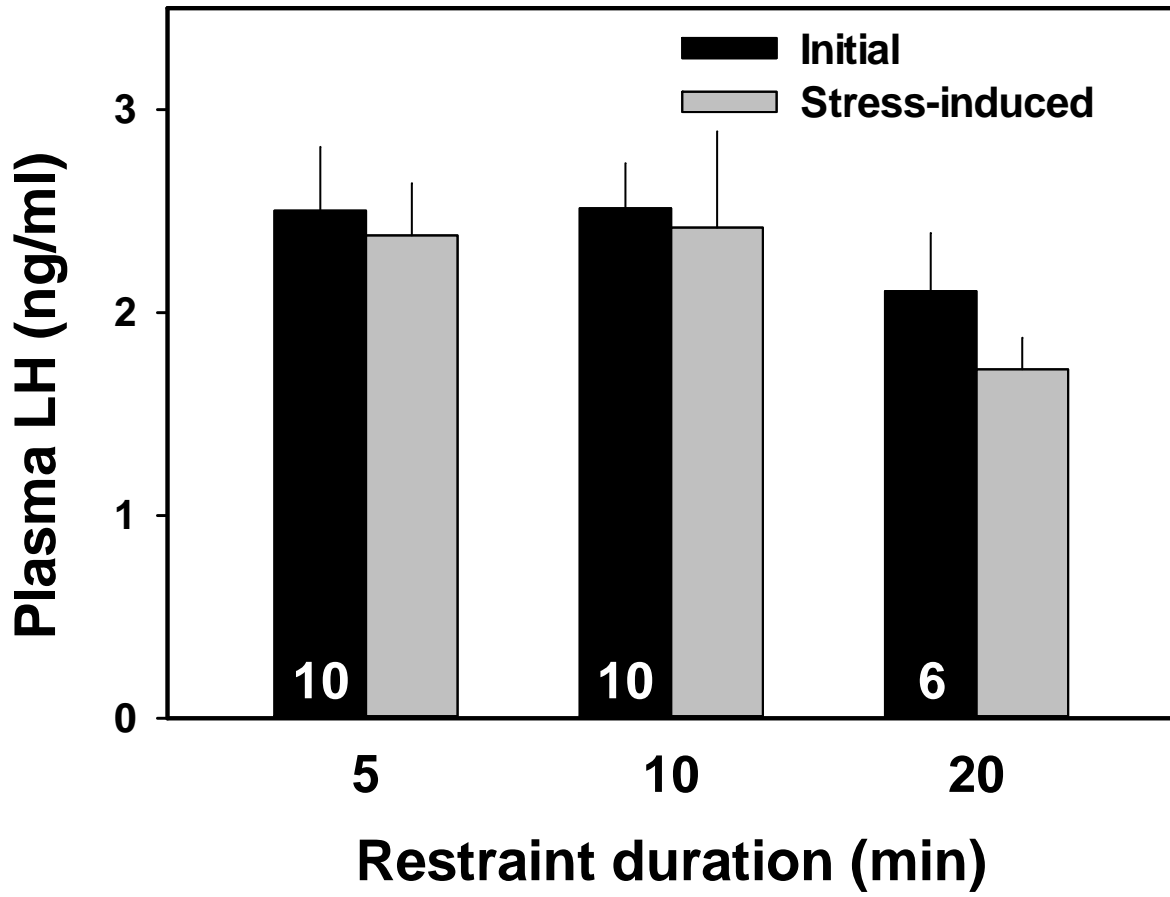


Figure 3.

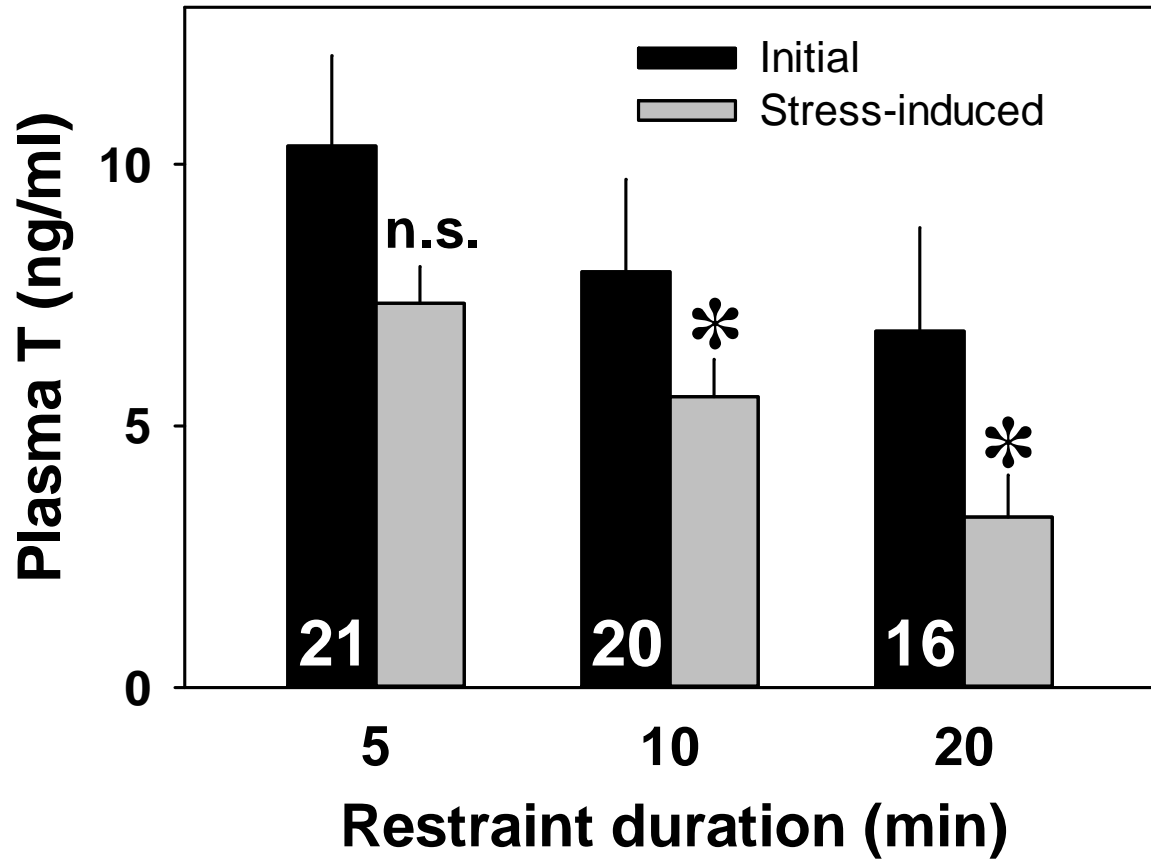


Figure 4.

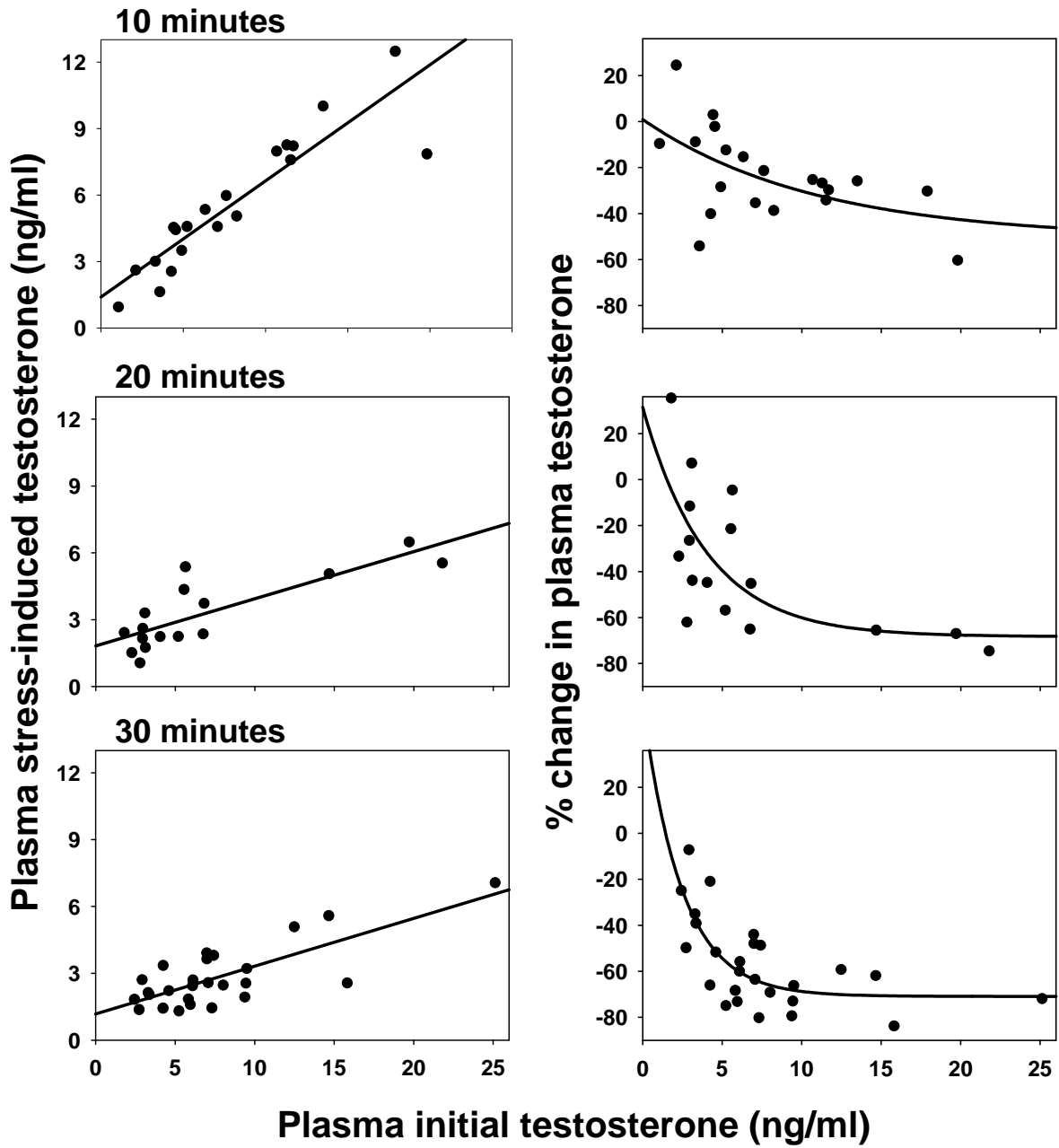


Figure 5.

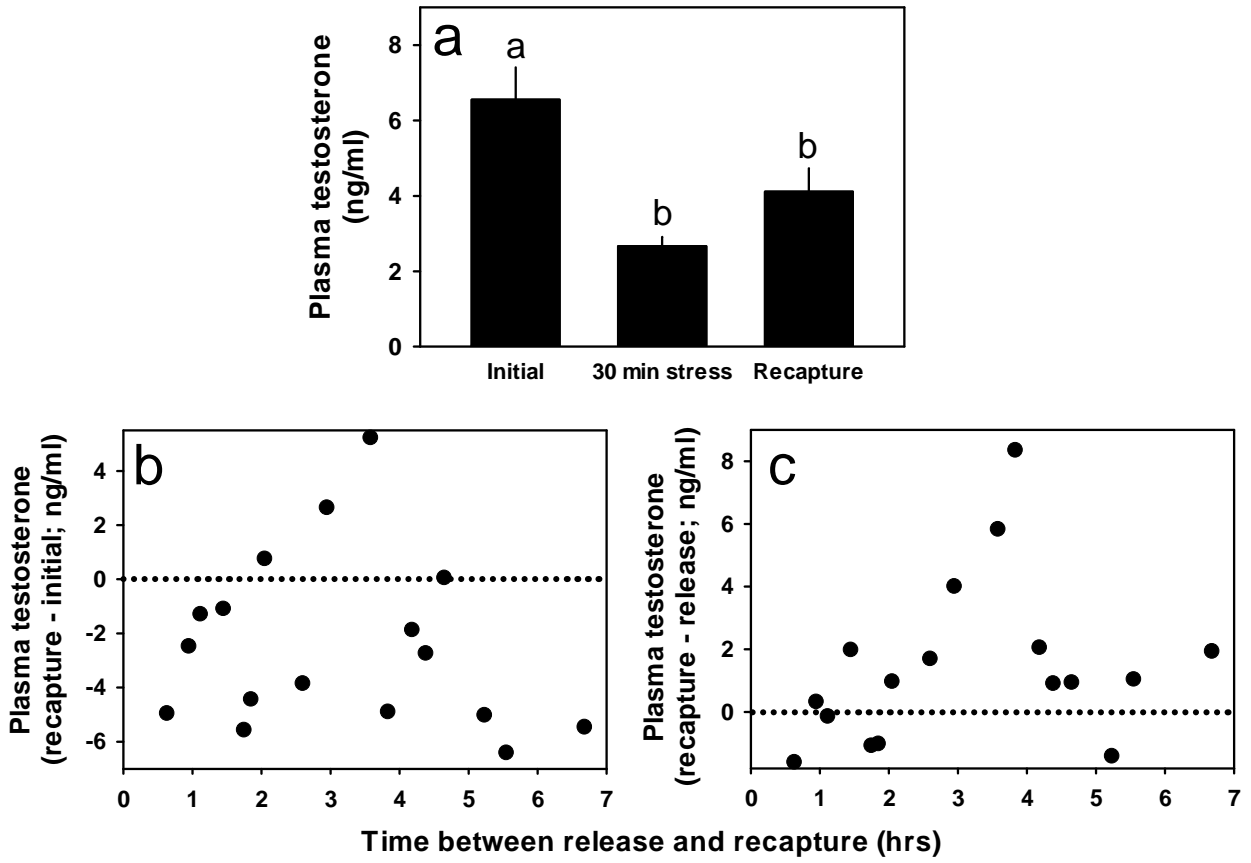


Table 1: Comparison (means \pm s.e.'s unless otherwise indicated) of various parameters between adult male rufous-winged sparrows, *Peucaea carpalis*, which were or not recaptured within hours of an initial capture in 2010 and 2011.

Year	Recapture?	<i>n</i>	Initial capture time (hrs, decimal) ^a	Plasma initial T (ng/ml) [*]	30 min stress plasma T (ng/ml) [*]	Wing chord (mm) ^a	Body mass (g)	Body condition index ^{**}
2010	Yes	8	9.9 \pm 3.3	4.61 \pm 1.22	2.80 \pm 0.55	62 \pm 1	15.7 \pm 0.1	-0.12 \pm 0.14
	No	4	7.8 \pm 2.2	6.09 \pm 1.53	N/A	61 \pm 0	15.2 \pm 0.3	-0.49 \pm 0.31
2011	Yes	17	6.8 \pm 0.9	6.55 \pm 0.86	2.66 \pm 0.25	62 \pm 1	15.9 \pm 0.2	-0.06 \pm 0.22
	No	9	11.7 \pm 2.6	9.53 \pm 2.25	3.06 \pm 0.65	62 \pm 1	16.3 \pm 0.2	0.44 \pm 0.21
Recaptured vs. not recaptured males			Recap. < Not Recap. (2011 only)	n.s.	n.s.	n.s.	n.s.	n.s.

*T = testosterone;

** body condition index expressed as the residual of a reduced major axis linear regression of wing chord over body mass;

^a: medians + 0.5 interquartile intervals;

n.s. = $p > 0.05$ (analysis of variance).