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1 **Title:** The effect of chronic and acute stressors, and their interaction, on gonadal function: an
2 experimental test during gonadal recrudescence.
3
4 **Running title:** The effect of chronic and acute stressors on gonads.
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6
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18 **Summary statement**

19 An acute stressor downregulated testosterone production, but this effect was absent in
20 chronically disturbed birds. The acute stressor had a strong effect on the gonadal transcriptome,
21 whereas chronic disturbance had a negligible effect.

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22 **Abstract**
23 Organisms are expected to invest less in reproduction in response to a stressor, but theory
24 predicts that this effect should depend on the frequency of stressors in the environment. Here
25 we investigated how an acute stressor affected gonadal function in a songbird, and how long-
26 term differences in the stress environment influenced these acute stress responses. We
27 exposed male Dark-eyed Juncos (*Junco hyemalis*) either to chronic or minimal (control)
28 disturbance during gonadal recrudescence, after which we measured baseline testosterone,
29 testosterone after an acute handling stressor, and the ability to elevate testosterone in response
30 to hormonal stimulation. In a 2x2 design, we then euthanized males from the two chronic
31 treatment groups either immediately or after an acute stressor to investigate the effect of these
32 treatments on the gonadal transcriptome. We found that chronically disturbed birds had
33 marginally lower testosterone. The acute stressor suppressed testosterone in control birds, but
34 not in the chronic disturbance group. The ability to elevate testosterone did not differ between
35 the chronic treatments. Surprisingly, chronic disturbance had a weak effect on the testicular
36 transcriptome, and did not affect transcriptomic response to the acute stressor. The acute
37 stressor, on the other hand, upregulated cellular stress response, and affected expression of
38 genes associated with hormonal stress-response. Overall, we show that both chronic and acute
39 stressors affect reproductive function, and that chronic stress changes how acute stressors
40 affect testosterone physiology. Our findings also suggest that acute and chronic stressors affect
41 testes differently, and that gonadal function is relatively robust to long-term stressors.

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42 **1. Introduction**

43 It is well-known that stressors can have a profound negative effect on reproductive physiology
44 and behavior (Chand and Lovejoy, 2011; Rivier and Rivest, 1991; Selye, 1946). This effect has
45 been demonstrated in relation to a variety of stressors, including food limitation (Lynn et al.,
46 2015), thermal stress (Hansen, 2009), and psychological stress (McGrady, 1984; Nargund,
47 2015). The main adaptive hypothesis for this suppressive effect states that reproduction is
48 inhibited because the physiological, energetic, and behavioral components of reproduction are
49 costly and may directly interfere with resource and time allocation to stress response (Breuner
50 et al., 2008). Because the stress response is an integral part of self-maintenance and survival,
51 the interaction between stress and reproduction constitutes an important aspect of the life-
52 history trade-off between current and future reproduction (Wingfield and Sapolsky, 2003).

53 Life history theory predicts that the effect of stress on reproduction should depend on the
54 costs and benefits of both of these functions (Wingfield and Sapolsky, 2003). For example, if
55 future reproductive success (residual reproductive value) is expected to be low, succeeding at
56 the current reproductive effort is crucial to the fitness of the organism. It is expected that under
57 these conditions reproduction should be more resistant to the suppressive effects of stressors
58 compared to cases when future reproductive success is expected to be high. Consistent with
59 this prediction, stress has been shown to be less suppressive of reproduction in semelparous
60 organisms and organisms that have short seasonal breeding seasons (Wingfield and Sapolsky,
61 2003). At an intraspecific level, stressors have been shown to have a reduced effect on
62 reproductive behaviors in populations that inhabit high-disturbance urban areas compared to
63 rural habitats (Abolins-Abols et al., 2016).

64 While these findings are consistent with the theoretical predictions, we lack experimental
65 studies that explicitly test these predictions and study which conditions lead to changes in the
66 suppressive effect of stress on reproduction. Revealing the conditions and mechanisms that
67 mediate effects of stress on reproduction is important for a better understanding of reproductive
68 physiology and ecology of animals (Calisi et al. 2017), especially in the context of the rapid
69 human-induced environmental change (Sih et al. 2011). Ultimately, such knowledge will aid in
70 developing a predictive understanding of how and why individuals or populations respond
71 differently to stressors.

72 We are addressing this question by focusing on the gonad, as one of the most important
73 reproductive organs. In vertebrate males, testes produce sperm and androgen hormones, such
74 as testosterone. Testosterone serves multiple important roles in reproduction: it regulates the
75 development of the sexual phenotype (Hau, 2007), and is important in mediating behaviors

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76 necessary for reproduction, such as courtship (Fusani et al., 2014; Hutchinson, 1967) and
77 territorial behavior (Soma, 2006; Wingfield et al., 1987). Testosterone synthesis is largely
78 regulated by the hypothalamic-pituitary-gonadal (HPG) axis, where gonadotropin releasing
79 hormone (GnRH) from hypothalamus causes the secretion of luteinizing hormone (LH) from the
80 pituitary, which in turn upregulates synthesis of testosterone by the Leydig cells in gonads
81 (Farner and Wingfield, 1980; London et al., 2006). However, testosterone synthesis by gonads
82 has also been shown to change in response to local signaling factors independent of brain
83 signaling (Nogueiras et al., 2004; Tena-Sempere et al., 2002). Testosterone levels can be
84 suppressed by both psychological (Deviche et al., 2010; Moore and Mason, 2000) as well as
85 physical stressors (Lynn et al., 2015; Nelson et al., 1989). This decrease may be mediated via
86 suppressive effects of stressors on GnRH or LH secretion (Breen and Karsch, 2006; Breen et
87 al., 2007; Nikolarakis et al., 1986) or by direct action of components of the physiological stress
88 response, such as the hypothalamic-pituitary-adrenal (HPA) axis, on gonads (Lynn et al., 2015;
89 McGuire et al., 2013).

90 Gonads have been shown to be especially sensitive to stressors and other perturbations
91 during development (Guillette Jr et al., 1994; Rhind et al., 2001; Zambrano et al., 2014). For
92 example, maternal stress during fetal gonadal development can result in reduced gonadal size
93 (Dahlöf et al., 1978), and exposure to glucocorticoids during fetal development has been shown
94 to reduce testosterone synthesis capacity *in vitro* (Page et al., 2001) and delay the onset of
95 puberty (Smith and Waddell, 2000). Such developmental programming has important
96 implications for pathology and reproductive rates in the wild (McMillen and Robinson, 2005;
97 Sheriff et al., 2010). If gonadal responses to developmental stress are adaptive, then the effect
98 of stressors on gonadal function should conform to the life history expectations, as outlined
99 above, wherein gonads are expected to be less sensitive to stressors in animals undergoing
100 development in a high-stress environment compared to animals developing in a benign
101 environment.

102 In this study we experimentally investigated how acute and chronic stressors
103 experienced during seasonal gonadal recrudescence (development) change gonadal function in
104 Dark-eyed Junco (*Junco hyemalis*), a small passerine. In particular, we asked if chronic
105 disturbance and acute stressors, and their interaction, changed baseline testosterone levels, the
106 overall ability to elevate testosterone following stimulation with exogenous GnRH, testicular
107 sensitivity to regulation by HPG and HPA axes, and testicular transcriptome in general. In
108 general, we expected both types of stressors to reduce reproductive function in animals with
109 actively recrudescing gonads, but that animals in high disturbance environment would show a

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110 reduced sensitivity of reproductive physiology to acute stressors, compared to animals in a low
111 disturbance (control) environment. Specifically, we predicted that both chronic and acute
112 stressors should decrease testosterone levels, but that the suppressive effect of an acute
113 stressor on testosterone synthesis would be dampened in chronically disturbed animals. We
114 further predicted that both chronic and acute stressors would negatively affect genes associated
115 with testosterone production and spermatogenesis, up-regulate expression of genes involved in
116 cellular and hormonal stress response, but that the effect of acute stressor on the gonadal
117 transcriptome would be dampened in the chronically disturbed animals. More specifically, we
118 expected that stressors would down-regulate steroidogenesis gene expression and up-regulate
119 receptors for stress hormones implicated in regulation of gonadal physiology, such as
120 glucocorticoid-receptor (GR)(McGuire et al., 2013) and gonadotropin-inhibitory hormone
121 receptor (GnIHR)(McGuire and Bentley, 2010).
122

123 **METHODS**

124 **2.1 Capture and housing of study organisms**

125 We captured 36 wintering male Dark-eyed Juncos *Junco hyemalis hyemalis* in Bloomington,
126 Indiana, in December 2013 using baited mistnets and walk-in traps. We determined the sex of
127 birds using plumage color and confirmed it with genetic sex markers (Griffiths et al., 1998).
128 Before the experiment, birds were housed in free-flying groups in indoor aviary rooms, reflecting
129 their natural winter flocking lifestyle. On January 15, 2014 we started to gradually (3 times per
130 week) increase day length from natural Indiana winter photoperiod (10:44 daylight hrs, **Figure**
131 **1**). On January 27 males were transferred from free flying flocks to individual 2x2 ft metal cages,
132 9 cages per room, with visual access to other birds, including females. Cages contained food,
133 water bowls, and perches. Food was provided *ad libitum* three times a week, and cages were
134 cleaned once a week. When the experiment began on February 3, animals were experiencing
135 13:23 hrs of daylight, and by the end of the experiment on February 26, birds were exposed to
136 16:03 hrs of daylight, reflecting a summer-like photoperiod. Males were singing during the last
137 week of the experiment.
138

139 **2.2. Chronic disturbance treatment**

140 We randomly assigned half of the birds (n=18) to a chronic disturbance treatment, while the
141 other half (n=18) were treated as controls. In the chronic disturbance treatment, birds were
142 exposed to either physical disturbance (hand-waving, cage-tapping) or to a predator mount for
143 30 min four times each day for three weeks. During physical disturbances, observers (one per

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144 room) either waved their hand inside the cage, or tapped on the cage for 30 sec, after which the
145 observer moved on to another cage in random order. For the predator stressor, we used
146 taxidermic study skins of Cooper's Hawk, Barn Owl, and Fox Squirrel, which were fixed to a
147 tripod and left in the room by the observer. All these treatments had previously been validated in
148 a pilot study and shown to result in an increase in corticosterone, a measure of a physiological
149 stress response (Hanauer et al. in prep). In the pilot study, physical disturbance caused the
150 highest increase in corticosterone, therefore birds in the chronic disturbance treatment received
151 at least one of these disturbances each day (average: 2.5 physical disturbances each day).
152 Otherwise, the choice of the stressors for each disturbance bout was random. The disturbance
153 bouts occurred at least 60 min apart for each room and were administered during daylight
154 hours. In the control group, we did not disturb the birds except for standard animal husbandry
155 (see above).
156
157 **2.3. Blood sampling and acute handling stressor**
158 We took blood samples from each bird to analyze testosterone and corticosterone
159 concentrations before (February 1-2) and after the three weeks (February 23-24) of chronic
160 disturbance or control treatment. In addition, blood was taken from birds one week after the
161 beginning of treatment for analysis of corticosterone levels (February 8-9). Results describing
162 corticosterone levels in response to treatments are reported in Hanauer et al. (in prep.). Each
163 bird was captured between 7 am and 12 pm, and a baseline blood sample (100 ul) was taken
164 from the brachial vein within 4 min of capture using a 26-gauge needle and microcapillary tubes.
165 After the sample was taken, each bird was kept in a brown paper bag for 30 min, a standard
166 acute handling stressor that is widely used to assess physiological stress response (Sheriff et
167 al., 2011). After 30 min in the paper bag, each bird was bled again (100 ul) to measure acute
168 stressor-induced hormone levels. Blood was stored at 4°C until centrifugation. Later the same
169 day, we centrifuged the microcapillary tubes to separate plasma and measure hematocrit,
170 following which plasma was removed using Hamilton syringe and stored in -20°C until analysis.
171
172 **2.4. GnRH injection following an acute stressor**
173 To assess the ability of gonads to increase T, during the last sampling round at the end of the
174 experiment (February 23-24) we injected birds with 50 ml of 25 ng/ul gonadotropin releasing
175 hormone (GnRH, in 1M PBS, American Peptide Company Inc., Sunnyvale, CA, product no. 54-
176 8-23) 35 min after capture (i.e. after the stress-induced 30 min blood sample was collected).
177 GnRH is the top regulator of testosterone synthesis that is naturally produced in hypothalamus

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178 (Ciechanowska et al., 2010). GnRH-induced testosterone levels have been shown to be
179 repeatable (Jawor et al., 2006) and are linked to a higher probability of survival (McGlothlin et
180 al., 2010) and morphological characteristics (Atwell et al., 2014). To administer GnRH, we
181 cleaned the pectoral muscle using an alcohol swab, and injected 50 ul of GnRH using a
182 Hamilton syringe. GnRH solutions were kept on ice before the injection to prevent degradation
183 of the peptide. We then collected blood (100 ul) 30 min after injection to measure the capacity of
184 gonads to secrete testosterone following an acute stressor.
185

186 **2.5. Morphological measurements**

187 After blood collection we used a ruler to measure the size of the cloacal protuberance by
188 measuring its length from body to the cloacal opening to the nearest 0.5 mm. We also
189 measured the pectoral muscle condition, fat score, and mass. The effects of the treatment on
190 condition, fat, and mass are reported elsewhere (Hanauer et al. in prep).
191

192 **2.6. Hormone assays**

193 We measured testosterone levels in the blood plasma using commercial enzyme immune assay
194 (EIA) from Enzo Life Sciences (Farmingdale, NY, product no. ADI-901-65) that has been
195 previously validated for use in this species (Clotfelter et al., 2004). Testosterone was extracted
196 from 20 ul of plasma using diethyl ether. Tritiated testosterone was added to the sample to
197 estimate extraction efficiency (average 94.8%). Extracted hormone was reconstituted in 50 ul of
198 98% ethanol, followed by 300 ul of assay buffer. We followed manufacturer's instructions for the
199 remaining procedures. We estimated hormone concentrations in reference to a 9-point standard
200 curve using a cure-fitting program (Microplate Manger, Bio-Rad Laboratories, Hercules, CA).
201 Samples were distributed randomly across and within plates, and all samples and standard
202 curves were run in duplicate. Within-plate variation was 7.01% and between-plate variation was
203 1.89%. We did not correct for either extraction efficiency or across-plate variation.
204

205 **2.7 Tissue collection**

206 One to two days after the final blood sampling (Feb 25-26), birds were euthanized using
207 isoflurane overdose in a two-way factorial design: birds from both chronic disturbance and
208 control treatments were euthanized either immediately after capture or after 90 min of being
209 held in a paper bag (acute handling stressor). Literature on immediate early gene (IEG)
210 expression and half-life suggests that 90 min following stimulus allows to capture both induction
211 as well as reduction in IEG expression (Maney and Goodson, 2011). Since IEGs include

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212 transcription factors, we estimated that gene expression 90 min after capture was likely to
213 capture both up-regulation and down-regulation of components of the gonadal transcriptome
214 compared to expression immediately following capture. Gonads were dissected and flash-frozen
215 on pulverized dry ice. To assess gonadal size, we measured gonadal mass (to the nearest mg)
216 before RNA extraction.
217
218 **2.8. Microarray hybridization and analyses**
219 RNA was extracted from gonads using Trizol method (Invitrogen, Carlsbad, CA) and quantified
220 using the Nanodrop ND-2000 (ThermoScientific, Waltham, MA). Sample integrity was verified
221 using Agilent Bioanalyzer or TapeStation (Agilent Technologies, Santa Clara, CA). Total RNA
222 was converted to double stranded cDNA using the TransPlex Complete Whole Transcriptome
223 Amplification Kit (Sigma-Aldrich, St. Louis, MO, product no. WTA2) according to manufacturers
224 instructions. We used a custom Nimblegen 12-plex microarray (Roche Nimblegen, Madison, WI)
225 for the Dark-eyed junco (Peterson et al., 2014) to analyze the effect of treatments on
226 transcriptome (see Supplementary Material for more detailed molecular methods). The
227 microarray contained 33,545 assembled sequencing reads (contigs) in triplicate covering 22,765
228 putative genes (isogroups), which were based on Dark-eyed Junco transcriptome sequencing
229 (Peterson et al., 2012). cDNA was hybridized to the microarray using a full round-robin design.
230 A series of pair-wise comparisons were tested to identify significant differences in gene
231 expression using R package limma (Smyth, 2005) (i.e. we compared chronic disturbance vs.
232 control within each acute handling treatment and pooled; and acute handing vs. unhandled
233 control within each chronic treatment and pooled). We calculated a global false-discovery rate
234 across all comparisons and used a q-value threshold of 0.05 for significance (see Peterson et
235 al. (2014) for further details). We used topGO (Alexa and Rahnenfuhrer, 2010) with the weight
236 algorithm (Alexa et al., 2006) to identify the gene ontology (GO) terms (Ashburner et al., 2000)
237 that were significantly over-represented among the significantly differentially expressed genes in
238 each comparison.
239
240 **2.9. qPCR**
241 The microarray did not include all of our candidate genes of interest. Therefore, we
242 subsequently performed quantitative polymerase chain reaction (qPCR) to test our a priori
243 predictions about genes related to testosterone synthesis and gonadal function. Specifically, we
244 quantified expression of steroid synthesis genes (luteinizing hormone receptor (LHR),
245 steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage (P450),

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246 cytochrome P450 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase/isomerase
247 (3βHSD), and 17β-hydroxysteroid dehydrogenase (17βHSD)), as well as genes that encode
248 receptors for hormones that may regulate gonadal function (sperm production: follicle-
249 stimulating hormone receptor (FSHR); response to stressors: glucocorticoid receptor (GR),
250 mineralocorticoid receptor (MR), gonadotropin inhibitory hormone receptor (GnIHR)). Most of
251 the primers, except 17βHSD and FSHR primers, had been previously validated in our system
252 (Bergeon Burns et al., 2014; Rosvall et al., 2016a; Rosvall et al., 2016b). 17βHSD and FSHR
253 primers were designed using Zebra finch and White-throated sparrow genomes, respectively,
254 using Primer-BLAST (Ye et al., 2012). Primer sequences and efficiencies are reported in the
255 Supplementary Material (**Table S1**). We ran qPCR reactions using PerfeCTa SYBR Green
256 SuperMix (Quantabio, Beverly, MA, product no. 95054) on the Roche LightCycler 480 platform
257 (Roche Holding AG, Basel, Switzerland) using the same cDNA samples from the microarray.
258 The cDNA concentration was similar between samples (17 to 22 ng/ul). We analyzed samples
259 in triplicate, calculated the relative gene expression in each sample in reference to a pooled
260 standard, and normalized this value against average expression of two housekeeping genes
261 (Peptidylprolyl Isomerase A (PPIA) and Ribosomal Protein L4 (RPL4)) using LightCycler 480
262 software (Roche Holding AG, release no. 1.5.1.62). PPIA and RPL4 are two of the most stable
263 housekeeping genes in testes of passerines (Zinzow-Kramer et al., 2014), and their expression
264 did not differ between chronic or acute treatments. Primers for PPIA and RPL4 were designed
265 using White-throated sparrow genome. Additional details for qPCR can be found in the
266 Supplementary Material.
267
268 **2.10. Statistical analysis**
269 We analyzed our data in R (R Core Team, 2013). When running parametric tests, we confirmed
270 that residuals satisfied the expectation of normality. If model residuals were not normal, we
271 transformed the data. We used linear (LM) and linear mixed effects models (LMM, package
272 nlme, Pinheiro et al. 2015) with marginal sums of squares to analyze the effect of chronic and
273 acute treatments on reproductive physiology (hormone levels) and morphology (testes mass
274 and CP size). We included time of sampling as a covariate in all models investigating hormone
275 levels. When testing for change in hormone levels between sampling rounds (beginning vs end
276 of the experiment) or the effect of acute (baseline vs acute stressor-induced), chronic
277 (disturbance vs control) treatment, and their interaction on hormone levels, we included
278 individual ID as random factor to account for repeated measures. If the interactions were not
279 significant, we report the main effects from models without the interaction term. Our sample size

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280 differed between sampling rounds and between bleeds because of low blood volume in some of
281 the samples.
282 We used two different multivariate approaches to analyze the effect of treatments on
283 candidate gene expression (determined by qPCR). Since the expression of steroidogenic genes
284 showed significant pairwise correlations, we used principal components analysis to summarize
285 the covariation in gene expression by creating independent principal components. We then
286 used the first principal component as a dependent variable in ANOVA to ask if chronic or acute
287 treatments, or their interaction affected steroidogenic gene expression. Following this, we used
288 ANOVAs to analyze each gene separately and corrected for multiple comparisons using the
289 Benjamini-Hochberg (BH) method.
290 Because the expression of receptors that may downregulate gonadal function (GR, MR,
291 and GnIHR) was not correlated to each other and they mediate different functions in animals,
292 we used MANOVA to analyze the effect of chronic and acute treatments on the overall
293 sensitivity to stress-signaling. Following MANOVA, we analyzed each gene independently using
294 ANOVA and corrected for multiple comparisons using the Benjamini-Hochberg (BH) method.
295 FSHR expression was analyzed independently using a linear model, because its function is not
296 know to relate to stress-signaling or steroidogenesis.
297

298 **3. RESULTS**

299 **3.1. Testosterone**

300 ***Pre-treatment***: At the outset of the experiment neither baseline testosterone (LM, n=20,
301 $F_{2,17}$=0.086, p=0.773) nor testosterone after acute handling stressor (LM, n=14, $F_{2,11}$=1.207,
302 p=0.295) differed between the chronic and control treatment groups, and acute handling
303 stressor did not reduce testosterone compared to baseline levels (LMM, n=33, $F_{1,10}$=0.226,
304 p=0.645, **Figure 2A**). Baseline testosterone increased throughout the experiment in both
305 treatments (LMM, n=46, $F_{1,12}$=15.716, p=0.002), consistent with recrudescence of gonads in
306 response to increasing day length.
307 ***Post-treatment***: After three weeks of treatment, birds from the chronic disturbance group had
308 marginally lower baseline testosterone levels than controls (LM, n=27, $F_{2,24}$=3.643, p=0.068).
309 Testosterone levels after 30 min of acute handling stressor did not differ between treatments
310 (LM, n=21, $F_{2,18}$= 0.584, p=0.455). There was a significant interaction between the two long-
311 term treatments (chronic and control) and acute treatments (handled vs unhandled) on
312 testosterone levels (LMM, n=48, $F_{1,17}$=5.935, p=0.026, **Figure 2B**). Post-hoc ANOVAS showed
313 that birds from the control treatment showed a significant reduction in their testosterone levels

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314 from baseline to post-acute handling stress (LMM, n=24, $F_{1,8}$=5.842, p=0.042), while birds from
315 the chronic disturbance treatment did not (LMM, n=24, $F_{1,9}$=0.793, p=0.396). Control and
316 chronically disturbed birds significantly increased their testosterone in response to GnRH-
317 injection compared to pre-injection levels (LMM, n=50, $F_{1,19}$=63.527, p<0.001), but the GnRH-
318 induced testosterone did not differ between treatment groups (LMM, n=50, $F_{1,18}$=0.274,
319 p=0.607, **Figure 2C**).
320
321 **3.2. Reproductive organs and relationship with physiology**
322 Testes mass was not affected by the chronic disturbance treatment (LM, n=35, $F_{1,33}$=0.873,
323 p=0.357, **Figure 3A**). Testes mass was positively related to testosterone levels after acute
324 handling stress (LM, n=21, $F_{2,18}$=6.620, p=0.019) as well as GnRH-induced testosterone levels
325 (LM, n=29, $F_{2,26}$=8.292, p=0.008), but was not related to baseline testosterone (LM, n=27,
326 $F_{2,24}$=0.435, p=0.516). The relationship between testes mass and testosterone was not
327 dependent on the chronic treatment (interaction between gonad mass and treatment on post-
328 handling T: LM, n=21, $F_{4,16}$=0.025, p=0.877; on GnRH-induced T: LM, n=29, $F_{4,24}$=1.201,
329 p=0.284). Cloacal protuberance (CP) size showed significant differences between treatments,
330 with chronically disturbed birds having larger CPs than control birds (LM, n=33, $F_{1,31}$=22.198,
331 p<0.001, **Figure 3B**).
332
333 **3.3. Gonadal transcriptome**
334 We found that the expression of 16 transcripts were significantly affected by the chronic
335 disturbance treatment after correcting for false discovery rate (out of 20390 total expressed in
336 gonads in this study, 0.078%; **Table S2**). GO analysis identified 3 terms that were
337 overrepresented among the significantly differentially expressed genes. These included:
338 electron transport chain, extracellular structure organization (biological process), and
339 glycosaminoglycan binding (molecular function) (**Table 1**). None of the genes that were
340 differentially expressed between tissues in response to chronic treatment are known to link
341 clearly to the stress response or reproductive function.
342 Acute handling stressor caused significant change in the expression of 168 transcripts
343 compared to unhandled controls (out of 20390 total expressed in gonads, 0.823%; **Table S3**).
344 Among genes that were contributing to these terms were a variety of heat shock proteins
345 (HSPB1, DNAJA4, HSPA4L, HSP90AA1), and genes associated with inflammation and cytokine
346 signaling (IL4R, PIK3AP1, MAP3K8). GO analysis identified a number of terms that were
347 overrepresented in the set of genes that showed significant treatment effect including: receptor

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348 signaling protein activity, intracellular steroid hormone receptor signaling pathway, cellular
349 protein complex assembly, response to stimulus, and 'de novo' posttranslational protein folding
350 (**Table 2**). One unannotated transcript with an unknown function showed a significant interaction
351 between the long-term treatments (chronic vs control) and acute treatments (handled vs
352 unhandled).
353

354 **3.4. Testosterone synthesis and HPG axis receptors**

355 Principal components analysis of six genes involved in testosterone synthesis (LH-R, STAR,
356 p450scc, CYP-17, 3bHSD, 17bHSD) showed that PC1 loaded negatively for all genes and
357 explained 45% of the variation in expression (**Table S4**). This suggests that the expression of
358 the testosterone synthesis genes co-varied in the same direction between individuals. However,
359 variation in PC1 was not explained by long-term disturbance (LM, n=35, $F_{3,31}$=0.261, p=0.613),
360 acute handling stressor (LM, n=35, $F_{3,31}$= 1.700, p=0.202), or the interaction of these treatments
361 (LM, n=35, $F_{3,31}$=0.231, p=0.634). Since testosterone levels decreased significantly in response
362 to acute handling stressor only in the control treatment, we conducted a separate PCA of only
363 the control individuals (see **Table S5** for loadings). This analysis showed that acute handling
364 stressor tended to reduce the expression of testosterone synthesis genes 90 min later, although
365 this effect was not significant (LM, n=17, $F_{1,15}$=3.157, p=0.096). Analysis of each of the genes
366 individually using ANOVA, showed that CYP17 levels were marginally lower in handled control
367 birds (LM, n=17, $F_{1,15}$=4.418, p=0.053, see **Table S6** for other genes) although this effect was
368 not significant after multiple comparison correction (p=0.317, **Figure S1**).
369 Neither handling (LM, n=35, $F_{2,32}$ = 2.051, p=0.162), nor chronic disturbance (LM, n=35,
370 $F_{2,32}$ = 2.733, p= 0.108), nor their interaction (LM, n=35, $F_{3,31}$ = 0.437, p= 0.514) affected
371 expression of FSH-R (**Figure S2**).
372

373 **3.5. Gonadal sensitivity to regulation by HPA**

374 MANOVA showed that acute handling stressor (n=35, Wilks=0.676, $F_{3,30}$=4.791, p=0.008), but
375 not chronic disturbance (n=35, Wilks=0.907, $F_{3,30}$=1.027, p=0.395), nor interaction between
376 chronic treatment and acute handling stressor (n=35, Wilks= 0.855, $F_{3,29}$=1.643, p=0.201)
377 affected expression of receptors for hormones that are known to suppress or inhibit testosterone
378 production. Post-hoc linear models comparing handled to unhandled birds showed that acute
379 handling stressor significantly down-regulated GR mRNA expression (LM, n=35, $F_{2,32}$=5.207,
380 p=0.029, **Figure 4A**) and marginally up-regulated of GnIHR expression (LM, n=35, $F_{2,32}$=3.517,

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381 p=0.070, **Figure 4C**), although these effects were not significant after correction for multiple
382 comparisons (see **Table S7**).
383

384 **4. DISCUSSION**

385 In this study we experimentally tested how chronic disturbance, acute handling stressor, and
386 their interaction affected gonadal function in a songbird during seasonal gonadal
387 recrudescence. We showed that birds in the chronic disturbance treatment had marginally lower
388 baseline testosterone compared to control animals. Acute handling stressor reduced
389 testosterone levels in control animals, but not in animals from the chronic disturbance treatment.
390 The chronic disturbance treatment did not affect the ability to produce testosterone in response
391 to exogenous stimulation from GnRH, and gonad size did not differ between chronic disturbance
392 and control treatments. Surprisingly, birds in the chronic disturbance treatment had significantly
393 larger cloacal protuberances than birds in the control treatment. Chronic disturbance had very
394 little effect on gonadal gene expression: neither steroidogenic enzyme expression, nor
395 expression of receptors associated with potential regulation of gonadal function showed
396 significant differences between the chronic disturbance and control treatments. Overall, chronic
397 disturbance had a significant effect on only a handful of genes in the transcriptome. Acute
398 handling stressor, on the other hand, had a comparatively strong effect on the gonadal
399 transcriptome, a marginal suppressive effect on steroidogenesis enzyme gene expression, a
400 suppressive effect on the expression of glucocorticoid receptor mRNA, and a marginal positive
401 effect on GnIH-R gene expression compared to unhandled animals. There was little evidence of
402 an interaction between chronic disturbance and acute stressor on the expression of gonadal
403 transcriptome. Collectively, these findings shed light on mechanisms by which short and long-
404 term stressors, and their interaction, affect reproductive function.
405

406 **4.1. Chronic stressor and gonadal function**

407 Birds from the chronic disturbance treatment had marginally lower baseline testosterone levels
408 compared to the control treatment. Our study adds to other reports in a variety of organisms that
409 have shown a decrease in testosterone in response to chronic disturbance (Pickering et al.
410 1987; Moore et al. 1991; Retana-Márquez et al. 2003) although some studies do not show this
411 effect (Armario and Castellanos 1984; Jones and Bell 2004). Long-term suppression of
412 testosterone levels in response to chronic stressor has important ecological implications.
413 Human-induced rapid environmental change is exposing animals to novel environments and
414 rapid changes in the disturbance regimes, which have been shown to have strong negative

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415 effects on foraging and survivorship (Kerley et al., 2002; Sih et al., 2011). If a decrease in
416 testosterone in response to chronic disturbance results in reduced investment in reproduction in
417 general, this may exacerbate the negative effects of disturbance on fitness.

418 Mechanistically, the differences in testosterone levels between the chronic disturbance
419 and control birds in this study were not explained by differences in gonadal physiology. Testes
420 mass and GnRH-induced testosterone levels did not differ between treatments, indicating that
421 the ability of the gonads to produce and elevate testosterone was not different. This suggests
422 that the marginally lower testosterone levels in the chronically disturbed animals and their
423 insensitivity to acute handling stress compared to control animals were not caused by long-
424 lasting differences in the ability of the testes to produce testosterone.

425 Differences in testosterone levels instead may be mediated by local or systemic
426 signaling that transiently suppresses or activates testosterone synthesis in the gonad or
427 testosterone metabolism in the liver. Experimental studies have shown that GnIH (McGuire and
428 Bentley, 2010) and corticosterone (McGuire et al., 2013) downregulate testosterone by acting
429 directly on gonadal tissue. This effect may be mediated by either changes in the levels of these
430 hormones or by changes in the expression of their receptors. Baseline corticosterone did not
431 differ between chronic disturbance and control treatments (Hanauer et al. in prep.). We could
432 not assess GnIH expression in gonads or brain, therefore we do not know if chronic stressors
433 upregulated GnIH synthesis. Our gene expression results showed that chronic disturbance did
434 not affect the expression of receptors for GnIH (GnIHR), corticosterone (GR, MR), or luteinizing
435 hormone (LHR), a top regulator of testosterone synthesis. Furthermore, chronic disturbance did
436 not have a significant effect on steroidogenic gene expression or the testicular transcriptome in
437 general, showing that gonadal function was nearly unaffected by the chronic disturbance
438 treatment. This suggests that local signaling at the level of the gonad is unlikely to be the cause
439 of lower testosterone levels or the decreased sensitivity to acute handling stressor that we
440 observed in the chronically disturbed animals. Future analysis of the post-translational effects of
441 stressors, as well as other tissues (e.g. hypothalamus, liver) collected from these animals may
442 elucidate the mechanisms that caused the differences in testosterone levels between
443 treatments (Lynn et al., 2015).

444 A surprising finding in this study was that the cloacal protuberances (CPs) were
445 significantly larger in the chronically disturbed birds compared to control animals. CPs in birds
446 are sperm storage organs that develop in males during the breeding season (Ray Salt, 1954)
447 and are regulated by testosterone (Witschi, 1961). Males with larger CPs store more sperm
448 (Tuttle et al., 1996) and larger CPs are hypothesized to allow faster copulation (Birkhead et al.,

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449 1993). Barring spurious results, our finding may indicate that birds in the chronic disturbance
450 group may be investing more in sperm production compared to the control animals, perhaps to
451 advance their reproductive readiness or enable more rapid copulation in an uncertain
452 environment. It is important to note that neither CPs nor testes reached their full size during this
453 study (i.e testes mass was 21% of mature reproductive size (Bergeon Burns et al., 2014)),
454 therefore our CP findings, coupled with measurement error, may reflect functionally irrelevant
455 differences.
456
457 **4.2. Acute stressor and gonadal function**
458 Birds in the control treatment showed a significant decrease in testosterone levels in response
459 to the acute handling stressor. Suppression of testosterone in response to acute stressors has
460 been shown in many other studies (Deviche et al., 2010; Deviche et al., 2012; Moore et al.,
461 1991). This effect may be due to reduction in testosterone synthesis, or an increase in
462 testosterone metabolism by liver (Lynn et al., 2015). We found a marginally significant decrease
463 in the expression of steroidogenic enzyme genes in birds that had experienced an acute
464 handling stressor compared to birds that were unhandled. Because the expression of
465 steroidogenic enzymes is positively correlated with testosterone levels (Rosvall et al., 2016b),
466 decrease in expression of testosterone synthesis genes may be responsible for suppression of
467 testosterone in response to handling.
468 Downregulation of steroidogenic enzyme gene expression in response to the acute
469 handling stressor may be due to either a change in the local or systemic signaling or because of
470 a change in sensitivity to this signaling (Ernst et al., 2015). We found a significant overall
471 change in hormone receptor gene expression, primarily downregulation of GR expression and
472 upregulation of GnIHR expression, in response to the acute handling stressor. This suggests
473 that gonads in acutely stressed birds may be less sensitive to regulation by glucocorticoids,
474 such as corticosterone, but more sensitive to another important hormone – GnIH – that has an
475 inhibitory effect on reproductive physiology (Tsutsui et al., 2010). MR expression was not
476 affected by acute stressor. While GR and MR are both involved in regulating the stress
477 response (Dickens et al., 2009) they fulfill different functional roles, with the high affinity MR
478 (Reul and Kloet, 1985) thought to be responsible regulating baseline functions when CORT
479 levels are low, while the low-affinity GR regulates stress-response when CORT levels are high
480 (Ulrich-Lai and Herman, 2009; Wingfield, 2012). Thus, the GR-specific reduction we observed in
481 response to an acute handling stressor is consistent with known differences between these two
482 types of CORT-binding receptors. We could not assess the expression of GnIH in the gonads or

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483 brain, therefore we do not know if acute or chronic stressors, in addition to GnIHR, also
484 upregulated GnIH synthesis. In another study, we validated that corticosterone levels increased
485 following this acute handling stressor (Hanauer et al. in prep).
486 Acute handling stress caused significant changes across the testicular transcriptome
487 compared to unhandled animals. The gene ontology (GO) analysis did not indicate that the
488 acute handling treatment affected testes-specific functions, such as spermatogenesis or
489 steroidogenesis, although we note that several candidate genes were not present on this array.
490 Instead, GO analysis suggested that acute handling stress induced cellular stress response,
491 resulting in upregulation of a handful of heat shock proteins. Heat shock proteins are molecular
492 chaperones that under normal conditions facilitate protein assembly and are upregulated in
493 response to stressors (Akerfelt et al., 2010). Heat shock protein expression in gonads increases
494 during spermatogenesis and oogenesis (Neuer et al., 2000) which may ensure that the
495 development of gametes is shielded from environmental stressors. Importantly, some of the
496 same genes (e.g. HSP90AA1, DNAJA4) upregulated in response to acute handling stress in this
497 study, were also upregulated in chicken testes in response to heat stress (Wang et al., 2015),
498 indicating that gonads may have a generalized cellular stress response that is upregulated in
499 response to a variety of stressors. Analysis of transcriptomic changes in other tissues will
500 indicate if the change in gonadal transcriptome represents testes-specific response to stress, or
501 if response to stress between tissues differs.
502
503 **4.3. Interaction between chronic and acute stressors**
504 Birds from chronic disturbance and control treatments responded differently to the acute
505 handling stressor: whereas handling reduced testosterone levels in control birds, it did not affect
506 testosterone levels in birds from the chronic disturbance treatment. This difference can be
507 interpreted in two non-exclusive ways.
508 First, the reduced impact of the acute stressor on testosterone in birds from the chronic
509 disturbance group might have arisen because their reproductive function was already
510 downregulated to a degree that prohibited further decrease ("floor effect" *sensu* (Sapolsky et al.,
511 1984)). In our study, however, both the baseline and post-handling testosterone levels of
512 chronically disturbed animals were significantly higher than testosterone levels at the beginning
513 of the experiment. This suggests that, mechanistically, testosterone levels could have shown a
514 further decrease below the levels observed in response to the acute handling stressor. It is
515 possible, however, that the lack of decrease in testosterone in response to handling in
516 chronically disturbed birds may be due to reduced testosterone metabolism by the liver. If this

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517 was the case, we would predict that acute stressors would start suppressing testosterone levels
518 later in chronically disturbed animals compared to control animals. Unfortunately, our
519 experimental design did not allow us to test this possibility due to the GnRH injection during
520 blood sampling.
521 Second, the difference in the effect of handling on testosterone levels between chronic
522 disturbance and control treatments could be explained by a lower sensitivity of the chronically
523 disturbed birds to stressors compared to the control individuals. We did not find any effect of
524 chronic disturbance on gonadal signaling that would explain this difference, suggesting that
525 differences in sensitivity to stress may exist in the HPG axis tissues that are upstream from the
526 gonad (pituitary, hypothalamus), or other hormonal targets that interact with testosterone
527 production.
528 From an ecological perspective, the first alternative (floor effect) could be interpreted as
529 a consequence of homeostatic overload (Romero et al., 2009). Homeostatic overload refers to
530 the cases where overexposure to stressors, and the associated increase in the frequency of
531 stress response, results in a pathological state that leads to compromised organismal function
532 (Romero et al., 2009). In our study, the high frequency of stressors may have have resulted in a
533 compromised ability to produce testosterone. However, because birds from both chronic and
534 control treatments elevated their testosterone in response to GnRH to the same degree, this
535 explanation seems unlikely. Instead, the floor effect could represent be a result of a "best of a
536 bad job" strategy, wherein individuals of low quality or in a bad environment maximize their
537 fitness by playing it safe (Sih and Bell, 2008). In this case, animals may opt to keep the
538 resources diverted from reproduction to maximize their ability to respond to stressors. This
539 strategy would be more likely to be adaptive in long-lived animals and in environments were the
540 disturbance frequency is likely to change. However, even in short-lived animals, strategies with
541 low investment in reproduction can have higher fitness compared to cases when animals invest
542 in reproduction at the cost of survival (e.g. sneaky copulations by alternative morphs in beetles
543 (Moczek and Emlen, 2000)).
544 The second alternative (differences in sensitivity to stress), is consistent with the life
545 history prediction that under conditions of low expected future reproductive success, stress
546 should have a less negative effect on reproduction compared to situations where expected
547 reproductive success is high (Wingfield and Sapolsky, 2003). Animals that develop in
548 environments with high frequency of stressors may change their physiology to reduce sensitivity
549 of HPG axis to stress, thus allowing them to maintain reproductive function during stressful
550 episodes, despite the possible cost to self-maintenance (Wingfield and Sapolsky, 2003). This

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551 prediction has been supported by a study in a natural system (Abolins-Abols et al., 2016), where
552 the reproductive phenotype of urban animals is less sensitive to stressors compared to rural
553 animals.
554 In contrast to our testosterone findings, we did not find and an interaction between
555 chronic and acute treatments on the gonadal transcriptome, although we note that the marginal
556 effects of the chronic treatment reduce our power to explore this interaction. This finding
557 nevertheless provides further evidence that chronic disturbance does not affect gonadal
558 capacity to produce testosterone or respond to stress. Instead, we hypothesize that the
559 differential effect of handling stress on testosterone levels in the chronic disturbance and control
560 treatments may be due to differences in upstream signaling from the pituitary or hypothalamus,
561 which regulates the activity of the molecular machinery in the gonad. Further work will be
562 needed to explore alternatives, such as post-translational effects of stress that are not captured
563 at the level of gene expression, or temporal dynamics wherein acute and chronic stressors
564 interact at only specific time-points following a stressor. Despite these uncertainties, our results
565 nevertheless demonstrate that the gonad is relatively robust to interacting effects of acute and
566 chronic stressors on gene expression.
567
568 **4.4. Summary**
569 Overall our results show that chronic and acute stressors suppress testosterone release by the
570 gonads, but that the effect of acute stressors differs depending on the frequency of stressors in
571 the environment. These differences in gonadal function between disturbance environments are
572 unlikely to be mediated by changes in gonadal gene expression, but are more likely to reside
573 upstream of the gonad. Whereas chronic treatment had a negligible effect on the gonadal
574 transcriptome, acute handling stress significantly upregulated major components of cellular
575 stress response and affected the expression of hormone receptors involved in downregulation
576 of testosterone production. These results suggest a potential mechanism for regulating
577 testosterone decrease in response to acute stressors. Furthermore, they show that transient
578 changes in gene expression in response to acute stressors are different from more permanent
579 responses to chronic stressors. An important future direction therefore is to identify the
580 mechanisms responsible for differences in testosterone levels between animals experiencing
581 different disturbance regimes.
582 This study is among the first to experimentally test the mechanisms by which acute and
583 chronic stressors interact to influence reproduction. We demonstrate patterns that, adaptive or
584 not, are doubtlessly important in understanding how animals respond to chronic and acute

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585 stressors. We therefore urge further study on the adaptive significance and mechanisms that
586 mediate the effect of chronic stressors on testosterone levels and other reproductive functions.
587

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599

600 **Competing interests**

601 Authors have no competing interests to declare.
602

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606

607 **Data availability**

608 Data will be published in the Dryad Digital Repository upon acceptance.
609

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825 **Table 1**: Gene ontology (GO) processes significantly affected by the acute handling treatment
826 (H) compared to unhanded control treatment (U) . Type= GO category; BP = biological process,
827 MF = molecular function; N = number of genes per GO category in microarray; DE = number of
828 genes per GO category significantly differentially expressed between treatments. Non-
829 underlined genes were up-regulated in the H treatment. Underlined genes were up-regulated in
830 C treatment. Group bias refers to direction of upregulation.

Gene ontology description	GO #	Type	N	DE	p value	Group bias	Genes
regulation of sodium ion transport	GO:0002028	BP	8	1	0.036	H	NEDD4
endothelium development	GO:0003158	BP	8	1	0.036	H	S1PR1
mitochondrial membrane organization	GO:0007006	BP	7	1	0.032	H	HSP90AA1
regulation of lamellipodium assembly	GO:0010591	BP	7	1	0.032	H	HSP90AA1
phosphatidylinositol 3-kinase signaling	GO:0014065	BP	11	1	0.050	H	NEDD4
negative regulation of blood coagulation	GO:0030195	BP	7	1	0.032	H	TMPRSS6
intracellular steroid hormone receptor signaling pathway	GO:0030518	BP	51	3	0.002	H	DNAJA4, FKBP4, NEDD4
TOR signaling	GO:0031929	BP	10	1	0.045	H	SLC7A1
negative regulation of actin filament bundle assembly	GO:0032232	BP	5	1	0.023	H	S1PR1
cellular protein complex assembly	GO:0043623	BP	69	2	0.040	H	HSP90AA1, FKBP4
response to stimulus	GO:0050896	BP	2099	20	0.007	H	TMPRSS6, HSP90AA1, SERPINH1, HSPB1, HSPH1, <u>CUBN</u>, DNAJB5, HSP90B1, DNAJA4, S1PR1, SLC7A1, HSPA4L, <u>PIK3AP1</u>, ERN1, FKBP4, <u>PTGR1</u>, <u>EPX</u>, NEDD4
'de novo' posttranslational protein folding	GO:0051084	BP	9	1	0.041	H	HSPH1
vitamin transport	GO:0051180	BP	5	1	0.023	U	<u>CUBN</u>
endoribonuclease activity	GO:0004521	MF	9	1	0.040	H	ERN1
receptor signaling protein activity	GO:0005057	MF	30	2	0.008	H	PIK3AP1, MAP3K8
amine transmembrane transporter activity	GO:0005275	MF	10	1	0.045	H	SLC7A1
macrolide binding	GO:0005527	MF	5	1	0.023	H	FKBP4
ion channel inhibitor activity	GO:0008200	MF	7	1	0.032	H	NEDD4
L-amino acid transmembrane transporter activity	GO:0015179	MF	6	1	0.027	H	SLC7A1
oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	GO:0016628	MF	7	1	0.032	U	<u>PTGR1</u>
intramolecular oxidoreductase activity, interconverting keto- and enol-groups	GO:0016862	MF	7	1	0.032	H	PDIA4
adenyl ribonucleotide binding	GO:0032559	MF	922	9	0.019	H	HSP90AA1, HSPH1, CLK4, HSP90B1, DNAJA4, HSPA4L, MAP3K8, ERN1, FKBP4
protein phosphorylated amino acid binding	GO:0045309	MF	8	1	0.036	H	NEDD4
lipoprotein particle receptor binding	GO:0070325	MF	9	2	0.001	H	HSP90B1, DNAJA4

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833 **Table 2**: Gene ontology processes significantly affected by chronic disturbance vs control
834 treatments. N = number of genes per GO category in microarray; DE = number of genes per GO
835 category significantly differentially expressed between treatments. C = control treatment.
836

Gene ontology description	GO #	Type	N	DE	p value	Group bias	Genes
electron transport chain	GO:0022900	BP	37	1	0.023	C	NDUFA3
extracellular structure organization	GO:0043062	BP	81	1	0.049	C	POSTN
glycosaminoglycan binding	GO:0005539	MF	48	1	0.027	C	POSTN

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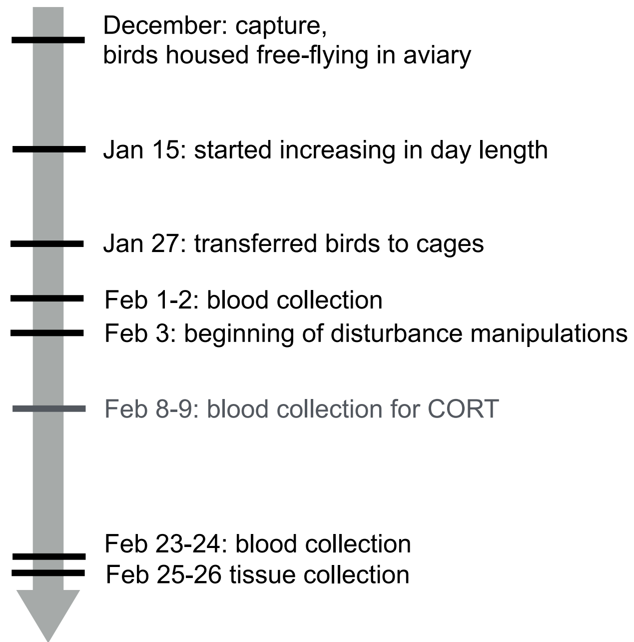
837 **Figure 1**: Timeline of the experiment



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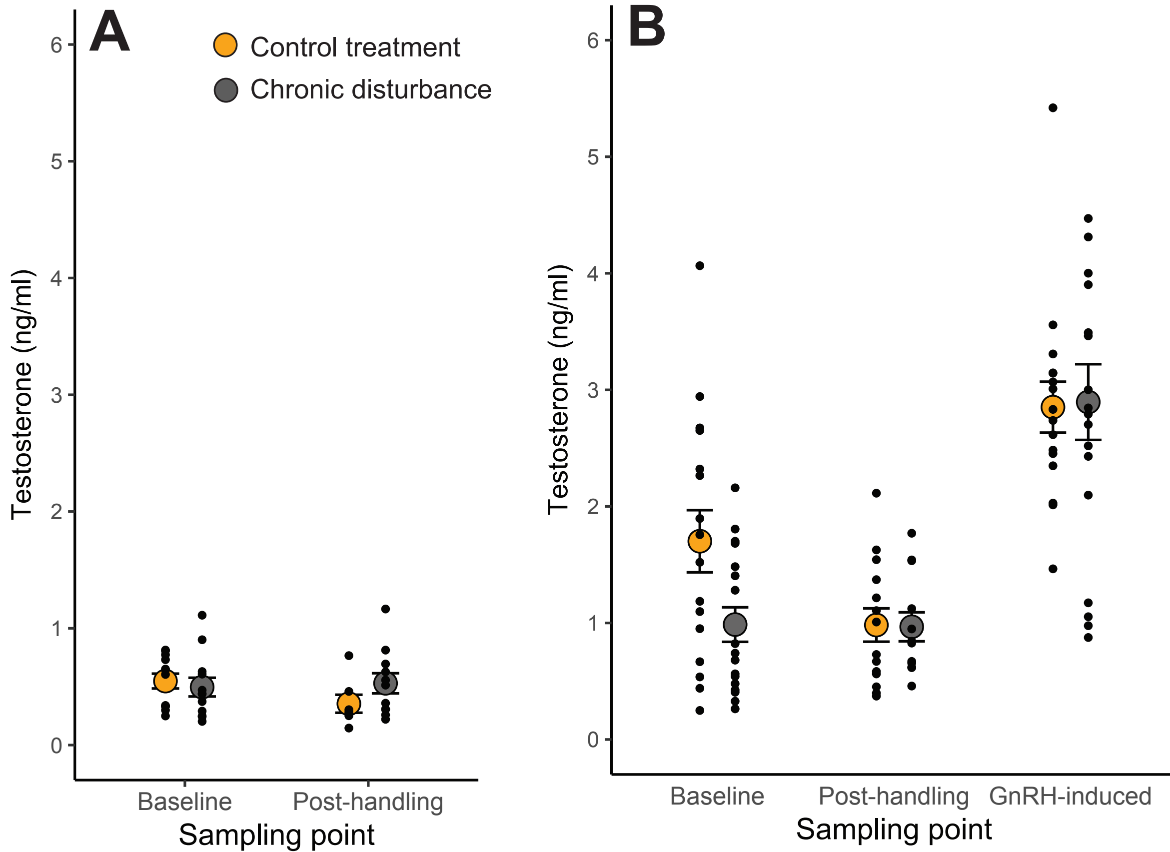
840 **Figure 2**. Effect of chronic disturbance and acute stressor on testosterone levels. A)
841 testosterone levels before the experiment; B) testosterone levels after after the
842 experiment. Shown are baseline testosterone levels, post-handling levels, and GnRH-
843 induced testosterone levels. GnRH-induced testosterone was not measured before the
844 experiment.
845



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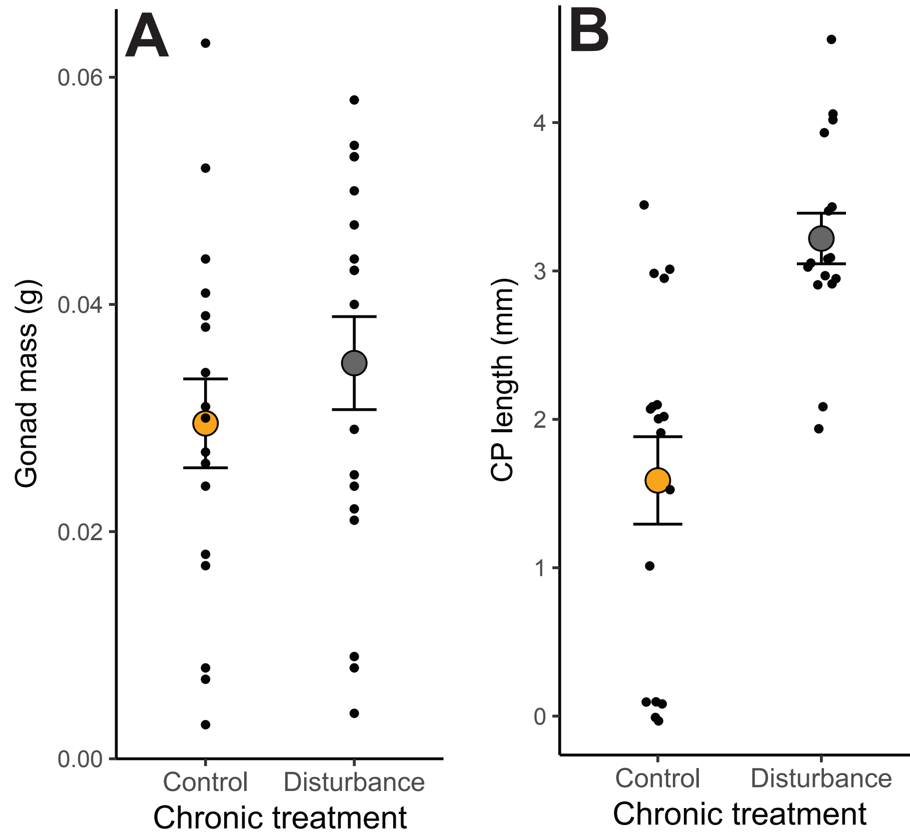
849 **Figure 3**: Effect of chronic disturbance on reproductive organs. A) Gonad mass; B)

850 Cloacal protuberance (CP). Points are jittered in B to show sample size.



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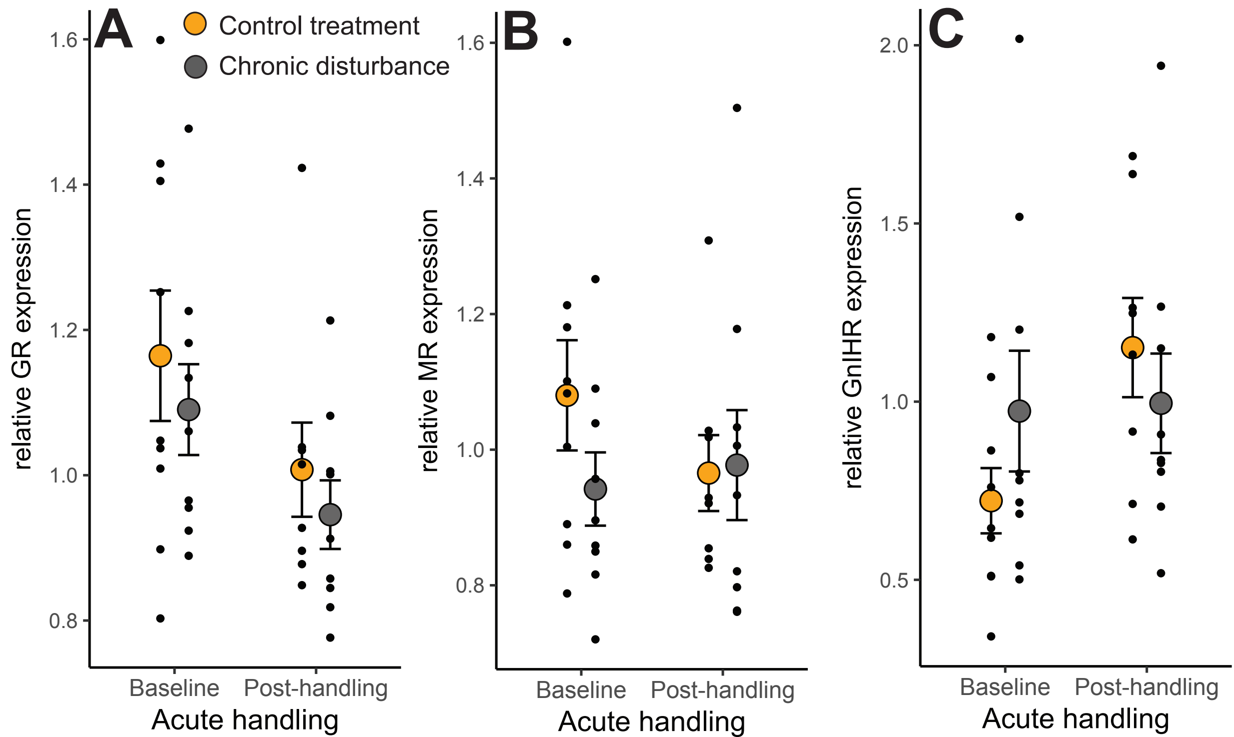
853 **Figure 4**. Effect of chronic disturbance and acute handling stressor on A) glucocorticoid
854 receptor (GR) expression; B) mineralocorticoid receptor (MR) expression; C)
855 gonadotropin inhibitory hormone receptor (GnIHR) expression in gonads.



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