© 2018. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2018) 221, jeb180869. doi:10.1242/jeb.180869

RESEARCH ARTICLE

The effect of chronic and acute stressors, and their interaction, on testes function: an experimental test during testicular recrudescence

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ABSTRACT

Organisms are expected to invest less in reproduction in response to a stressor, but theory predicts that this effect should depend on the frequency and duration of stressors in the environment. Here, we investigated how an acute stressor affected testes function in a songbird, and how chronic stressors influenced the acute stress response. We exposed male dark-eyed juncos (*Junco hyemalis*) either to chronic or minimal (control) disturbance during testicular recrudescence, after which we measured baseline testosterone, testosterone after an acute handling stressor, and capacity to produce testosterone after hormonal stimulation. In a 2×2 design, we then killed males from the two chronic treatment groups either immediately or after an acute stressor to investigate the effect of long- and short-term stressors on the testicular transcriptome. We found that chronically disturbed birds had marginally lower baseline testosterone. The acute stressor suppressed testosterone in control birds, but not in the chronic disturbance group. The ability to elevate testosterone did not differ between the chronic treatments. Surprisingly, chronic disturbance had a weak effect on the testicular transcriptome, and did not affect the transcriptomic response to the acute stressor. The acute stressor, on the other hand, upregulated the cellular stress response and affected expression of genes associated with hormonal stress response. Overall, we show that testicular function is sensitive to acute stressors but surprisingly robust to long-term stressors, and that chronic disturbance attenuates the decrease in testosterone in response to an acute stressor.

KEY WORDS: Stress, Reproduction, Gonad, Testosterone, Transcriptome, Songbird

INTRODUCTION

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

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

While these findings are consistent with the theoretical prediction that low expected future reproductive success should reduce the effect of stressors on reproduction, we lack experimental studies that explicitly test this prediction and study which conditions lead to changes in the suppressive effect of stress on reproduction. Revealing the conditions and mechanisms that mediate effects of stress on reproduction is important for a better understanding of reproductive physiology and ecology of animals (Calisi et al., 2018), especially in the context of the human-induced rapid environmental change (Sih et al., 2011). Ultimately, such knowledge will aid in developing a predictive understanding of how and why individuals or populations respond differently to stressors.

Here, we manipulated the disturbance environment (long-term stressors) to simulate environments with different expected future reproductive success, and asked whether animals exposed to high and low disturbance differed in the degree to which an acute stressor affected their testicular function. In male vertebrates, testes produce sperm and androgen hormones, such as testosterone. Testosterone mediates multiple important reproductive functions: it regulates the development of the sexual phenotype (Hau, 2007) and is important in mediating behaviors necessary for reproduction, such as courtship (Fusani et al., 2014; Hutchinson, 1967) and territorial behavior (Soma, 2006; Wingfield et al., 1987). Testosterone synthesis is largely regulated by the hypothalamic–pituitary–gonadal (HPG) axis, where secretion of gonadotropin releasing

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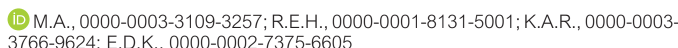
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Received 20 March 2018; Accepted 29 June 2018

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hormone (GnRH) from the hypothalamus causes the secretion of luteinizing hormone (LH) from the pituitary, which in turn upregulates synthesis of testosterone by the Leydig cells in the testes (Farner and Wingfield, 1980; London et al., 2006). Testosterone synthesis by testes has also been shown to change in response to local signaling factors independent of brain signaling (Nogueiras et al., 2004; Tena-Sempere et al., 2002). Testosterone levels can be suppressed by both psychological (Deviche et al., 2010; Moore and Mason, 2000) as well as physical (Lynn et al., 2015; Nelson et al., 1989) stressors. This decrease may be mediated via suppressive effects of stressors on GnRH or LH secretion (Breen and Karsch, 2006; Breen et al., 2007; Nikolarakis et al., 1986), or by direct action of components of the physiological stress response, such as the hypothalamic–pituitary–adrenal (HPA) axis, on gonads (Deviche et al., 2012, 2017; Lynn et al., 2015; McGuire et al., 2013).

Gonads have been shown to be especially sensitive to stressors and other perturbations during growth (Guillette et al., 1994; Rhind et al., 2001; Zambrano et al., 2014). For example, maternal stress during fetal gonadal development can result in reduced gonadal size (Dahlöf et al., 1978), and exposure to glucocorticoids during fetal development has been shown to reduce testosterone synthesis capacity *in vitro* (Page et al., 2001) and delay the onset of puberty (Smith and Waddell, 2000). Such developmental programming has important implications for pathology and reproductive rates in the wild (McMillen and Robinson, 2005; Sheriff et al., 2010). If gonadal responses to stress during development are adaptive, then the effect of stressors on gonadal function should conform to the life history expectations (Sheriff and Love, 2013), as outlined above, wherein gonads are expected to be less sensitive to stressors in animals undergoing gonadal growth in a high-stress environment compared with animals developing in a benign environment.

In this study, we experimentally investigated whether a long-term disturbance environment during seasonal testicular recrudescence affected testicular physiology and gene expression in the dark-eyed junco [*Junco hyemalis* (Linnaeus, 1758)], a small passerine. Testicular recrudescence in seasonally breeding animals shares many parallels with development of testes in the embryo in that, during recrudescence, undifferentiated fibroblast tissue develops into mature testes with fully differentiated cell types (Nicholls and Graham, 1972). In particular, we investigated whether chronic disturbance and acute stressors, and their interaction, changed baseline testosterone levels, the overall ability to elevate testosterone following stimulation with exogenous GnRH, expression of hormone receptors that mediate testes function, and the testicular transcriptome in general. We predicted that both chronic and acute stressors should decrease testosterone levels (Deviche et al., 2010; Moore et al., 1991; Retana-Márquez et al., 2003), but that the suppressive effect of an acute stressor on testosterone synthesis would be dampened in chronically disturbed animals. We further predicted that both chronic and acute stressors would negatively affect gene expression associated with testosterone production and spermatogenesis, but upregulate expression of genes involved in cellular and hormonal stress responses, such as glucocorticoid receptor (GR) (McGuire et al., 2013) and gonadotropin-inhibitory hormone receptor (GnIHR) (McGuire and Bentley, 2010). Lastly, we predicted that the effect of an acute stressor on the testicular transcriptome would be dampened in the chronically disturbed animals.

MATERIALS AND METHODS

Capture and housing of study organisms

This study was conducted in accordance with Indiana University Animal Care and Use Committee guidelines, protocol #12-050-08. We captured 36 wintering male dark-eyed juncos (*Junco hyemalis hyemalis*) in Bloomington (latitude: 39.144887, longitude: −86.535839), IN, USA, in December 2013 using baited mistnets and walk-in traps. We determined the sex of birds by plumage color and confirmed it by analysis of genetic sex markers (Griffiths et al., 1998). The age of birds was unknown. Before the experiment, birds were housed in free-flying groups in indoor aviary rooms, reflecting their natural winter flocking lifestyle. On 15 January 2014, we started to gradually (3 times per week) increase day length from the natural Bloomington winter photoperiod (10 h 44 min of daylight, Fig. 1). On 27 January 2014, all males were transferred to 60×60 cm metal cages (1 individual per cage, 10 cages per room, 4 rooms in total) with visual access to other birds, including females. Cages contained food, water bowls and perches. Food was provided *ad libitum* 3 times a week, and cages were cleaned once a week. When the experiment began on 3 February 2014, animals were experiencing 13 h 23 min of daylight and, by the end of the experiment on 26 February 2014, birds were exposed to 16 h 3 min of daylight, reflecting a summer-like photoperiod. Males were singing during the last week of the experiment.

Chronic disturbance treatment

We randomly assigned half of the birds (n=18, 2 separate rooms) to a chronic disturbance treatment, while the other half (n=18, 2 separate rooms) were treated as controls. Chronic disturbance and control rooms were separated in space to reduce the possibility that control birds were affected by the noises from the disturbance treatment. In the chronic disturbance treatment, birds were exposed to either physical disturbance (hand-waving, cage-tapping) or to a predator mount for 30 min 4 times each day for 3 weeks. During physical disturbances, observers (1 per room) either waved their hand inside the cage or tapped on the cage for 30 s, after which the observer moved on to another cage in random order. For the

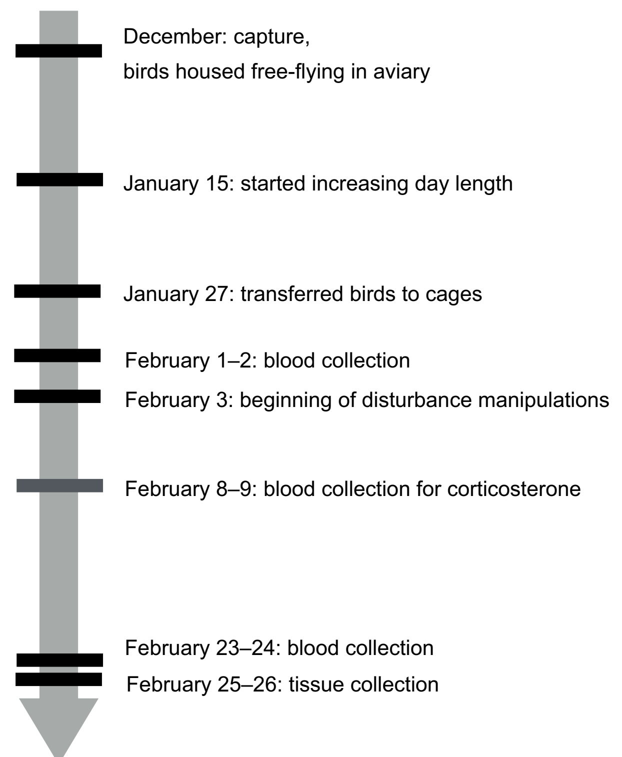

Fig. 1. Timeline of the experiment.

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predator stressor, we used taxidermic study skins of Cooper's hawk (*Accipiter cooperii*), barn owl (*Tyto alba*) and fox squirrel (*Sciurus niger*), which were fixed to a tripod and left in the room by the observer. We had validated these treatments in a pilot study in which we investigated whether juncos exposed to hand waving or an owl mount for 30 min had higher baseline corticosterone levels than control birds (Hanauer, 2017). The pilot experiment was conducted between 17 and 21 December 2013, a month before the main experiment, on individually caged birds kept on the natural winter photoperiod. Birds used for the pilot experiment were not used in the main study. In the pilot study, hand waving (n=8) and owl presentation (n=8) treatments were carried out on different dates, with control animals (n=8 for each treatment) sampled on the same date as the treatment. Birds that experienced hand waving had significantly higher corticosterone levels [mean (x)=14.75 ng μl^{-1}, s.e.m.=2.48] than control birds (x=4.51 ng μl^{-1}, s.e.m.=0.20). Similarly, birds that were exposed to owl had significantly higher corticosterone levels (x=6.25 ng μl^{-1}, s.e.m.=0.83) than control birds (x=3.23 ng μl^{-1}, s.e.m.=0.38).

Because hand waving caused the largest increase in corticosterone in our pilot study, birds in the chronic disturbance treatment received at least 1 of these disturbances each day (average: 2.5 physical disturbances each day). Otherwise, the choice of the stressors for each disturbance bout was random. The disturbance bouts occurred at least 60 min apart for each room and were administered during daylight hours. In the control group, we did not disturb the birds except for standard animal husbandry (see above). We did not measure corticosterone increase in response to hand waving or predator presentation in the birds in the chronic disturbance treatment. We do not know, therefore, whether these birds habituated to these repeated stressors during the experiment. However, after 3 weeks of treatment, birds from the chronic disturbance treatment had significantly lower condition (mass relative to body size) and significantly lower fat deposits compared with control birds (Hanauer, 2017), an indication of chronic stress (Dickens and Romero, 2013).

Blood sampling and acute handling stressor

We took blood samples from each bird to analyze testosterone and corticosterone concentrations before (1–2 February 2014) and after (23–24 February 2014) the 3 weeks of chronic disturbance or control treatment. In addition, blood was taken from birds 1 week after the beginning of treatment for analysis of corticosterone levels (8–9 February 2014). Each bird was captured between 7:00 and 12:00 h, and a baseline blood sample (100 μl) was taken from the brachial vein within 4 min of capture using a 26-gauge needle and microcapillary tubes. The timer for blood collection was started when we entered the room in which birds were housed. Multiple birds (up to 5) were captured at once by 1 of up to 5 observers. In order to exclude indirect effects from the capture of other birds in the study, we turned off the lights during capture and did not sample the remaining birds from the same room until the following day.

After the blood sample was taken, each bird was put in a brown paper bag, a standard acute handling stressor that is widely used to assess physiological stress response in birds (Sheriff et al., 2011). After 30 min, each bird was bled again (100 μl) to measure acute-stressor-induced hormone levels (Deviche et al., 2010; Schoech et al., 1999). Blood was stored at 4°C for up to 6 h until centrifugation. Following centrifugation, plasma was removed using a Hamilton syringe and stored at −20°C until analysis.

Both chronically disturbed and control birds increased their corticosterone levels in response to the acute stressor, but chronically disturbed birds elevated their corticosterone levels to a lesser extent (Hanauer, 2017).

GnRH injection following an acute stressor

To assess the ability of testes to increase testosterone, during the last sampling round at the end of the experiment (23–24 February 2014), we injected birds with 50 μl of 25 ng μl^{-1} GnRH (chicken-derived GnRH I in 1 mol l^{-1} PBS, American Peptide Company Inc., Sunnyvale, CA, USA, product no. 54-8-23) 35 min after capture (i.e. after the post-acute-stressor 30 min blood sample was collected). GnRH is the top regulator of testosterone synthesis and is naturally produced in hypothalamus (Ciechanowska et al., 2010). GnRH-induced testosterone levels have been shown to be repeatable (Jawor et al., 2006), and are linked to a higher probability of survival (McGlothlin et al., 2010) and with morphological characteristics, such as plumage color (Atwell et al., 2014). To administer GnRH, we cleaned the pectoral muscle using an alcohol swab, and injected GnRH using a Hamilton syringe. GnRH solutions were kept on ice before the injection to prevent degradation of the peptide. A total of 100 μl of blood were collected 30 min after injection.

Morphological measurements

After blood collection, we used a ruler to measure the size of the cloacal protuberance (CP) by measuring its length from body to the cloacal opening, to the nearest 0.5 mm. We also measured the pectoral muscle condition, fat score, and mass. Birds from the chronic disturbance treatment had significantly lower condition (mass relative to body size) and significantly lower fat deposits compared with control birds (Hanauer, 2017), an indication of chronic stress (Dickens and Romero, 2013). To minimize potential measurement bias, we randomized the order in which birds from different treatments were measured.

Hormone assays

We measured testosterone levels in the blood plasma using a commercial enzyme immune assay (EIA) from Enzo Life Sciences (Farmingdale, NY, USA, product no. ADI-901-65) that has been previously validated for use in this species (Clotfelter et al., 2004). Testosterone was extracted from 20 μl of plasma using diethyl ether. Tritiated testosterone was added to the sample to estimate extraction efficiency (average 94.8%). Extracted hormone was reconstituted in 50 μl of 98% ethanol, followed by 300 μl of assay buffer. We followed the manufacturer's instructions for the remaining procedures. We estimated hormone concentrations in reference to a 9-point standard curve (range 0.78–200 pg well^{-1}) using a curve-fitting program (Microplate Manger, Bio-Rad Laboratories, Hercules, CA, USA). The detection limit of the assay was 0.099 ng ml^{-1} of testosterone in plasma. Samples were distributed randomly across and within plates, and all samples and standard curves were run in duplicate. Within-plate variation was 7.01% and between-plate variation was 1.89%. We did not correct for either extraction efficiency or across-plate variation.

Tissue collection

At 1–2 days after the final blood sampling (25–26 February 2014), birds were killed using isoflurane overdose in a 2-way factorial design: birds from both chronic disturbance and control treatments were killed either immediately after capture (baseline treatment) or after 90 min of being held in a paper bag (acute handling treatment). We selected this timing for several reasons. First, we wanted to allow sufficient time for stress-induced changes in gene expression.

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Second, we had preliminary data to suggest that steroidogenic gene expression is sensitive to other hormonal stimuli 90 min after administration [e.g. steroidogenic acute regulatory protein (*StAR*) mRNA abundance after GnRH injection; K.A.R. and E.D.K., unpublished data]. Third, literature on immediate early gene (IEG) expression and half-life suggests that assessment at 90 min following a stimulus allows us to capture both induction as well as reduction in IEG expression (Maney and Goodson, 2011). Since IEGs include transcription factors, we estimated that gene expression 90 min after capture was likely to capture both up- and downregulation of components of the testicular transcriptome, compared to expression immediately following capture. Testes were dissected and flash frozen on pulverized dry ice. To assess testes size, an observer who was unaware of our hypotheses measured testes mass (to the nearest mg) before RNA extraction.

Microarray hybridization and analyses

We used a custom Nimblegen 12-plex microarray (Roche Nimblegen, Madison, WI, USA) for the dark-eyed junco (Peterson et al., 2014) to analyze the effect of treatments on the transcriptome. RNA was extracted from testes using the Trizol method (Invitrogen, Carlsbad, CA, USA) and quantified using the Nanodrop ND-2000 (ThermoScientific, Waltham, MA, USA). Sample integrity was verified using TapeStation (mean RIN: 9.3; range 8.6–9.7; Agilent Technologies, Santa Clara, CA, USA). Total RNA was converted to double-stranded cDNA using the TransPlex Complete Whole Transcriptome Amplification Kit (Sigma-Aldrich, St Louis, MO, USA, product no. WTA2) according to the manufacturer's instructions. Labeled cDNA was synthesized using a NimbleGen Dual-Color DNA Labeling Kit (Roche NimbleGen, Inc.) as described in the standard NimbleGen CGH Analysis Procedure. Briefly, 500 ng of cDNA was denatured at 98°C for 10 min. The denatured sample was snap chilled on ice and incubated with 50 units of Klenow fragment (New England Biolabs, Ipswich, MA, USA) and 10 mmol l^{-1} dNTP mix for 2 h at 37°C. Reactions were terminated with 0.5 mol l^{-1} EDTA and precipitated with isopropanol, rinsed in cold 80% ethanol and desiccated using a Savant DNA 120 SpeedVac Concentrator (ThermoScientific). Pellets were resuspended in nuclease-free water. Cy-labeled product was quantified using a Nanodrop ND-2000 (ThermoScientific) and 8 µg (4 µg each of Cy-3 and Cy-5) labeled product was pooled and desiccated for subsequent hybridization. The labeled product was resuspended using Sample Tracking Controls (Roche NimbleGen, Inc.). Components from the Hybridization Kit, LS (Roche NimbleGen, Inc.) were combined to prepare the hybridization solution. This solution was added to each subarray pool for a total volume of 8 µl. The sample was denatured at 95°C for 5 min and allowed to hybridize on the microarray for 18 h at 42°C in a NimbleGen hybridization system. The post-hybridization wash was conducted using a Wash Buffer Kit (Roche NimbleGen, Inc.). Arrays were agitated consecutively in Wash Buffer I (2 min), Wash Buffer II (1 min) and Wash Buffer III (15 s). The arrays were dried for 1 min using a High-Speed Microarray Centrifuge (Arrayit Corp., Sunnyvale, CA, USA). Image acquisition was attained using the NimbleGen MS 200 Microarray Scanner (Roche NimbleGen, Inc.) at 2 µm resolution. cDNA was hybridized to the microarray using a full round-robin design.

The microarray contained 33,545 assembled sequencing reads (contigs) in triplicate covering 22,765 putative genes (isogroups), which were based on dark-eyed junco transcriptome sequencing (Peterson et al., 2012). We tested for significant differences in gene expression between treatment groups (and their interaction) using R package limma (Smyth, 2005) (i.e. we compared pooled chronic disturbance versus control, and pooled acute handing versus baseline control). We calculated a global false-discovery rate across all comparisons and used a q-value threshold of 0.05 for significance (see Peterson et al., 2014 for further details). We used topGO (Alexa and Rahnenfuhrer, 2016) with the weight algorithm (Alexa et al., 2006) to identify the Gene Ontology (GO) terms (Ashburner et al., 2000) that were significantly over-represented among the significantly differentially expressed genes in each comparison.

qPCR

The microarray did not include all of our candidate genes of interest. Therefore, we subsequently performed quantitative polymerase chain reaction (qPCR) to test our *a priori* predictions about genes related to testosterone synthesis and testicular function. Specifically, we quantified expression of steroid synthesis genes [luteinizing hormone receptor (*LHR*), *StAR*, cytochrome P450 side-chain cleavage (*P450*), cytochrome P450 17α-hydroxylase (*CYP17*), 3β-hydroxysteroid dehydrogenase/isomerase (*3βHSD*); we did not measure the expression of *17βHSD*, a gene that catalyzes the last step in testosterone synthesis, because its isoforms are poorly characterized in birds (London and Clayton, 2010)], as well as genes that encode receptors for hormones that may regulate testicular function [sperm production: follicle-stimulating hormone receptor (*FSHR*); response to stressors: *GR*, mineralocorticoid receptor (*MR*), *GnIHR*]. Most of the primers, except *FSHR* primers, had been previously validated in our system (Bergeon Burns et al., 2014; Rosvall et al., 2016a,b). *FSHR* primers were designed using white-throated sparrow genomes using Primer-BLAST (Ye et al., 2012). Primer sequences and efficiencies are reported in Table S1. We ran qPCR reactions using PerfeCTa SYBR Green SuperMix (Quantabio, Beverly, MA, USA, product no. 95054) on the Roche LightCycler 480 platform (Roche Holding AG, Basel, Switzerland) using the same cDNA samples from the microarray. The cDNA concentration was similar between samples (17–22 ng µl^{-1}). All sample and calibrator cDNA samples for genes of interest and housekeeping genes were run in triplicate on clear LightCycler 480 384-well plates (product no. 05102430001, Roche Holding AG). Prior to sample analysis, we measured the efficiency of each primer by amplifying sequential 1:4 dilutions of a pooled cDNA sample. cDNA was diluted 1:40 in double-distilled water from the Millipore system (Merck Millipore) prior to plating. Each well contained 8 µl of PerfeCTa SYBR Green SuperMix and 2 µl of the 1:40 cDNA dilution. Plates were sealed with a clear plastic seal and centrifuged at 1200 ***g*** for 4 min to remove bubbles. The qPCR cycle program was as follows: initial denaturation for 10 min at 95°C was followed by amplification for 40 cycles (denaturation for 30 s at 95°C; annealing for 60 s at 60°C; extension for 30 s at 70°C; fluorescence measurement for 2 s at 78°C).

Following sample amplification, we investigated product size and quality using a melting curve (60 s at 95°C, 30 s at 55°C, followed by a ramp from 55–95°C at 0.11°C s^{-1}). Samples with multiple melting peaks were excluded from analysis. We observed a small extra peak with low melting temperature for most samples, which likely represents conspecific amplification or primer dimers. To preclude the possibility of this affecting our gene expression measurement, following the guidelines from the manufacturer, we moved the fluorescence measurement to 78°C (see above). This ensured that the fluorescence measurements were collected at

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temperatures at which the short double-stranded non-specific products were denatured.

We calculated the relative gene expression in each sample in reference to a pooled standard, and normalized this value against average expression of 2 housekeeping genes [peptidylprolyl isomerase A (*PPIA*) and ribosomal protein L4 (*RPL4*)] using LightCycler 480 software (Roche Holding AG, release no. 1.5.1.62). *PPIA* and *RPL4* are 2 of the most stable housekeeping genes in testes of passerines (Zinzow-Kramer et al., 2014), and their expression did not differ between chronic or acute treatments. Primers for *PPIA* and *RPL4* were designed using the white-throated sparrow genome. To calculate amplification efficiencies, we used the 'Abs Quant/2nd Derivative Max' method. For analysis of relative gene expression in our samples, we used the 'Advanced Relative Quantification' method, specifying the 'High Confidence' option under 'Abs/Quant/2nd Derivative Max', choosing the 'All to All' pairing rule, and using the efficiency to control for differences in amplification. We inspected all replicates for samples and the calibrator, and excluded the replicates that deviated from others by more than 0.7 crossing points (Cps). We then verified that the target/reference ratios calculated using the 2 housekeeping genes separately identified quantitatively similar relationships between the samples. If so, we reran the analysis using the 'All to Mean' pairing rule, which uses the mean Cp of both housekeeping genes to calculate the final target/reference ratio for each sample.

Statistical analysis

We analyzed our data in R (http://www.R-project.org/). We used linear (LM) and linear mixed effects models (LMM, package nlme, https://CRAN.R-project.org/package=nlme) to analyze the effect of chronic and acute treatments on reproductive physiology (hormone levels) and morphology (testes mass and CP size). We tested whether the time of day of hormonal sampling explained variation in hormone levels and used it as a continuous covariate in models where this effect was significant. We included individual as a random factor in all mixed effects models that included more than one measurement from the same individual (denoted as LMMs). When testing whether the change in testosterone, as a result of acute handling stress, as a result of GnRH injection, or between the beginning and end of the experiment differed between the two long-term treatments, we included an interaction term between the long-term treatment and testosterone sampling time points. To test whether the relationship between testes mass and testosterone differed between treatments, we included an interaction term between the chronic disturbance treatment and testes mass. Interactions were removed from models if they were not significant. Our sample size differed between sampling rounds and between bleeds because of low blood volume in some of the samples. When necessary, the response variables were transformed to meet model assumptions of normality. Full model tables for models with interaction terms are reported in the Supplementary Information.

We used 2 different multivariate approaches to analyze the effect of treatments on candidate gene expression (determined by qPCR). Since the expression of steroidogenic genes showed significant pairwise correlations (Table S8), we used principal components analysis (PCA) to summarize the covariation in gene expression by creating independent principal components. We then used the first principal component as a dependent variable in ANOVA to investigate whether chronic or acute treatments, or their interaction, affected steroidogenic gene expression. Following this, we used ANOVAs to analyze each gene separately and corrected for multiple comparisons using the Benjamini–Hochberg (BH) method.

Because the expression of receptors that may downregulate testicular function (*GR*, *MR* and *GnIHR*) was not correlated to each other and they mediate different functions in animals, we used MANOVA to analyze the effect of chronic and acute treatments on the overall sensitivity to stress signaling. Following MANOVA, we analyzed each gene independently using ANOVA and corrected for multiple comparisons using the BH method. *FSHR* expression was analyzed independently using an LM, because its function is not known to relate to stress signaling or steroidogenesis.

RESULTS

Testosterone

Pre-treatment

At the outset of the experiment, neither baseline testosterone (LM, $n_{disturb}$=12, $n_{control}$=12, β=−0.142, t=−7.21, P=0.479) nor testosterone after the acute handling stressor (LM, $n_{disturb}$=11, $n_{control}$=6, β=0.158, t=1.172, P=0.230) differed between the chronic and control treatment groups, and the acute handling stressor did not reduce testosterone compared to baseline levels (LMM, n=41, β=−0.049, t=−0.616, P=0.549; Fig. 2A).

Post-treatment

Baseline testosterone increased throughout the experiment in both disturbance treatments (LMM, $n_{disturb}$=29, $n_{control}$=28, β=0.401, t=3.964, P<0.001; Table S2), consistent with recrudescence of testes in response to increasing day length. After 3 weeks of treatment, there was a significant interaction between the long-term disturbance treatment and acute handling treatment on testosterone levels (LMM, n=48, β=0.296, t=2.265, P=0.034; Table S3, Fig. 2B). *Post hoc* tests showed that birds from the long-term disturbance group had marginally lower baseline testosterone levels than controls (LM, $n_{disturb}$=14, $n_{control}$=13, β=−0.482, t=−1.909, P=0.068) but that testosterone levels after 30 min of the acute handling stressor did not differ between the disturbance treatments (LM, $n_{disturb}$=12, $n_{control}$=14, β=−0.015, t=−0.078, P=0.938). *Post hoc* analyses also showed that birds from the control treatment showed a significant reduction in their testosterone levels from baseline to post-acute-handling stress (LMM, $n_{handled}$=16, $n_{unhandled}$=14, β=−0.702, t=−2.894, P=0.015), while birds from the chronic disturbance treatment did not (LMM, $n_{handled}$=17, $n_{unhandled}$=12, β=−0.031, t=−0.187, P=0.855). Both control and chronically disturbed birds significantly increased their testosterone in response to GnRH injection compared with pre-injection levels (LMM, n_{pre}=26, n_{post}=34, β=0.214, t=9.077, P<0.001), but the GnRH-induced testosterone did not differ between treatment groups (LMM, treatment×injection interaction, n=60, β=0.016, t=0.037, P=0.971; Table S4, Fig. 2C).

Reproductive organs and their relationship with testosterone

Testes mass was not affected by the chronic disturbance treatment (LM, $n_{disturb}$=18, $n_{control}$=17, β=0.005, t=0.935, P=0.357; Fig. 3A). There was no relationship between testes mass and baseline testosterone levels (LM, n=27, β=7.120, t=0.949, P=0.352), but testes mass was positively related to testosterone levels after acute handling stress (LM, n=25, β=12.393, t=2.838, P=0.010) as well as to GnRH-induced testosterone levels (LM, n=34, β=34.627, t=3.127, P=0.004; Fig. 4). The relationship between testes mass and testosterone did not differ between disturbance treatments (Table S5). CP size showed significant differences between disturbance treatments, with chronically disturbed birds having larger CPs than control birds (LM, $n_{disturb}$=16, $n_{control}$=17, β=1.631, t=4.711, P<0.001; Fig. 3B).

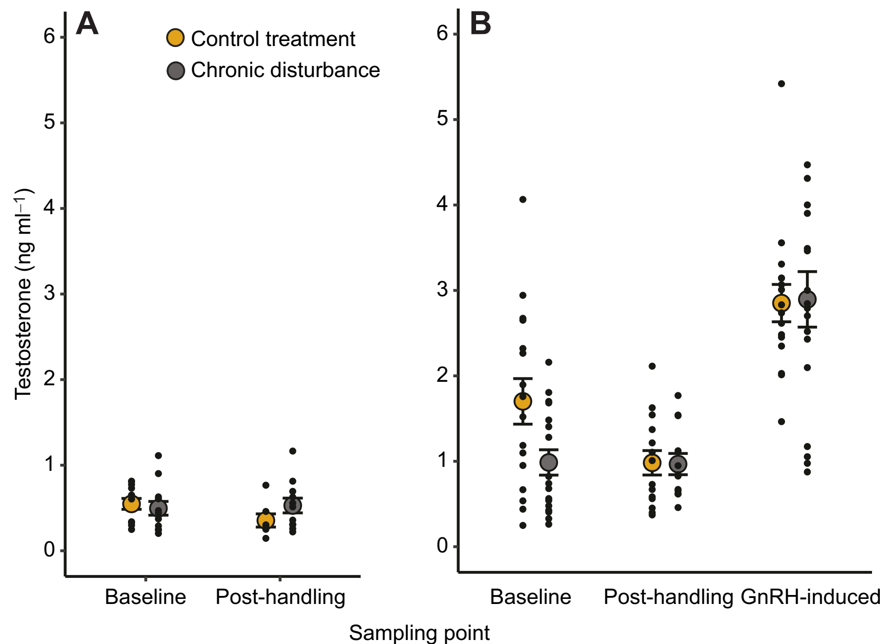

Fig. 2. Effect of chronic disturbance and acute stressor on testosterone levels. (A) Testosterone levels before the long-term disturbance treatments began (see Fig. 1). (B) Testosterone levels after 3 weeks of long-term treatment. Shown are baseline testosterone levels, post-handling levels and GnRH-induced testosterone levels. GnRH-induced testosterone was not measured before the experiment. Shown are group means (orange and gray circles)±s.e.m.; black points represent individual measurements.

Testicular transcriptome

We found that the expression of 16 transcripts was significantly affected by the chronic disturbance treatment after correcting for false discovery rate (out of 20,390 total expressed in testes in this study, 0.078%; Table S6). GO analysis identified 3 terms that were overrepresented among the significantly differentially expressed genes. These were: electron transport chain, extracellular structure organization (biological process) and glycosaminoglycan binding (molecular function) (Table 1). None of the genes that were differentially expressed between tissues in response to chronic treatment are known to link clearly to the stress response or reproductive function.

The acute handling stressor caused significant changes in the expression of 168 transcripts compared with baseline treatment (out of 20,390 total expressed in testes, 0.823%; Table S7). Among genes that were contributing to these terms were a variety of heat shock proteins (*HSPB1*, *DNAJA4*, *HSPA4L*, *HSP90AA1*), and genes associated with inflammation and cytokine signaling (*IL4R*, *PIK3AP1*, *MAP3K8*). GO analysis identified a number of terms that were overrepresented in the set of genes that showed significant treatment effect, including: receptor signaling protein activity, intracellular steroid hormone receptor signaling pathway, cellular protein complex assembly, response to stimulus, and '*de novo*' posttranslational protein folding (Table 2). One unannotated transcript with an unknown function showed a significant interaction between the long-term treatments (chronic versus control) and acute treatments (handled versus baseline).

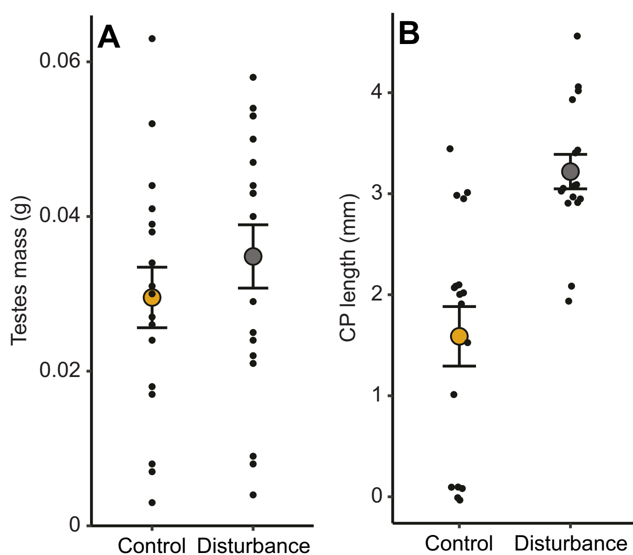

Fig. 3. Effect of chronic disturbance on reproductive organs. (A) Testes mass. (B) Cloacal protuberance (CP). Shown are group means (orange and gray circles) ±s.e.m.; black points (jittered in B to show sample size) represent individual measurements.

Testosterone synthesis and HPG axis receptors

The expression of 5 genes (*LHR*, *StAR*, *p450scc*, *CYP-17*, *3bHSD*) involved in testosterone synthesis was positively correlated (Table S8). In PCA, all of these genes loaded negatively on PC1, which explained 50% of the variation in expression (Table S9). However, variation in PC1 was not explained by long-term disturbance (LM, n_{disturb}=18, n_{control}=17, β=−0.294, t=−0.540, P=0.593), acute handling stressor (LM, n_{handled}=17, $n_{\text{unhandled}}$=18, β=−0.653, t=−1.201, P=0.239) or the interaction of these treatments (LM, n=35, β=0.670, t=0.609, P=0.547). Since testosterone levels decreased significantly in response to the acute handling stressor only in the control treatment, we conducted a separate PCA of only the control individuals (see Table S10 for loadings). This analysis showed that the acute handling stressor tended to reduce the expression of testosterone synthesis genes 90 min later, although this effect was not significant (LM, n_{handled}=8, $n_{\text{unhandled}}$=9, β=−1.226, t=−1.828, P=0.088). Analysis of each of the genes individually using ANOVA showed that *CYP17* levels were marginally lower in handled control birds (LM, n=17, t=2.102, P=0.053; see Table S11 for other genes), although this effect was not significant after multiple comparison correction (P=0.265; Fig. S1).

Neither handling (LM, n_{handled}=17, $n_{\text{unhandled}}$=18, β=0.103, t=1.432, P=0.162) nor chronic disturbance (LM, n_{disturb}=18,

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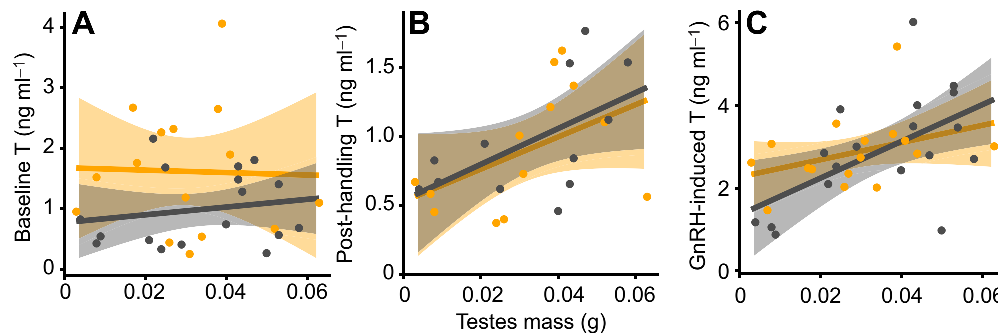

Fig. 4. Relationship between testes mass and testosterone (T) levels before handling (baseline), post-handling, and after a GnRH injection. Orange data points represent control birds; gray data points indicate chronically disturbed birds; shaded areas denote 95% confidence interval for the slope of the regression line.

$n_{control}$=17, β=−0.119, *t*=−1.653, *P*=0.108), nor their interaction (LM, *n*=35, β=−0.096, *t*=−0.661, *P*=0.514), affected expression of *FSHR* (Fig. S2).

Sensitivity of testes to regulation by the HPA axis

MANOVA showed that the acute handling stressor (*n*=35, Wilks=0.676, $F_{3,30}$=4.791, *P*=0.008), but not chronic disturbance (*n*=35, Wilks=0.907, $F_{3,30}$=1.027, *P*=0.395), nor interaction between chronic treatment and the acute handling stressor (*n*=35, Wilks=0.855, $F_{3,29}$=1.643, *P*=0.201), affected expression of receptors (*GR*, *MR*, *GnIHR*) for hormones that are known to suppress or inhibit testosterone production. *Post hoc* linear models comparing handling to baseline treatment showed that the acute handling stressor significantly downregulated *GR* mRNA expression (LM, $n_{handled}$=17,

Table 1. Gene Ontology (GO) processes significantly affected by the acute handling treatment (H) compared with the unhandled control treatment (U)

GO description	GO #	GO category	*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*

BP, biological process; MF, molecular function; *N*, number of genes per GO category in microarray; DE, number of genes per GO category significantly differentially expressed between treatments; TOR, target of rapamycin; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate. Non-underlined genes were upregulated in the H treatment. Underlined genes were upregulated in U treatment. Group bias refers to direction of upregulation.

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Table 2. GO processes significantly affected by chronic disturbance versus control treatments

GO description	GO #	GO category	*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*

N, number of genes per GO category in microarray; DE, number of genes per GO category significantly differentially expressed between treatments; C, control treatment.

$n_{unhandled}$=18, β=0.137, t=2.282, P=0.029; Fig. 5A) and marginally upregulated *GnIHR* expression (LM, $n_{handled}$=17, $n_{unhandled}$=18, β=−0.262, t=−1.875, P=0.070; Fig. 5C), although these effects were not significant after correction for multiple comparisons (Table S12).

DISCUSSION

In this study, we experimentally tested how chronic disturbance, an acute handling stressor and their interaction affected testes function in a songbird during seasonal testicular recrudescence. We showed that birds in the chronic disturbance treatment had marginally lower baseline testosterone compared with control animals. An acute handling stressor reduced testosterone levels in control animals, but not in animals from the chronic disturbance treatment. The chronic disturbance treatment did not affect the ability to produce testosterone in response to exogenous stimulation from GnRH, and testes size did not differ between chronic disturbance and control treatments. Surprisingly, birds in the chronic disturbance treatment had significantly larger CPs than birds in the control treatment. Chronic disturbance had very little effect on gene expression in the testes: neither steroidogenic enzyme expression nor expression of receptors associated with potential regulation of testes function showed significant differences between the chronic disturbance and control treatments. Overall, chronic disturbance had a significant effect on only a handful of genes in the transcriptome. The acute handling stressor, by contrast, had a comparatively strong effect on the testicular transcriptome, a marginally suppressive effect on steroidogenesis enzyme gene expression, a marginally suppressive effect on the expression of *GR* mRNA, and a marginally positive effect on *GnIHR* gene expression compared with baseline treatment. There was little evidence of an interaction between chronic disturbance and the acute stressor on expression of the testicular transcriptome. Collectively, these findings shed light on mechanisms by which short- and long-term stressors, and their interaction, affect reproductive function.

Chronic stressor and testes function

Birds from the chronic disturbance treatment had marginally lower baseline testosterone levels compared with the control treatment, agreeing with other reports in a variety of organisms that show a decrease in testosterone in response to chronic disturbance (Pickering et al., 1987; Moore et al., 1991; Retana-Márquez et al., 2003; but see Armario and Castellanos, 1984; Jones and Bell, 2004). Suppression of testosterone levels in response to a chronic stressor in the wild may result in reduced investment in reproduction, exacerbating the negative effects of disturbance on fitness.

Mechanistically, the marginal difference in testosterone levels between the chronic disturbance and control birds in this study was not explained by differences in testicular physiology: testes mass and the GnRH-induced testosterone levels were not different between the disturbance treatments, suggesting that the difference in testosterone was not caused by long-lasting differences in the ability of the testes to produce testosterone.

Furthermore, chronic disturbance did not have a significant effect on steroidogenic gene expression, expression of receptors (*GnIHR*, *GR*, *MR*) for potential inhibitory hormones, or the testicular transcriptome in general, suggesting that testes function was nearly unaffected by the chronic disturbance treatment. This suggests that

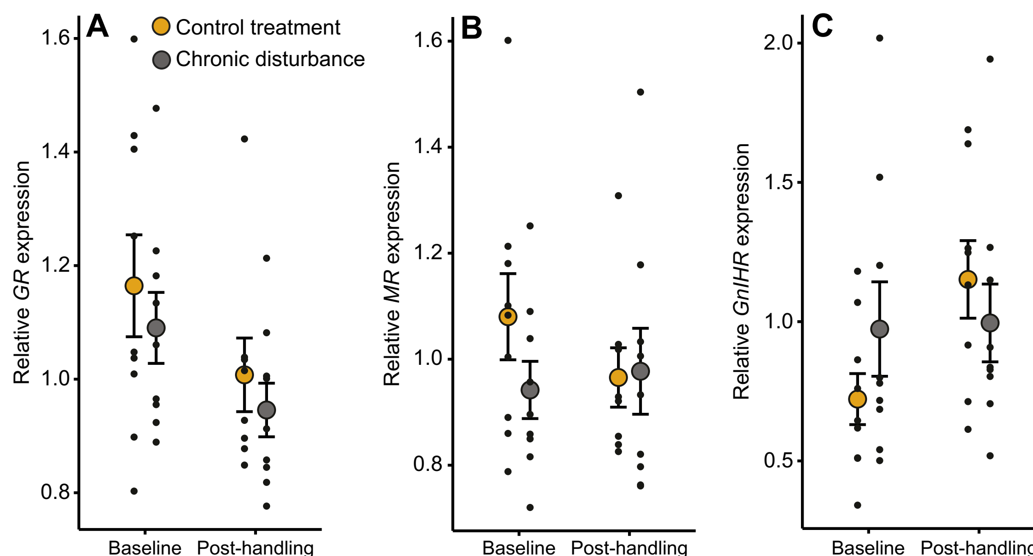

Fig. 5. Effect of chronic disturbance and an acute handling stressor on hormone receptor expression in testes. (A) Glucocorticoid receptor (*GR*) expression, (B) mineralocorticoid receptor (*MR*) expression and (C) gonadotropin inhibitory hormone receptor (*GnIHR*). Shown are group means (orange and gray circles)±s.e.m.; black points represent individual measurements.

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local signaling at the level of the testes is unlikely to be the cause of lower testosterone levels or the decreased sensitivity to an acute handling stressor that we observed in the chronically disturbed animals. Instead, potential long-term differences in testosterone may be mediated by systemic signals that inhibit testicular function, such as GnIH (McGuire and Bentley, 2010) or corticosterone (McGuire et al., 2013). However, birds from the chronic disturbance treatment did not show higher corticosterone levels (Hanauer, 2017), suggesting that variation in circulating corticosterone was not responsible for differences in testosterone between the disturbance and control treatments.

The lack of effect of chronic disturbance on the testicular transcriptome may also have been due to an unintended long-term modulation of gene expression by the acute stressor treatments during blood collection in the days preceding killing. Future analysis of the post-translational effects of stressors, as well as other tissues (e.g. hypothalamus, liver) collected from these animals, may elucidate the mechanisms that caused the differences in testosterone levels between the disturbance treatments (Lynn et al., 2015).

A surprising finding in this study was that the CPs were significantly larger in the chronically disturbed birds compared to control animals. CPs in birds are sperm storage organs that develop in males during the breeding season (Salt, 1954) and are regulated by testosterone (Witschi, 1961). Males with larger CPs store more sperm (Tuttle et al., 1996) and larger CPs are hypothesized to allow faster copulation (Birkhead et al., 1993). Barring spurious results, our finding may indicate that birds in the chronic disturbance group may be investing more in sperm production compared to the control animals, perhaps to advance their reproductive readiness or enable more rapid copulation in an uncertain environment.

It is important to note that the birds in this study had not reached full reproductive capacity. Testes and CPs did not reach their full size during this study. Testes mass was 21% of mature reproductive size (Bergeon Burns et al., 2014). The capacity of birds to produce testosterone following GnRH injection was slightly lower than in wild animals during the breeding season (Rosvall et al., 2014). However, baseline testosterone concentration was similar to that in the wild (Rosvall et al., 2014), indicating that patterns observed in this study are likely to persist later in the breeding season.

Acute stressor and testes function

Before the disturbance treatment, testosterone did not decrease in response to acute stress. However, testosterone levels at this stage were low and near the assay sensitivity limit; therefore, the absence of the effect may be due to the limitations of the hormone assay. After the treatment, birds in the control treatment showed a significant decrease in testosterone levels in response to the acute handling stressor. Suppression of testosterone in response to acute stressors has been shown in many other studies (Deviche et al., 2010, 2012; Moore et al., 1991). This effect may be due to a reduction in testosterone synthesis, or an increase in testosterone metabolism by the liver (Lynn et al., 2015). We found a marginally significant decrease in the expression of *CYP17*, an enzyme involved in steroidogenesis, in birds that had experienced an acute handling stressor compared with baseline treatment. Because the expression of steroidogenic enzymes is positively correlated with testosterone levels (Rosvall et al., 2016b), a decrease in expression of testosterone synthesis genes may be responsible for suppression of testosterone in response to handling.

Downregulation of steroidogenic enzyme gene expression in response to the acute handling stressor may be due to either a change in the local or systemic signaling, or because of a change in sensitivity to this signaling (Ernst et al., 2015). We found a significant overall change in hormone receptor gene expression, primarily downregulation of *GR* expression and upregulation of *GnIHR* expression, in response to the acute handling stressor. This suggests that testes in acutely stressed birds may be less sensitive to downregulation by glucocorticoids, such as corticosterone, but more sensitive to another important hormone – GnIH – that has an inhibitory effect on reproductive physiology (Tsutsui et al., 2010). Indeed, while corticosterone levels increased following the acute handling stressor (Hanauer, 2017), other studies have shown that corticosterone is unlikely to be the cause for rapid decrease in testosterone in response to acute stress in birds (Davies et al., 2016; Deviche et al. 2017). Testosterone and corticosterone levels in birds are often either unrelated or positively correlated (Klukowski et al., 1997; Buchanan et al., 2003), showing that corticosterone and testosterone do not covary in a way that is consistent with testosterone suppression by corticosterone. We could not assess the expression of GnIH in the testes or brain; therefore, we do not know whether acute or chronic stressors, in addition to GnIHR, also upregulated GnIH synthesis.

Acute handling stress caused significant changes across the testicular transcriptome compared with baseline treatment. The GO analysis did not indicate that the acute handling treatment affected testes-specific functions, such as spermatogenesis or steroidogenesis, although we note that several candidate genes were not present on this array. Instead, GO analysis suggested that acute handling stress induced a cellular stress response, resulting in upregulation of a handful of heat shock proteins. Heat shock proteins are molecular chaperones that, under normal conditions, facilitate protein assembly and are upregulated in response to stressors (Åkerfelt et al., 2010). Heat shock protein expression in testes increases during spermatogenesis and oogenesis (Neuer et al., 2000), which may ensure that the development of gametes is shielded from environmental stressors. Importantly, some of the same genes (e.g. *HSP90AA1*, *DNAJA4*) that were upregulated in response to acute handling stress in this study were also upregulated in chicken testes in response to heat stress (Wang et al., 2015), indicating that testes may have a generalized cellular stress response that is upregulated in response to a variety of stressors. Because we could only measure gene expression at one time point, we do not know the duration of the observed changes in gene expression, or whether they are linked to the observed changes in hormone levels (measured on a shorter timescale). Our data nevertheless indicate that acute handling stress has marked effects on hormone-related gene expression in the testes in the short term.

Interaction between chronic and acute stressors

Birds from chronic disturbance and control treatments responded differently to the acute handling stressor: whereas handling reduced testosterone levels in control birds, it did not affect testosterone levels in birds from the chronic disturbance treatment. This difference can be interpreted in 2 non-exclusive ways: first, the reduced impact of the acute stressor on testosterone in birds from the chronic disturbance group might have arisen because their reproductive function was already downregulated to a degree that prohibited further decrease ['floor effect' *sensu* (Sapolsky et al., 1984)]. Second, the difference in the effect of handling on testosterone levels between chronic disturbance and control treatments could be explained by a lower sensitivity of the chronically disturbed birds to stressors compared to the control individuals. We did not find any effect of chronic disturbance

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on signaling in the testes that would explain this difference, suggesting that differences in sensitivity to stress may exist in the HPG axis tissues that are upstream from the testis (pituitary, hypothalamus), or other hormonal targets that interact with testosterone production.

From an ecological perspective, the first alternative (floor effect) could be interpreted as a consequence of homeostatic overload (Romero et al., 2009). Homeostatic overload refers to the cases where overexposure to stressors, and the associated increase in the frequency of stress response, results in a pathological state that leads to compromised organismal function (Romero et al., 2009). In our study, the high frequency of stressors may have resulted in a compromised ability to produce testosterone. However, because birds from both chronic and control treatments elevated their testosterone in response to GnRH to the same degree, this explanation is unlikely. Instead, the floor effect could be a result of a 'best of a bad job' strategy, wherein individuals of low quality or in a bad environment maximize their fitness by playing it safe (Sih and Bell, 2008). In this case, animals may opt to keep the resources diverted from reproduction to maximize their ability to respond to stressors.

The second alternative (differences in sensitivity to stress), is consistent with the life history prediction that, under conditions of low expected future reproductive success, stress should have a less negative effect on reproduction compared to situations where expected reproductive success is high (Wingfield and Sapolsky, 2003). Animals that grow their reproductive tissues in environments with a high frequency of stressors may change their physiology to reduce sensitivity of the HPG axis to stress, thus allowing them to maintain reproductive function during stressful episodes, despite the possible cost to self-maintenance (Wingfield and Sapolsky, 2003). This prediction has been supported by a study in a natural system (Abolins-Abols et al., 2016), where the reproductive phenotype of urban animals is less sensitive to stressors compared to that of rural animals.

In contrast to our testosterone findings, we did not find an interaction between chronic and acute treatments on the testicular transcriptome, although we note that the marginal effects of the chronic treatment reduce our power to explore this interaction. This finding nevertheless provides further evidence that chronic disturbance does not affect testicular capacity to produce testosterone or respond to stress. Instead, we hypothesize that the differential effect of handling stress on testosterone levels in the chronic disturbance and control treatments may be due to differences in upstream signaling from the pituitary or hypothalamus, which regulates the activity of the molecular machinery in testes. Further work will be needed to explore alternatives, such as post-translational effects of stress that are not captured at the level of gene expression. Despite these uncertainties, our results nevertheless demonstrate that testes are relatively robust to interacting effects of acute and chronic stressors on gene expression.

Correlation between testes mass and testosterone

We found that testes mass was positively correlated with testosterone levels after acute handling stress and after GnRH injection, but not with baseline testosterone. A significant positive relationship between testes mass and the capacity of testes to produce testosterone following GnRH injection is likely due to the variation in testicular growth rate or stage in junco males: individuals with more recrudesced testes likely had more or larger Leydig cells capable of producing more testosterone (Temple, 1974). A more interesting observation is that testicular mass was positively correlated with testosterone levels after an acute stressor but not at baseline levels. This suggests that, while baseline testosterone is responsive to various environmental and social factors and stressors, these effects are superimposed on 'true' baseline production of testosterone that is insensitive to stimuli and is proportional to the size of the testes. In other words, suppression of testosterone may lower testosterone levels, but larger testes will nevertheless keep producing more testosterone than small testes as a result of baseline activity of the synthesis machinery.

Summary

Overall, our results show that chronic and acute stressors suppress testosterone release by the testes, but that the effect of acute stressors differs depending on the frequency of stressors in the environment. These differences in testes function between disturbance environments are unlikely to be mediated by changes in testicular gene expression, but are more likely to reside upstream of the testes. Whereas chronic treatment had a negligible effect on the testicular transcriptome, acute handling stress significantly upregulated major components of the cellular stress response and affected the expression of hormone receptors involved in the downregulation of testosterone production. These results suggest a potential mechanism for regulating testosterone decrease in response to acute stressors. Furthermore, they show that transient changes in gene expression in response to acute stressors are different from more permanent responses to chronic stressors. An important future direction is therefore to identify the mechanisms responsible for differences in testosterone levels between animals experiencing different disturbance regimes.

This study is among the first to experimentally test the mechanisms by which acute and chronic stressors interact to influence reproduction. We demonstrate patterns that, adaptive or not, are doubtlessly important in understanding how animals respond to chronic and acute stressors. We therefore urge further study on the adaptive significance and mechanisms that mediate the effect of chronic stressors on testosterone levels and other reproductive functions.

Acknowledgements
We thank A. Kimmitt, S. Slowinski, H. Kassab, A. Brenner, A. McKay, C. Taylor and N. Fletcher for assistance with disturbance manipulations, animal care and sampling. We thank L. Sloan, who extracted RNA, M. Stephens, who ran the microarray at the University of Notre Dame Genomics & Bioinformatics Core Facility, and D. Rusch and A. Fellows from Indiana University's Center for Genomics and Bioinformatics for assistance with microarray data analysis. We thank C. Bergeon-Burns for providing us with primer sequences and advice on lab work, R.A. Stewart for assistance with molecular sex determination, and the Center for the Integrative Study of Animal Behavior at Indiana University for providing laboratory facilities and equipment. We also thank the two anonymous reviewers for their feedback on the earlier drafts of this manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.A.-A., R.E.H.; Methodology: M.A.-A., R.E.H., K.A.R.; Validation: M.A.-A., R.E.H.; Formal analysis: M.P.P.; Investigation: M.A.-A., K.A.R.; Resources: E.D.K.; Writing - original draft: M.A.-A.; Writing - review & editing: M.A.-A., R.E.H., K.A.R., M.P.P., E.D.K.; Visualization: M.A.-A.; Funding acquisition: E.D.K. and K.A.R.

Funding
This study was funded by the National Institutes of Health grant R21HD073583 to K.A.R. and the National Science Foundation grant IOS-1257474 to E.D.K.

Data availability
Data described and analyzed in this article are available from the figshare digital repository: doi:10.6084/m9.figshare.6987023

Supplementary information
Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.180869.supplemental

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Table S1: qPCR 1 primer sequences, design and efficiencies. WTSP = White throated sparrow (*Zonotrichia albicollis*); ZF = Zebra finch (*Taeniopygia guttata*).

Gene	Primer	Sequence	Reference	Genome	Product length	Efficiency
FSHR	FWD REV	ACGCCACCGAGCTGAGATTTGT TCAACAAGTCCTGTGAAAGCTCCCT	Designed by CMBB	WTSP	73	1.996
RPL4	FWD REV	GTCTAAAGGCCACCGCATTGA CGCTGGGAGGCGTAAACCTT	Designed by CMBB	WTSP	150	1.996
PPIA	FWD REV	TCCCGAAGACAGCAGAAAACT CCATTGTGGCGTGTGAAGTC	Designed by CMBB	WTSP	130	1.954
GR	FWD REV	TGAAGAGCCAGTCCCTGTTCGAG CAACCACATCATGCATAGAGTCCAGCA	Rosvall et al. 2016b	see ref	see ref	1.985
MR	FWD REV	AAGAGTCGGCCAAACATCCTTGTTCT AAGAAACGGGTGGTCCTAAAATCCCAG	Rosvall et al. 2016b	see ref	see ref	1.998
GnIHR	FWD REV	TGGCCCTTTGACAACATCACGTGCAA ACAGCAATGGCGACCAGCGTGAAA	Rosvall et al. 2016b	see ref	see ref	2.034
LHR	FWD REV	TCTCAGAGCGACTCCCTG, TCCGTCCTCAATGTGCAAC	Burgeon-Burns et al. 2014	see ref	see ref	1.994
StAR	FWD REV	TGGGCCAGCACATGCTAGTTAAGCAA AGCAGGGCTTCCTTCAGGAACCAAGTAT	Rosvall et al. 2016a	see ref	see ref	1.983
P450	FWD REV	GACCGCGAGAAGATGCTGAAA TCTCCTTGATGGTGGCCTTGAG	Rosvall et al. 2016a	see ref	see ref	2.045
CYP17	FWD REV	CATCAACCTCTGGTCTGTGCAC AAGCGGCCAGGATTGAACT	Rosvall et al. 2016a	see ref	see ref	1.93
3βHSD	FWD REV	ATGAGCTACGCTGACCTGAA CAGCAGCAGCGAGAAGTAATAG	Rosvall et al. 2016a	see ref	see ref	2.016

Table S2: Change in baseline testosterone over the course of experiment. SE = standard error. Interaction (in italic font) was not significant and was taken out of the final model.

Linear mixed model

Fixed variable	**Estimate**	**SE**	**t**	**p**
Sampling round (after vs before)	0.401	0.077	5.219	**<0.001**
Long-term treatment (disturbance)	-0.373	0.183	-2.038	**0.049**
Sampling round x Long-term treatment (disturbance)	*-0.195*	*0.150*	*-1.301*	*0.210*

Sample size	
Group	**n**
Before long-term treatment	24
After long-term treatment	33

Table S3: Effect of disturbance treatment on change in testosterone in response to handling. SE = standard error.

Linear mixed model

Fixed variable	Estimate	SE	t	p
Acute stressor (handling)	-0.702	0.207	-3.395	**0.003**
Long-term treatment (disturbance)	-0.714	0.251	-2.846	**0.008**
Acute stressor (handling) x long-term treatment (disturbance)	0.669	0.296	2.265	**0.034**

Sample size

Group	n
Long-term control, unhandled baseline	16
Long-term control, acute handling stress	14
Long-term disturbance, unhandled baseline	17
Long-term disturbance, acute handling stress	12

Table S4: Effect of disturbance treatment on testosterone increase in response to GnRH injection. SE = standard error. Interaction (in italic font) was not significant and was taken out of the final model.

Linear mixed model

Fixed variable	Estimate	SE	t	p
GnRH injection	1.941	0.214	9.077	**<0.001**
Long-term treatment (disturbance)	0.016	0.266	0.059	0.953
GnRH injection x Long-term treament (disturbance)	*0.016*	*0.435*	*0.037*	*0.971*

Sample size

Group	n
Long-term control, pre-injection	14
Long-term control, post-injection	16
Long-term disturbance, pre-injection	12
Long-term disturbance, post-injection	18

Table S5: Relationship between baseline testosterone and testes mass. SE = standard error. Interactions that were not significant (in italic font) were taken out of the final model.

Linear model					**Sample size**	
Variable	**Estimate**	**SE**	**t**	**p**	**Group**	**n**
	Baseline testosterone					
Testes mass	7.120	7.500	0.949	0.352	Long-term control	13
Long-term treatment (disturbance)	-0.513	0.255	-2.010	0.056	Long-term disturbance	14
Time of sampling	0.004	0.002	2.692	**0.013**		
Testes mass x Long-term treatment (disturbance)	*22.128*	*14.623*	*1.513*	*0.144*		
	Post-handling testosterone					
Testes mass	12.393	4.367	2.838	**0.010**	Long-term control	13
Long-term treatment (disturbance)	0.051	0.153	0.333	0.742	Long-term disturbance	12
Testes mass x Long-term treatment (disturbance)	*1.311*	*8.935*	*0.147*	*0.885*		
	GnRH-induced testosterone					
Testes mass	34.627	11.072	3.127	**0.004**	Long-term control	12
Long-term treatment (disturbance)	-0.189	0.364	-0.518	0.608	Long-term disturbance	14
Testes mass x Long-term treatment (disturbance)	*23.579*	*22.439*	*1.051*	*0.302*		

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Table S6. Significantly differentially expressed genes in response to chronic disturbance treatment (excel file available separately).

Click here to Download Table S6

Table S7. Significantly differentially expressed genes in response to acute handling treatment (excel file available separately).

Click here to Download Table S7

Table S8. Correlation coefficients between genes involved in steroidogenesis. Coefficients are below the diagonal, p-values are above the diagonal.

Gene	*LHR*	*StAR*	*P450*	*CYP17*	*3βHSD*
LHR		0.004	0.251	0.024	0.438
StAR	0.48		0.002	0.017	0.121
P450	0.20	0.50		0.001	0.001
CYP17	0.38	0.40	0.54		0.301
3βHSD	0.14	0.27	0.56	0.18	

Table S9. Variance and loadings of principal components for genes involved in testosterone synthesis

	PC1	PC2	PC3	PC4	PC5
Proportion of variance explained	0.498	0.209	0.138	0.103	0.052
Standard deviation	1.578	1.021	0.831	0.719	0.508
Genes			*Loadings*		
LHR	-0.377	0.601	-0.437	0.474	-0.285
StAR	-0.486	0.234	-0.260	-0.741	0.304
P450	-0.520	-0.370	0.241	-0.154	-0.715
CYP17	-0.461	0.195	0.707	0.285	0.411
3βHSD	-0.372	-0.640	0.429	0.349	0.382

Table S10. Variance and loadings of principal components for genes involved in testosterone synthesis from animals in the control treatment only.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Proportion of variance explained	0.411	0.213	0.195	0.124	0.057
Standard deviation	1.434	1.033	0.987	0.787	0.532
Genes			*Loadings*		
LHR	-0.486	0.612			0.619
StAR	-0.536	0.240	0.422	-0.342	-0.600
P450	-0.394	-0.657		-0.541	0.337
CYP17	-0.351		-0.841	0.166	-0.376
3βHSD	-0.445	-0.370	0.317	0.750	

Table S11. Effect of acute handling treatment on steroidogenesis gene expression in birds from the long-term control treatment (acute handling treatment n=8; unhandled controls n=9). Positive coefficient indicates higher gene expression in unhandled birds. SE = standard error

Gene	n	Coefficient	SE	t	p value	adjusted p value
LHR	17	0.202	0.142	1.424	0.175	0.438
StAR	17	0.132	0.170	0.777	0.449	0.562
P450	17	0.117	0.200	0.583	0.568	0.568
CYP17	17	0.163	0.077	2.102	0.053	0.265
3βHSD	17	0.133	0.131	1.015	0.326	0.544

Table S12. Effect of chronic and acute handling treatment of *GR*, *MR*, and *GnIHR* (sample sizes: chronic disturbance & acute handling n=9; chronic disturbance & unhandled control n=9; chronic control & acute handling n=9; chronic control & unhandled control n=8). SE = standard error. Interactions that were not significant (in italic font) were taken out of the final model.

Fixed effect	coefficient	SE	t	p value	adjusted p value
			GR		
Chronic treatment (disturbance)	-0.057	0.060	-0.956	0.346	0.5192
Acute treatment (unhandled)	0.137	0.060	2.282	0.029	0.0879
Chronic x acute treatment	*0.006*	*0.122*	*0.050*	*0.960*	*na*
			MR		
Chronic treatment (disturbance)	-0.067	0.066	-1.022	0.315	0.5192
Acute treatment (unhandled)	0.037	0.066	0.556	0.582	0.5822
Chronic x acute treatment	*-0.127*	*0.132*	*-0.963*	*0.343*	*na*
			GnIHR		
Chronic treatment (disturbance)	0.058	0.140	0.413	0.682	0.6822
Acute treatment (unhandled)	-0.262	0.140	-1.875	0.070	0.1049
Chronic x acute treatment	*0.418*	*0.274*	*1.524*	*0.138*	*na*

Figure S1: Effect of chronic disturbance and acute handling stressor on the expression of genes involved in steroidogenesis; A) *LHR*, B) *StAR*, C) *P450*, D) *CYP17*, E) *3βHSD*. Shown are group means (open circles) +/- standard error; points represent individual measurements.

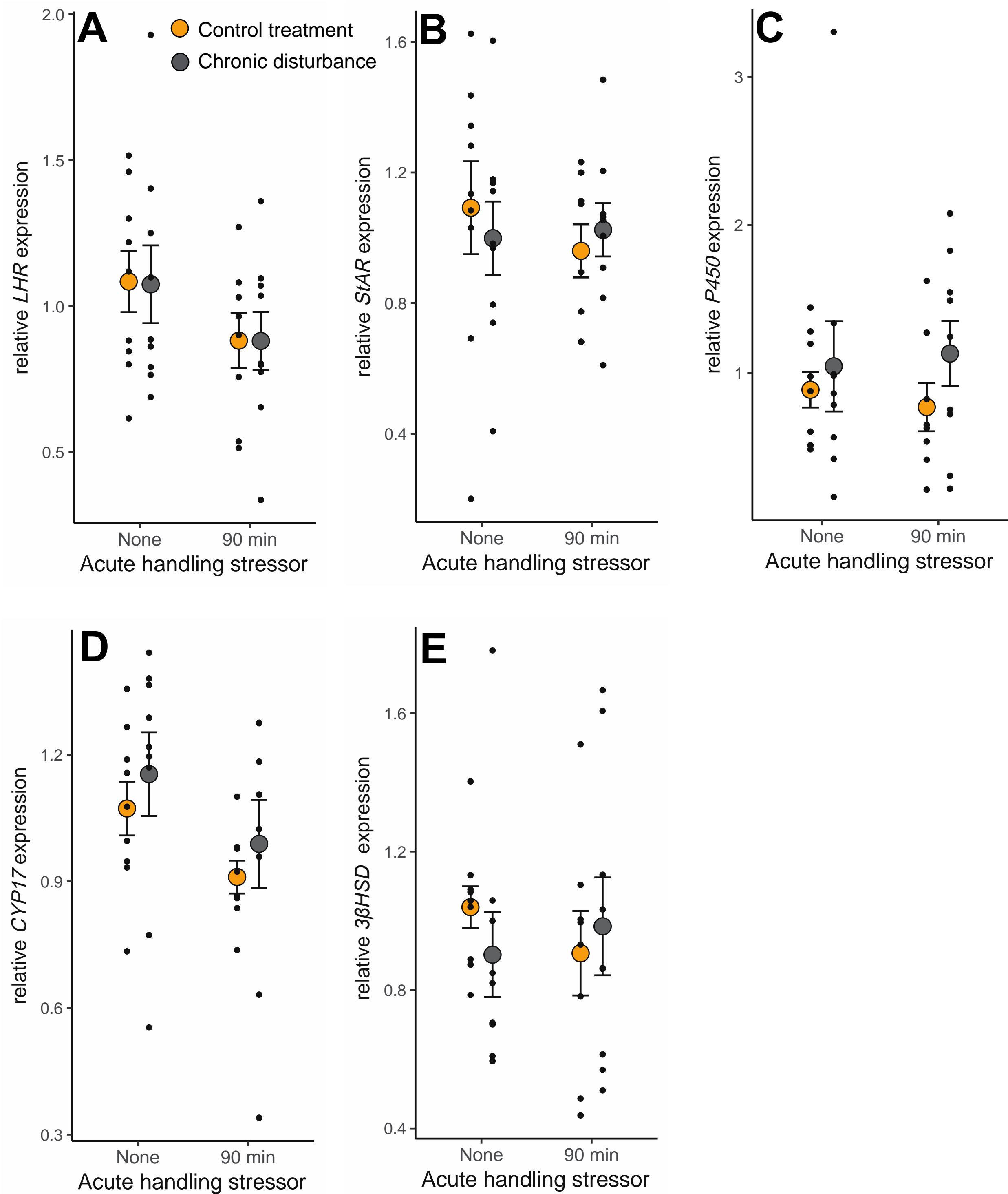
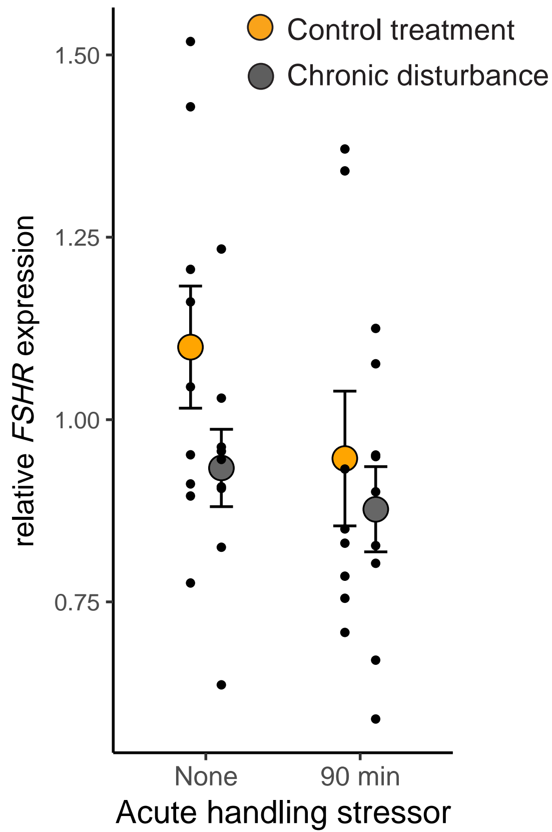


Figure S2: Effect of chronic disturbance and acute handling stressor on the expression of *FSHR*. Shown are group means (open circles) +/- standard error; points represent individual measurements.



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