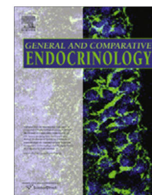
General and Comparative Endocrinology 169 (2010) 82–90

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: Potential site of action and mechanism

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ARTICLE INFO

Article history:
Received 21 March 2010
Revised 13 July 2010
Accepted 22 July 2010
Available online 29 July 2010

Keywords:
Aimophila
GnRH
Gonadotropin
NMA
N-methyl-D,L-aspartate
Passerine
Pituitary gland
Rufous-winged sparrow
Sonoran desert
Testis

ABSTRACT

We used a free-ranging, seasonally breeding adult male songbird, the rufous-winged sparrow, *Aimophila carpalis*, to investigate the effects of acute stress-induced by capture followed by restraint, on the hypothalamo-pituitary–testicular axis. Intra- and interindividual comparisons revealed that males decreased their plasma testosterone (T) by 37–52% in response to acute stress. The decrease occurred within 15 min of capture and persisted for at least another 15 min. Within 15 min, the decrease in plasma T was not associated with a reduction in plasma luteinizing hormone (LH). Thirty minutes after capture and restraint, the decrease in plasma T either was likewise not associated with decreased plasma LH (intra-individual comparison) or concurred with a reduction in plasma LH (interindividual comparison). These observations indicate that effects of stress may have been mediated at the pituitary gland and also directly at the testicular levels. To address this question, we measured the hormonal response to an injection of the glutamate receptor agonist *N*-methyl-D,L-aspartate (NMA) to stimulate to stimulate the release of gonadotropin-releasing hormone (GnRH) or of GnRH to stimulate the release of LH. Treatment with NMA did not change plasma LH, presumably because the birds were in breeding condition and already secreting GnRH at a maximum rate. Administration of GnRH increased plasma LH equally in birds that were or were not stressed before the treatment. An injection of purified ovine LH (oLH) increased plasma T equally in birds that were or were not acutely stressed before the hormone injection. Thus, the observed acute stress-induced decrease in plasma T was apparently not mediated by decreased responsiveness of the pituitary gland to GnRH or of the testes to LH. Decreased plasma T following stress may involve a direct impairment of the testicular endocrine function.

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1. Introduction

It has long been recognized that stressful events can disrupt reproductive functions (Hardy et al., 2005; Wingfield and Sapolsky, 2003; Deviche, 1983). One characteristic physiological response to stress is the activation of the hypothalamo-pituitary–adrenal axis that results in increased plasma glucocorticoids, generally cortisol or corticosterone (CORT), depending on the species (Norris, 2007). Stress in mammals exerts complex effects on the hypothalamo-pituitary–gonadal (HPG) axis. At the level of the hypothalamus, stress or glucocorticoids decrease the production of gonadotropin-releasing hormone (GnRH; Tanebe et al., 2000; Clarke and Pompolo, 2005; Calogero et al., 1999) and increase the production of a RFamide-related neuropeptide (ortholog to avian gonadotropin-inhibitory hormone: GnIH; Kirby et al., 2009). At the level of the pituitary gland, a study found in the male rat that glucocorticoids *in vitro* do not influence the basal secretion of gonadotropins by pituitary gland cells, but increase the responsiveness of luteinizing hormone- (LH) and follicle-stimulating hormone- (FSH) secreting cells to GnRH, and the cell content of these hormones (Suter and Schwartz, 1985). By contrast, another study found that the *in vivo* or *in vitro* administration of a glucocorticoid receptor agonist reduces the response of LH cells to GnRH (Briski and Sylvester, 1991). At the level of the gonads, mammalian Leydig cells have been shown to contain glucocorticoid receptors (GR; Ge et al., 1997; Hu et al., 2008; Martin and Tremblay, 2008; Stalker et al., 1991; Weber et al., 2000) and these may mediate direct inhibitory effects of glucocorticoids on testosterone (T) production without affecting plasma LH (Orr and Mann, 1992; Hardy et al., 2005). Consistent with this view, *in vitro* treatment of rodent Leydig cells with CORT decreases T production (Dong et al., 2004; Monder et al., 1994a). An additional mechanism

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doi:10.1016/j.ygcen.2010.07.009

may operate at the level of the gonads to mediate a direct inhibitory effect of stress on reproductive function. As shown in the rat, the mammalian testes are innervated by an inhibitory neural pathway originating in the brain that is rapidly activated in response to stress (James et al., 2008). In mammals, stress or glucocorticoids can, therefore, inhibit reproductive functions by a direct action on the gonads through mechanisms that do not involve either the GnRH system or the pituitary gland.

In birds, as in mammals, chronic stress or long-term exposure to CORT can inhibit the reproductive system (Schoech et al., 2009; Cyr and Romero, 2007; Marin and Satterlee, 2004; Salvante and Williams, 2003; Deviche and Hendrick, 1981). However, limited information exists on effects of acute stress on avian reproductive hormones. Plasma LH has been reported to decrease in response to serial bleeding over a 2-h long period in the chicken, *Gallus domesticus* (Wilson and Sharp, 1975), but not following 2 h of immobilization in the turkey, *Meleagris gallopavo* (El Halawani et al., 1985). In the male Gambel's white-crowned sparrow, *Zonotrichia leucophrys gambelii*, sampled during the breeding period, plasma LH is not affected by capture and handling for 1 h, but plasma T levels are negatively correlated with time after capture (Wingfield et al., 1982). A more complex picture emerged from a study on semipalmated sandpipers, *Calidris pusilla* (Gratto-Trevor et al., 1991). In this species plasma T in response to capture and handling increases or decreases depending upon whether plasma T is initially low or high, respectively. Similarly, plasma T increases in response to repeated blood collection in some roosters, but decreases in others (Wilson et al., 1979). As in mammals, the avian testes contain glucocorticoid receptors (Kwok et al., 2007) and consequently stress may also directly inhibit gonadal function in birds. However, with the exception of a study on the white-crowned sparrow (Wingfield et al., 1982), there are no reports in birds measuring the responses of both plasma gonadotropin and gonadal steroids to stress in the same study.

We addressed this issue using free-ranging adult male rufous-winged sparrows, *Aimophila carpalis*, a seasonal flexible breeder in which plasma LH and T increase in response to factors associated with local summer precipitation (Deviche et al., 2006a; Small et al., 2007). A first objective of the study was to determine effects of acute stress-induced by capture and restraint on plasma LH and T. A second objective was to determine where along the HPG axis stress influences the secretion of reproductive hormones. For this, we treated birds either with *N*-methyl-D,L-aspartate (NMA), a centrally acting neuroexcitatory glutamate receptor agonist that stimulates GnRH secretion (Lee et al., 1993; Bonavera et al., 1998; Yin et al., 2007), with GnRH, or with LH, in each case before or after a standard period of restraint. We reasoned that this approach would enable us to evaluate the effects of restraint at the hypothalamic (LH response to NMA treatment), pituitary gland (LH response to GnRH treatment), and testicular (T response to LH treatment) levels of the HPG axis. We predicted that if acute stress attenuates the GnRH response to NMA treatment or the LH response to GnRH treatment, plasma LH would increase less in birds receiving these treatments after than before restraint. If stress decreases the testicular sensitivity to LH, we predicted that plasma T would increase less in sparrows receiving LH after than before restraint.

2. Methods

2.1. Study location and bird capture

The effect of capture and restraint on plasma CORT was determined in 14 adult male rufous-winged sparrows caught at the Santa Rita Experimental Range (SRER; 31°42′N; 110°56′ W; Pima Co., AZ, USA) on 21 July ($n = 7$) and 5 August ($n = 7$) 2005. This study was part of an investigation aimed at determining seasonal changes in plasma baseline and stress-induced CORT over a 15-month long period in birds of the same population (Deviche et al., in preparation). As this investigation found, adult male rufous-winged sparrows rapidly increase their plasma CORT secretion in response to capture and restraint throughout the year and the magnitude of this increase is independent of the reproductive condition.

All the other studies used a total of 114 adult male rufous-winged sparrows caught at the SRER in summer 2009 (see below for details). Plasma T and LH in male rufous-winged sparrows increase during the summer in association with the seasonal monsoon, and birds in mid-July to mid-August are in full breeding condition, with males having seasonally elevated plasma LH and T (Deviche et al., 2006a; Small et al., 2007). As the onset of the monsoon varies from one year to another, the timing of peak plasma hormone levels also varies interannually (Deviche et al., 2006a). To circumvent potential confounding effects of this variation and of within-season changes in hormone levels, we conducted studies during a same breeding season (summer 2009) and over a short period (six consecutive days: 28 July to 2 August). Sparrows caught in 2009 for plasma LH and T analysis were, therefore, sampled at the same time of the year as sparrows caught in 2005 for plasma CORT analysis.

Sparrows were caught individually using Japanese mist nets and conspecific song playbacks, and between 06:05 AM and 15:20 PM (Pacific Standard Time; local sunrise and sunset times: 05:40 AM and 19:20 PM). Song playback duration before capture varied from less than 1 min to approximately 30 min and we previously found no association between this duration and plasma T or CORT (Deviche et al., 2006a; Deviche, personal observation). Sex and age were determined by behavior (singing in males only) and morphology (developed cloacal protuberance in adult males only; incubation patch in adult females only; Pyle, 1997).

2.2. Blood sample collection

Blood (each sample = 0.1 ml) was collected from the jugular vein using heparinized syringes. Two blood samples (total: 0.2 ml/bird, corresponding to 1.4% of the average body mass (15 g)) were collected from each bird (see below for details). Samples were placed on ice until centrifuged later the same day. Plasma was harvested using a Hamilton glass syringe and frozen until assayed for CORT, LH, or T (see below).

After the second sample collection, each sparrow received an intramuscular (i.m.; pectoral muscle) injection of saline solution (0.9% NaCl in water) to compensate for the volume of blood removed and then a uniquely numbered US Geological Survey aluminum tarsal band, and it was released on site. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and were conducted under appropriate scientific collecting permits issued by the Arizona Game and Fish Department and the US Fish and Wildlife Service.

2.3. Experimental groups

2.3.1. Control groups

Control groups included all the sparrows caught in 2005 and 16 birds caught in 2009. A first blood sample was collected within 3 min of capture (= *Unstressed* condition). Sparrows were then placed into a breathable cloth bag for 30 min and bled again (= 30 min *stress* condition). Samples from birds caught in 2005 were assayed for CORT and samples from birds caught in 2009 were assayed for LH or T. The relatively small size of rufous-winged sparrows makes it impossible to collect enough blood from a same bird on a same day to measure both LH and T concentrations.

Therefore, *Control* birds in 2009 were randomly divided into two groups: plasma samples from 8 birds were assayed for LH and samples from the other 8 birds were assayed for T.

2.3.2. NMA-, GnRH-, and LH-treated groups

Depending on treatment, birds either were bled within 3 min of capture (= *Unstressed* groups) or were placed into a breathable cloth bag immediately after capture for 30 min before the first bleed (= 30 min *stress* groups; see Fig. 1 for depiction of the experimental design). In both cases the first bleed (= pre-injection; *Preinj*) was followed within 2 min with an injection of NMA, GnRH, oLH or control saline solution (= *Saline* groups), and then with a second bleed (= post-injection (*Postinj*); see below for details). NMA, GnRH, and oLH were dissolved in saline solution so that the injection volume was in all cases equal to 0.1 ml. Plasma samples from the NMA and GnRH treatment groups were assayed for LH; samples from the LH treatment groups were assayed for T.

2.3.2.1. NMA-treated groups. After the *Preinj* bleed, sparrows immediately received an i.m. injection either of NMA (0.9 mg, corresponding to 60 mg/kg body mass; Sigma Chemicals Co., MO, USA; *Unstressed*: $n = 8$; 30 min *stress*: $n = 8$) or of saline solution (*Unstressed*: $n = 8$; 30 min *stress*: $n = 8$) and were bled 15 min later. This dose of NMA is higher than used in a previous study on white-crowned sparrows (Meddle et al., 1999). It is, however, comparable to that administered to European starlings, *Sturnus vulgaris* (Dawson, 2005) and Cassin's sparrows, *Aimophila cassinii* (Deviche et al., 2008). In these species, the effect of NMA administration on plasma LH varied as a function of the reproductive condition, with birds being less sensitive to the agonist when in non-breeding than in breeding condition.

2.3.2.2. GnRH-treated groups. After the *Preinj* bleed, sparrows immediately received an intrajugular injection either of chicken GnRH-I (10 ng, corresponding to 0.67 μg/kg body mass; Sigma Chemical Co., MO, USA; *Unstressed*: $n = 8$; 30 min *stress*: $n = 8$) or of saline solution (*Unstressed*: $n = 8$; 30 min *stress*: $n = 9$) and they were bled 2 min later. This protocol is identical to that used by Deviche et al. (2006a). In this study, an injection of GnRH elevated plasma LH in free-ranging rufous-winged sparrows within 2 min.

2.3.2.3. oLH-treated groups. After the *Preinj* bleed, sparrows immediately received an intrajugular injection either of purified ovine LH (oLH; 3.75 μg, corresponding to 250 μg/kg body mass; National Hormone and Peptide Program, Harbor-UCLA Medical Center, CA, USA; *Unstressed*: $n = 9$; 30 min *stress*: $n = 8$) or of saline solution (*Unstressed*: $n = 8$; 30 min *stress*: $n = 8$) and they were bled 15 min later. Previous work demonstrated that the *in vitro* potency of oLH to stimulate avian testicular T secretion is similar to that of avian LH (Chase, 1982).

2.4. Hormone assays

Plasma CORT was assayed using an enzyme-linked immunoassay (AssayDesigns, Inc., Ann Arbor, MI, USA) according to the manufacturer's specifications. Plasma was diluted 8× in assay buffer before dispensing into assay wells. Samples were part of a large assay ($n = 220$ samples) involving six assay plates and they were randomly assigned to one of these plates. A plasma dilution curve was parallel to a standard curve run on the same plate. The primary antibody used in the assay has less than 5% crossreactivity with progesterone, aldosterone, T, and 17β-estradiol (manufacturer's specifications). The intra-assay coefficient of variation was 2.26%, the interassay coefficient of variation was 9.03% (two samples assayed on each plate), and the minimum detectable dose ranged from 9 to 470 pg/ml depending on the plate.

Plasma T was also measured by a validated enzyme-linked immunoassay (AssayDesigns, Inc., Ann Arbor, MI, USA; Deviche et al., 2006a) according to the manufacturer's specifications. Plasma was diluted 10× in assay buffer before dispensing into assay wells. Samples were assigned randomly to one of four assay plates except that samples from a same sparrow were assayed on a same plate. The primary antibody used in the assay has less than 5% crossreactivity with 17β-estradiol, dihydrotestosterone, CORT, and progesterone (manufacturer's specifications). The intra-assay coefficient of variation was 2.6% ($n = 90$ samples), the inter-plate coefficient of variation was 7.7% ($n = 2$ samples assayed on each plate), and the average minimum detectable dose was 1.62 pg/ml, as calculated using the manufacturer's recommendation.

Plasma LH concentrations were determined using a micromodification of the radioimmunoassay described previously (Sharp et al., 1987). The assay has been previously validated for use in rufous-winged sparrows (Deviche et al., 2006a). Briefly, the reaction volume was 60 μl, comprising 20 μl of plasma sample or standard, 20 μl of primary rabbit LH antibody, and 20 μl of I^{125}-labeled LH. The primary antibody was precipitated to separate free and bound I^{125} label using 20 μl of donkey anti-rabbit precipitating serum and 20 μl of non-immune rabbit serum. All samples were measured in

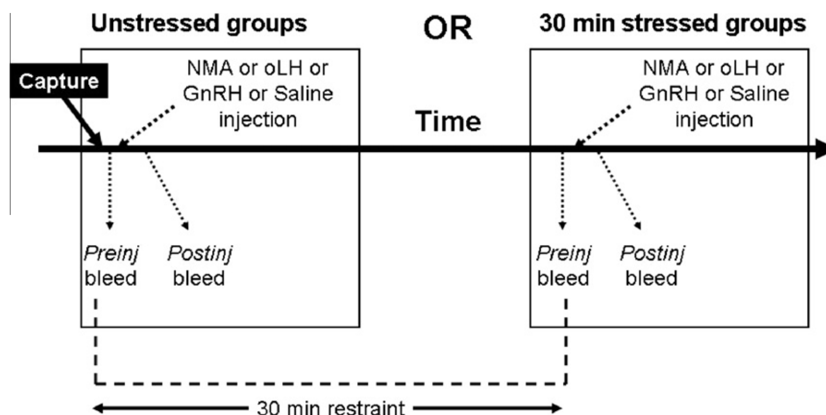

Fig. 1. Experimental design used to determine the effect of an injection of *N*-methyl-D,L-aspartate (NMA) or of gonadotropin-releasing hormone (GnRH) on plasma luteinizing hormone (LH), or of an ovine LH (oLH) injection on plasma testosterone, in adult male rufous-winged sparrows, *Aimophila carpalis*. Birds were bled (*Preinj* bleed) immediately after capture, injected with one of the above agents or with saline solution, and then bled again 2 min (GnRH treatment) or 15 min (NMA and oLH treatments) later (*Postinj* bleed). Other sparrows underwent the same experimental treatments after restraint for 30 min to determine the effect of acute stress on the responsiveness to NMA, GnRH, or oLH administration. See text for additional information.

a single assay. The intra-assay coefficient of variation was 7.9% and the minimum detectable dose was 0.09 ng/ml.

2.5. Statistical analyses

The effects of sampling date and stress (*Unstressed* vs. 30 min *stress*) on plasma CORT in sparrows sampled in 2005 were analyzed using two-way analysis of variance (ANOVA) for repeated measures. Data were normalized before analysis by transformation into square root.

We analyzed changes in plasma LH and T in response to capture and 30 min of restraint through intraindividual as well and *interindividual* comparisons. For intraindividual comparisons, we compared hormone levels immediately after capture and 30 min later in *Control* sparrows (Section 2.3.1; $n = 8$ birds for each hormone) using paired Student's *t*-tests. For *interindividual* comparisons of LH data, we combined *Preinj* data from *Unstressed* NMA and GnRH treatment groups (NMA, GnRH, and 2 *Saline* groups: $n = 33$; Section 2.3.2) and compared these data with *Preinj* data from the corresponding 30 min *stress* group (id: $n = 32$) using an unpaired Student's *t*-test. Plasma LH values were not normally distributed and were ranked before analysis (Conover and Iman, 1981). For *interindividual* comparison of T data, we likewise used an unpaired Student's *t*-test to compare *Preinj* plasma T in *Unstressed* oLH treatment groups (oLH and *Saline*; $n = 17$) with data from the corresponding 30 min *stress* groups (oLH and *Saline*: $n = 16$).

Over the 6-day long period of sampling, birds in 2009 were caught between 6 AM and 3:30 PM (Section 2.1). We examined whether plasma baseline LH and T during this period undergo a diurnal cycle which, if this were the case, would require inclusion of this factor in the analysis of the experimental treatment effects. To this goal, we used linear regression to analyze the relationship between time of day and plasma baseline LH (*Unstressed* data from *Control* birds ($n = 8$) plus *Preinj* data from *Unstressed* NMA and GnRH groups (NMA, GnRH, and two corresponding *Saline* groups: $n = 32$; total $n = 40$). We performed a similar analysis to determine the relationship between time of day and plasma baseline T (*Unstressed* data from *Control* birds ($n = 8$) plus *Preinj* data from *Unstressed* oLH and corresponding *Saline* groups ($n = 17$); total $n = 25$).

We used multiple factor analyses of variance (MANOVA) to determine effects of NMA or GnRH injection on plasma LH, and effects of oLH injection on plasma T. Plasma LH data for these analyses were not normally distributed and were log-transformed before analysis. Independent factors included in the MANOVAs included stress (*Unstressed* vs. 30 min *stress*), treatment (NMA, GnRH, or oLH vs. corresponding *Saline* group), time (*Preinj* vs. *Postinj*), and interactions between these variables. The average capture times of birds in the four GnRH treatment groups did not differ (Kruskal–Wallis analysis of variance on ranks: $p = 0.067$), but there were differences between average times of capture of birds in the four NMA groups (id, $p < 0.001$). These times varied between 07:20 AM (30 min *stress*, NMA injection group) and 14:00 PM (*Unstressed*, *Saline* group). To account for this variation, we included individual capture time as a covariate in the statistical MANOVA model used to analyze plasma LH of the four NMA treatment groups.

3. Results

3.1. Effect of capture and restraint on plasma corticosterone

Plasma CORT increased in response to capture and restraint (ANOVA: $F_{1,27} = 127.51$, $p < 0.001$; Fig. 2). Hormone levels did not differ between birds caught on 25 July and 5 August and there was no statistical interaction between stress and sampling date.

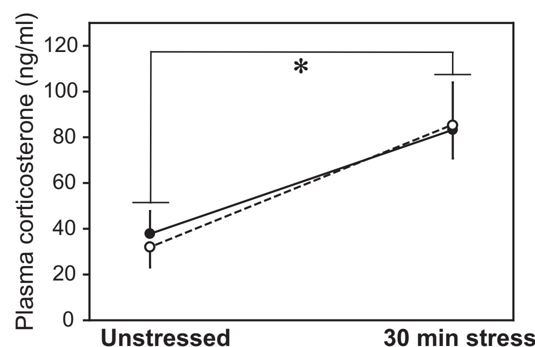

Fig. 2. Plasma corticosterone (means ± SE) of free-ranging adult male rufous-winged sparrows, *Aimophila carpalis*, sampled within 3 min of capture (Unstressed) and after 30 min of restraint (30 min stress) on 21 July (black circles; $n = 7$) and 5 August (white circles; $n = 7$) 2005. $^{*}p < 0.001$.

3.2. Daily change in plasma hormone concentrations

Plasma baseline LH concentrations increased during the daily period of sampling (linear regression: coefficient of determination: $r^2 = 0.134$, $p = 0.020$; $n = 40$). Close examination of the data, however, revealed that the data set used for this analysis included one statistical outlier (plasma LH: 6.81 ng/ml; group average: 2.80 ± 0.23 ng/ml; Chauvenet's criterion outlier test: $p < 0.05$). When this outlying value was excluded, no daily change in plasma baseline LH was apparent ($r^2 = 0.086$, $p = 0.069$; $n = 39$).

A similar analysis for plasma baseline T revealed no daily change in hormone level ($r^2 = 0.018$, $p = 0.17$; $n = 25$).

3.3. Effect of acute stress on plasma LH and testosterone

3.3.1. Intraindividual comparison: effect within Control groups

Within individuals, plasma LH after 30 min of restraint did not differ from pre-restraint level (9.7% increase; $p > 0.2$; Fig. 3A). However, plasma T was significantly lower after than before restraint (31.4% decrease; $t = 2.999$, df = 8: $p = 0.020$; Fig. 3A).

3.3.2. Interindividual comparison: effect across Control NMA, GnRH, and oLH treatment groups

Across individuals, *Preinj* plasma LH and T decreased by 29.6% and 52.5%, respectively, in response to capture and restraint for 30 min (30 min *stress* vs. corresponding *Unstressed* groups: LH: $t = 2.334$, df = 63: $p = 0.023$; T: $t = 3.352$, df = 31: $p = 0.002$; Fig. 3B).

3.4. Effect of NMA injection on plasma LH

Average capture times of NMA group birds differed (Table 1) and when this variable was accounted for in the MANOVA model, treatment with NMA did not change the concentration of plasma LH (Fig. 4 and Table 1). There was no effect of capture and restraint on the concentration of LH or on the change in plasma LH over the 15 min separating the *Preinj* and *Postinj* bleeds, and no interaction between these variables.

3.5. Effect of GnRH injection on plasma LH

Plasma LH changed in the four GnRH treatment groups between the *Preinj* and *Postinj* bleeds ($p = 0.0017$). An increase in plasma LH was a function of whether or not birds received GnRH (treatment × time interaction: $p < 0.001$: Fig. 5 and Table 1). As was the case for NMA, restraint did not alter plasma LH and there was no interaction between restraint and the increase in LH observed after GnRH treatment (Table 1).

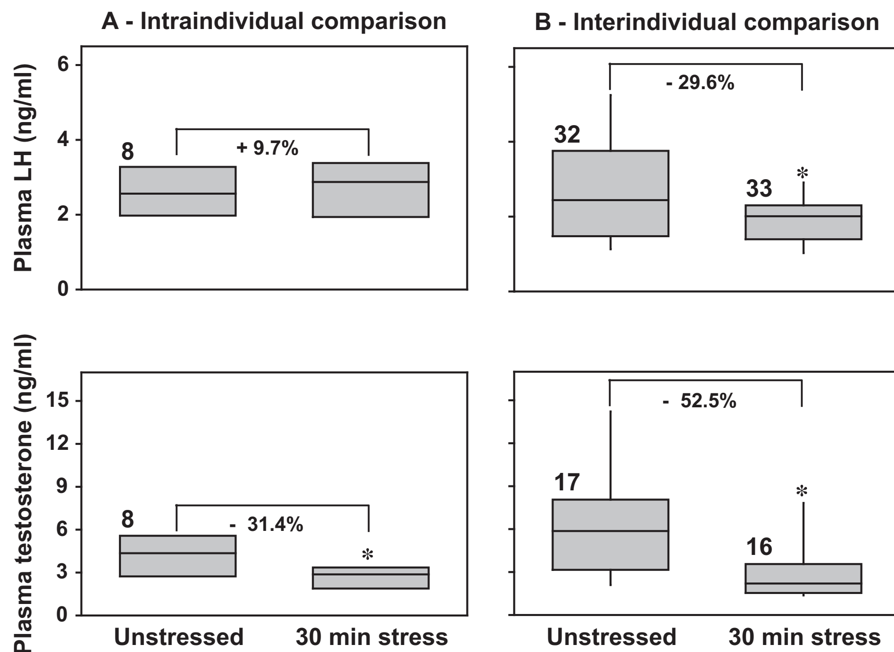

Fig. 3. Plasma luteinizing hormone (LH) and testosterone of free-ranging adult male rufous-winged sparrows, *Aimophila carpalis*, sampled within 3 min of capture (Unstressed) and again after 30 min (30 min stress) of restraint. The box plots show, for each group, the median and 25% and 75% percentiles. (B) also shows 10% and 90% percentiles. Data are from two independent studies: (A) intraindividual comparison: same birds sampled before and after capture; (B) interindividual comparison: different birds sampled before and after capture. Sample sizes are indicated above columns. $^{*}p < 0.05$, paired (A) or unpaired (B) Student's *t*-test.

Table 1
Multiple analysis of variance (MANOVA) results showing the effects in adult male rufous-winged sparrows, *Aimophila carpalis*, of an injection of *N*-methyl-D,L-aspartate (NMA), gonadotropin-releasing hormone (GnRH), ovine LH (oLH) or control saline on plasma LH (NMA and GnRH treatments) or testosterone (oLH treatment), The table presents *F* values and corresponding probabilities for the effects of each treatment given immediately after capture or 30 min later (stress effect). Plasma hormone levels were compared in samples collected from each bird before (*Preinj*) or after (*Postinj*; time effect) an injection of hormone or of control saline solution (treatment effect), Time of day that a bird was captured was factored into the NMA MANOVA, See text for additional details,

Factor	NMA			GnRH			oLH		
	DF	*F* value	Probability	DF	*F* value	Probability	DF	*F* value	Probability
(Intercept)	1,29	95.845	<0.0001	1,29	657.214	<0.0001	1,29	148.692	<0.0001
Treatment	1,28	0.209	n.s.	1,29	15.638	0.0005	1,29	5.638	0.0244
Stress (Unstressed vs. 30 min stress)	1,28	0.875	n.s.	1,29	1.536	n.s.	1,29	8.888	0.0058
Time (*Preinj* vs. *Postinj*)	1,29	3.674	n.s,	1,29	11.972	0.0017	1,29	21.977	0.0001
Capture time	1,28	7.484	0.0107	n.t.			n.t.		
Stress × treatment	1,28	1.418	n.s.	1,29	1.575	n.s.	1,29	0.321	n.s.
Stress × time	1,29	1.369	n.s.	1,29	0.568	n.s.	1,29	2.435	n.s.
Treatment × time	1,29	1.859	n.s.	1,29	29.712	<0.0001	1,29	87.464	<0.0001
Stress × treatment × time	1,29	1.341	n.s.	1,29	1.680	n.s.	1,29	0.534	n.s.

DF, degrees of freedom; n.s., not significant ($p > 0,05$); n.t,, not tested.

3.6. Effect of oLH injection on plasma T

Capture and restraint for 30 min decreased plasma T (Stress factor effect: $p = 0.006$; Fig. 6 and Table 1). The lack of statistical interaction between stress and other variables under study indicates that this decrease occurred independently of whether birds received oLH.

Plasma T changed over the 15 min separating the *Preinj* and *Postinj* bleeds (time factor effect: $p = 0.0001$; Table 1), but the magnitude of this change depended upon whether sparrows were oLH- or control vehicle-treated (treatment × time interaction: $p < 0.0001$). Sparrows receiving an injection of oLH increased plasma T by 189% (*Unstressed*) and 302% (30 min *stress*). By contrast, plasma T in *Saline* groups decreased by 38% (*Unstressed*) and 27% (30 min *stress*; Fig. 6), thus demonstrating a rapid (within 15 min of capture/vehicle injection/restraint) inhibitory effect of acute stress on this hormone.

4. Discussion

Effects of stress on avian reproduction have been investigated extensively (e.g., Cyr and Romero, 2007; Ellenberg et al., 2007; Marin and Satterlee, 2004; Salvante and Williams, 2003), but the mechanisms mediating these effects and their time course are incompletely understood. To determine if acute stress inhibits the secretion of LH and T, we compared plasma levels of these hormones prior to and in response to acute non-invasive stress for 15 or 30 min. Capture, injection of vehicle solution, and then restraint for 15 min decreased plasma T (*Saline* control groups, Fig. 6) but not LH (*Saline* control groups, Fig. 4). The response to capture and restraint for 30 min was more complex than when birds were exposed to this stress for 15 min. During one study, a 30-min long period of restraint after capture reduced plasma T but not LH (intraindividual comparison; Fig. 3A). In another study, however,

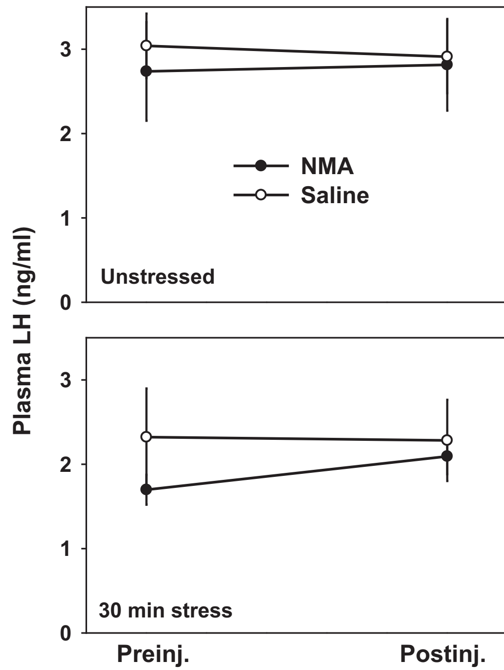

Fig. 4. Plasma luteinizing hormone (LH; mean ± SE; $n = 8$ per group) of free-ranging adult male rufous-winged sparrows, *Aimophila carpalis*, immediately before (*Preinj*) and 15 min after (*Postinj*) an intramuscular injection of *N*-methyl-D,L-aspartate (NMA; 60 mg/kg body mass) or of control saline solution. Birds either were bled (*Preinj*) and then injected within 3 min of capture (Unstressed) or were restrained for 30 min before the initial bleed and an injection (30 min stress).

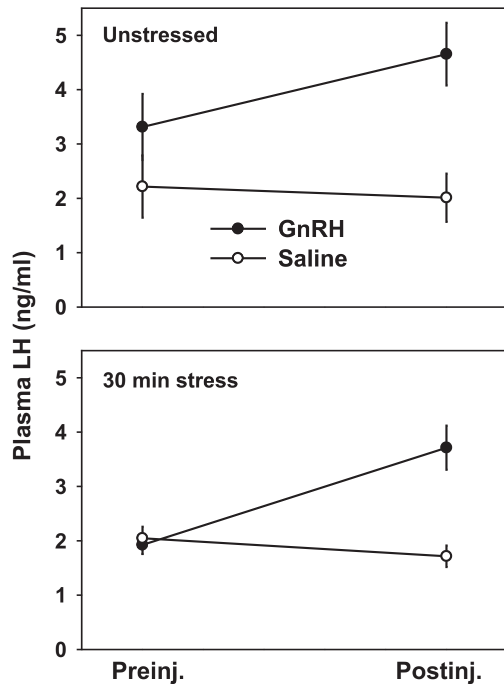

Fig. 5. Plasma luteinizing hormone (LH; means ± SE; $n = 8$ or 9 per group) of free-ranging adult male rufous-winged sparrows, *Aimophila carpalis*, immediately before (*Preinj*) and 2 min after (*Postinj*) an intravenous injection of gonadotropin-releasing hormone (GnRH; 0.67 µg/kg body mass) or of control saline solution. Birds either were bled (*Preinj*) and then injected within 3 min of capture (Unstressed) or were restrained for 30 min before the initial bleed and an injection (30 min stress).

this manipulation decreased the plasma concentrations of both hormones (interindividual comparison; Fig. 3B). To investigate the site(s) mediating the stress-induced inhibition, we measured

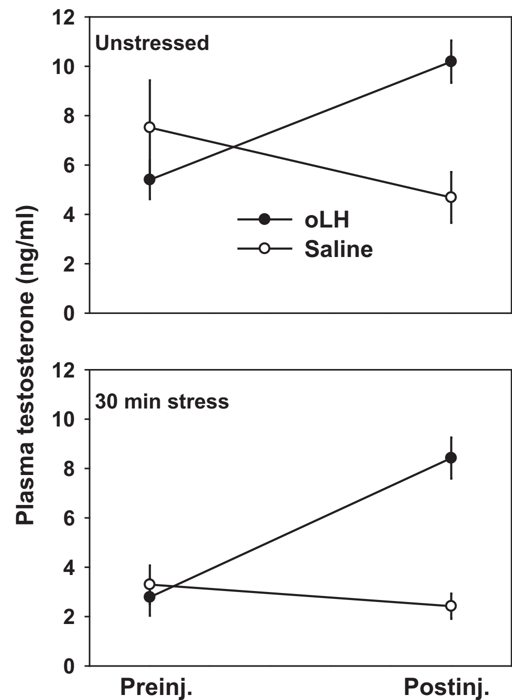

Fig. 6. Plasma testosterone (means ± SE; $n = 8$ or 9 per group) of free-ranging adult male rufous-winged sparrows, *Aimophila carpalis*, immediately before (*Preinj*) and 15 min after (*Postinj*) an intravenous injection of purified ovine luteinizing hormone (oLH; 250 µg/kg body mass) or of control saline solution. Birds either were bled (*Preinj*) and then injected within 3 min of capture (Unstressed) or were restrained for 30 min before the initial bleed and an injection (30 min stress).

plasma LH and T concentrations before and after stress, and in each case following an injection of NMA, GnRH, or oLH. Administration of NMA did not influence plasma LH, but an injection of GnRH or oLH increased plasma LH or T, respectively. These increases were similar irrespective of whether the birds were stressed before hormone treatment. Collectively, the results suggest inhibitory effects of stress on the pituitary gland secretion of LH as well as an inhibition of testicular endocrine function that may be LH-independent. The latter hypothesis is consistent with findings in the Gambel's white-crowned sparrow (Wingfield et al., 1982). In this species, plasma T in breeding males is negatively correlated with time (up to 1 h) after capture but plasma LH does not change for at least 12 h after capture.

4.1. Site of stress action

Administration of NMA to rufous-winged sparrows did not increase plasma LH. The absence of a stimulatory effect of NMA on plasma LH may be a consequence of the birds being in full breeding condition. This conclusion is supported by the observation that the stimulatory the effects of NMA administration on plasma LH is related to reproductive condition and tends to be lost or reduced in breeding white-crowned sparrows (Meddle et al., 1999), European starlings (Dawson, 2005), and Cassin's sparrows, a rufous-winged sparrow congener (Deviche et al., 2008). Since no stimulatory effect of NMA on plasma LH was observed in breeding rufous-winged sparrows irrespective of whether they were stressed, no firm conclusion can be drawn regarding potential effects of acute stress on the GnRH system of this species. The lack of effect of NMA treatment on plasma LH in this study may have resulted from GnRH being secreted at the maximum rate. This hypothesis is consistent with findings suggesting in this species that elevated plasma LH during the summer monsoon is associated with increased

GnRH production and secretion (Small et al., 2008). Stress might, however, affect the LH response to NMA in birds that are not in full breeding condition. In this case, a decrease in the stimulatory effect of NMA administration on plasma LH might be predicted as shown in stressed rats (Akema et al., 1995; Srivastava et al., 1995).

In mammals, glucocorticoids mediate the inhibitory effect of some types of stress on the reproductive system (Breen and Karsch, 2006) and these effects are thought to involve actions of glucocorticoids on the pituitary gland. A pituitary gland-mediated inhibitory role of glucocorticoids on plasma LH is supported by the observation that administration of a glucocorticoid receptor agonist dose-dependently reduces plasma LH and decreases the *in vitro* LH response to GnRH (Briski and Sylvester, 1991). Further supporting this proposition, the mammalian pituitary gland expresses glucocorticoid receptors (rat: Spiga and Lightman, 2009; dog: Reul et al., 1990; pig: Weaver et al., 2000) which, upon activation, can rapidly decrease the gland's responsiveness to GnRH (Breen et al., 2008). The chicken anterior pituitary gland also contains glucocorticoid receptors (Porter et al., 2007; Kwok et al., 2007) and we observed an inhibitory effect of acute stress on plasma LH in one study (interindividual comparison, Fig. 3B). As in mammals, this inhibition may have involved a glucocorticoid-mediated impairment of the pituitary gland ability to secrete LH.

The present results also suggest direct inhibitory effects of stress – whether mediated by elevated plasma glucocorticoids or by other factors – on testicular function. Indeed, capture and then restraint for 15 min decreased plasma T but not LH (Figs. 4 and 6). Within individuals, acute stress for 30 min likewise decreased plasma T without affecting plasma LH (Fig. 3A). In addition, GnRH administration rapidly stimulated plasma LH secretion and the magnitude of this stimulation was similar irrespective of whether the birds were stressed before treatment with the peptide. Finally, the rapid (within 15 min) and large (38%) decrease in plasma T in response to stress indicates that it may have begun immediately after capture and restraint. This depression in plasma T did not appear to be due to a loss of responsiveness to LH, since LH treatment stimulated T release equally in stressed and unstressed birds. Collectively, these observations suggest that stress rapidly inhibited plasma T through a LH-independent mechanism involving actions directly at the testicular level.

4.2. Mechanism of stress action within the testes

Three mutually non-exclusive mechanisms may mediate rapid inhibitory effects of stress on testicular function. One such mechanism involves actions of glucocorticoids on testicular Leydig cells. Autoradiography, immunocytochemistry, and gene expression studies have identified specific glucocorticoid receptors in mammalian, avian, as well as amphibian testes (Denari and Ceballos, 2006; Ortlip et al., 1981; Kwok et al., 2007; Stalker et al., 1989, 1991; Schultz et al., 1993). Receptors in mammals are located, in particular, in Leydig cells. Testosterone production in rodents decreased within 30 min of *in vitro* administration of CORT to Leydig cells (Dong et al., 2004) and the decrease was prevented by a glucocorticoid receptor blocker (Monder et al., 1994b). These observations suggest that elevated glucocorticoids during stress can inhibit Leydig cell T biosynthesis through a fast non-genomic mechanism, a hypothesis that is well supported experimentally (Hardy et al., 2005; Monder et al., 1994a; Morris et al., 2003; Hu et al., 2008). A same mechanism, if present in birds, would suffice to account for the rapid (i.e., within 15–30 min) decline in plasma T that is associated with acute stress, as seen in the present investigation.

A second mechanism by which acute stress may decrease plasma T involves a role for the inhibitory peptide GnIH (see Section 1). Avian testes including the interstitium, express GnIH and its receptors (Bentley et al., 2008; McGuire and Bentley, 2010). The latter study found in house sparrows, *Passer domesticus* that GnIH decreases the *in vitro* testicular secretion of T, suggesting paracrine effects of the peptide. Of interest, this decrease occurred with, but not without the incubation medium containing exogenous LH and FSH. Plasma levels of LH in the rufous-winged sparrows used for the present study were elevated and a testicular action of GnIH may, therefore, likewise have mediated inhibitory effects of stress on T secretion. This hypothesis postulates that stress upregulates the testicular GnIH system. The observation that acute stress increases the brain expression of RFamide-related peptides (RFRPs, which are related to avian GnIH) in rats (Kirby et al., 2009) and possibly also of GnIH in birds (Calisi et al., 2008), is compatible with this hypothesis, and so is the recent finding that avian hypothalamic GnIH-expressing cells have glucocorticoid receptors (Calisi et al., 2010). However, it remains to be determined whether stress influences gonadal GnIH in any species and whether the peptide influences testicular function *in vivo*. It should also be pointed out that GnIH inhibited avian testicular T secretion *in vitro* after 2 h of tissue exposure to the peptide (McGuire and Bentley, 2010), but effects of shorter incubation times were not examined. Further work is warranted to evaluate whether glucocorticoids regulate gonadal GnIH and whether this peptide plays a physiological role in mediating acute inhibitory effects of stress on testicular function.

Finally, stress may inhibit testicular function by rapidly activating a neural brain–testicular pathway that functions independently of the GnRH system and the pituitary gland. Based on studies involving intratesticular injection of the retrograde transganglionic tracer pseudorabies virus, James et al. (2008) recently identified a neural multisynaptic pathway that is centrally activated by corticotropin-releasing factor and catecholamines and terminates in the testes to rapidly inhibit T synthesis (also see Herman and Rivier (2006)). This pathway has not been identified in non-mammalian vertebrates. If present in birds, it may likewise induce a rapid decrease in T secretion following stressful events.

4.3. Significance

Decreased T secretion in response to acute stress may protect against the potentially detrimental effects of elevated plasma glucocorticoids. Avian blood contains binding globulins (CBG) that bind T and CORT competitively and with high affinity (Deviche et al., 2001; Fokidis et al., 2009). According to a widely, although not universally accepted hypothesis (free hormone hypothesis: Adams, 2005; Ekins, 1992; Mendel, 1989; Rosner, 2006), only free (i.e., unbound) plasma steroids can penetrate cells and, therefore, influence these cells by interacting with their intracellular receptors. The competitive binding of T and CORT to plasma CBG presumably results in a decrease in plasma total T, as observed during stress, causing more CBG binding sites to become available for CORT. This should, in turn, promote the binding of CORT to CBG and consequently reduce the concentration of plasma free CORT that is available for intracellular receptor binding. This reduction may mitigate cellular effects of stress-induced elevated plasma CORT. Whether this decrease would be sufficiently large to be physiologically meaningful, however, warrants further research for two reasons. First, plasma CBG concentration is large relative to plasma CORT or T concentrations, resulting in a large proportion (70–90%) of these hormones being normally bound to CBG in circulation (Deviche et al., 2001). Second, T binds to CBG with lower affinity than CORT (Deviche et al., 2001; Fokidis et al., 2009). It, therefore, remains to be determined whether changes in plasma T of magnitude such as measured in the present study decrease plasma free CORT to a physiologically significant extent.

In conclusion, we found that acute stress decreases plasma T but this decrease is not necessarily associated with an inhibition

of plasma LH. In addition, stress did apparently not compromise the capacity of LH to stimulate T secretion. These observations suggest that T secretion may rapidly increase to pre-stress level following relief from stress. This would be the case particularly if, as mammalian studies indicate (see above), acute stress in birds impairs T secretion through non-genomic actions involving either glucocorticoids or an inhibitory neural pathway terminating in the testes. Rapidly restoring plasma T to pre-stress level may be particularly important during the reproductive period, when this hormone regulates the expression of behaviors and physiological processes including gonadal function (Deviche et al., 2006b) that are important to the success of the breeding effort.

Acknowledgments

We are indebted to the managers of the Santa Rita Experimental Range for their assistance and continued support.

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