Fasting Increases Aggression and Differentially Modulates Local And Systemic Steroid Levels in Male Zebra Finches

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Aggression enables individuals to obtain and retain limited resources. Studies of the neuroendocrine regulation of aggression have focused on territorial and reproductive contexts. By contrast, little is understood concerning the neuroendocrine regulation of aggression over other resources, such as food. Here, we developed a paradigm to examine the role of steroids in food-related aggression. In groups of male zebra finches, a 6-hour fast decreased body mass and increased aggressive interactions among subjects that competed for a point source feeder. Fasting also dramatically altered circulating steroid levels by decreasing plasma testosterone but not estradiol (E_2). By contrast, both plasma corticosterone and dehydroepiandrosterone (DHEA) concentrations were elevated with fasting. Interestingly, short-term access to food (15 minutes) after fasting normalized circulating steroid levels. Fasting increased corticosterone levels in a wide range of peripheral tissues but increased DHEA levels specifically in adrenal glands and liver; these effects were quickly normalized with refeeding. DHEA can be metabolized within specific brain regions to testosterone and E_2, which promote the expression of aggression. We measured E_2 in microdissected brain regions and found that fasting specifically increased local E_2 levels in 3 regions: the periaqueductal gray, ventral tegmental area, and ventromedial nucleus of the hypothalamus. These regions are part of the vertebrate social behavior network and regulate the expression of aggression. Together, these data suggest that fasting stimulates secretion of DHEA from the adrenals and liver and subsequent conversion of DHEA to E_2 within specific brain regions, to enable individuals to compete for limited food resources. **(*Endocrinology* 154: 4328–4339, 2013)**

Aggression is an ancient behavior that evolved to enable organisms to cope with limited resources. Aggression is context dependent, has multiple causes, and involves the coordination of peripheral endocrine signals and central neural processes. Sex steroids are well-known modulators of aggression within the context of dyadic encounters, such as resident-intruder interactions for territorial defense (1) or male-male competition for mates (2, 3). Aggression in these contexts is typically attributed to the actions of gonadal T, which can bind to neural androgen receptors (4, 5) or be converted locally to 17β-estradiol (E_2) by the enzyme aromatase and bind to neural estrogen receptors (6, 7). Importantly, aggression occurs in other contexts, and its most ubiquitous expression is the defense and monopolization of food resources (8, 9), but little is understood of the physiologic regulation of aggression in this context.

In social species, limited food availability or fasting can spur aggressive interactions within the group or decreased social stability (10, 11). Fasting induces a myriad of effects on steroid profiles, including adrenal secretion of glucocorticoids (eg, corticosterone [CORT]) to mobilize energy reserves (12, 13) and increase food intake (14, 15). Increased CORT secretion also suppresses gonadal sex ste-

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Abbreviations: BST, bed nucleus of stria terminalis; CB, cerebellum; CORT, corticosterone; DHEA, dehydroepiandrosterone; E_2, 17β-estradiol; HP, hippocampus; HSD, hydroxysteroid dehydrogenase; LS, lateral septum; PAG, periaqueductal gray; POA, preoptic area; VMH, ventromedial nucleus of the hypothalamus; VTA, ventral tegmental area.

roid (eg, T and E_2) secretion (16). Because fasting decreases sex steroid secretion, it is unlikely that circulating T or E_2 levels directly promote the heightened aggression seen in fasted animals.

Alternatively, the androgen precursor dehydroepiandrosterone (DHEA) can maintain aggression, even when circulating sex steroid levels are low (17). Secreted by the adrenal glands, as well as the liver, the prohormone DHEA can be locally converted to active T and/or E_2 within behaviorally relevant neural circuits that possess the necessary steroidogenic enzymes (17, 18). Adrenal DHEA secretion increases with acute stress in humans (19, 20) and with injections of ACTH in red squirrels (21). In contrast, in song sparrows (*Melospiza melodia*), both acute restraint stress and social stress do not affect DHEA levels in the brachial vein, but modulate DHEA levels in the jugular vein (22–24). Fasting increases serum DHEA levels in humans and mice (25) as well as hepatic DHEA secretion in mice (25, 26). Because circulating DHEA levels change with fasting and DHEA promotes agonistic behavior, it is a good candidate for a steroid regulator of food-related aggression.

Here, we examine the effects of fasting on aggression, circulating steroid levels, and local steroid levels in peripheral tissues and specific brain regions in a songbird, the zebra finch (*Taeniopygia guttata*). Songbirds are excellent models for understanding the links between food availability, behavior, and steroids. Compared with laboratory rodents, songbirds have higher circulating DHEA concentrations (17), higher rates of DHEA conversion and neurosteroid synthesis in the brain (18, 27), higher mass-specific metabolic rates (28, 29), and more robust responses to even mild fasts (30, 31). Highly gregarious zebra finches are the most extensively studied songbird. Short-term fasting (ie, 2–6 hours) increases plasma CORT and decreases plasma T levels and alters courtship behavior and singing rates (32, 33). A suite of steroidogenic enzymes is expressed within the zebra finch brain (27, 34–36), thus indicating the capacity for de novo sex steroid synthesis.

If aggression over food is regulated by neurally synthesized sex steroids, then we predict that a fast would increase aggression, decrease circulating sex steroid levels, and increase circulating DHEA and CORT levels and/or local sex steroid levels in behaviorally relevant brain regions such as the bed nucleus of stria terminalis (BST), lateral septum (LS), ventromedial nucleus of the hypothalamus (VMH), periaqueductal gray (PAG), and the ventral tegmental area (VTA). In particular, E_2 is a key regulator of male aggression (17, 37), and we measured local E_2 levels within the social behavior network, a highly conserved circuit of brain regions (including those listed above) in vertebrates that regulates a variety of social behaviors (38, 39). To our knowledge, this is the first study to investigate the role of steroids in food-related aggression in any species.

Materials and Methods

All methods complied with the Canadian Council on Animal Care and University of British Columbia guidelines for animal care and use.

Experiment 1: effects of an acute fast on aggressive behavior

This experiment tested the hypothesis that acute fasting increases aggressive interactions within a group. Adult male zebra finches that had not previously interacted were housed overnight in groups of 4 with ad libitum access to food (50:50 panicum and white millet, ~12% protein, 4.7% lipid; Just for Birds) and water. Subjects were held on a 14-hour light, 10-hour dark photoperiod. Prior to lights on the following day, all food was removed to prevent subjects from feeding and accumulating seeds in their crop, an alimentary structure that stores food prior to digestion. Subjects were transferred as a group to a testing room. Subjects were weighed (±0.25 g), their furcular fat and pectoralis muscle scores (both 0–5) were recorded, and an empty crop was confirmed via palpation. All subjects were marked on their breast feathers with a blue or green washable marker for individual identification. Zebra finch plumage lacks these colors, and thus they are unlikely to influence social interactions.

On either side of the testing cage (38 ½" × 19 ¾" × 19", Corner's Ltd) were 2 wooden perches, a single food dish for general feeding, a water tower, and a point source feeder that allows only one subject to access food at a time. Groups of 4 subjects were randomly assigned to either 1) ad libitum food (control) with a full food dish or 2) a 6-hour fasting treatment (fasted) with an empty food dish (n = 15 groups per treatment, 60 subjects per treatment, 120 subjects total). All cages began with an empty point source feeder, which was replaced with full one at the onset of the behavioral trial. Each testing room housed 2 groups (one control, one fasted). Subjects were left undisturbed for 6 hours, after which food dishes (empty or full) were removed from all cages, and subjects were video-recorded for 15 minutes. Then empty point source feeders were replaced with full ones, and subjects were video recorded for an additional 30 minutes. Birds were then captured, reweighed, rescored for fat and muscle, and returned to the colony. Several subjects (n = 19) were tested multiple times, but with at least 6 days between tests.

Videos were scored by a single observer (HBF) that was blind with respect to treatment, and various behaviors were assessed within 2 separate time blocks: 1) from 0–15 minutes and 2) from 15–30 minutes after introduction of the full point source feeder. Behaviors were categorized into those in which aggression focused on accessing the feeder and those not directly related to the feeder. The former category includes displacements of one subject by another from the point source feeder, the total amount of time each subject spent at the point source feeder, and the order in which subjects accessed the point source feeder. The latter category includes aggression away from the point source feeder, such as chases, displacements from a perch, and bill fencing

(rapid exchanges of facial pecks). We quantified the total number of behaviors from both categories that were initiated as well as received by an individual.

Experiment 2: effects of an acute fast on circulating steroid levels

This experiment tested the hypothesis that acute fasting decreases circulating sex steroid levels and increases circulating CORT and DHEA levels. Adult male zebra finches were treated as described above (Experiment 1), but here subjects were tested in pairs. Pair housing allowed blood samples to be collected from all subjects within a short time frame to avoid steroid changes resulting from handling stress, while simultaneously avoiding isolation stress. Subjects were again weighed and scored for fat and muscle.

Subjects were then exposed to 1 of 3 treatments: 1) ad libitum food (control); 2) a 6-hour fast (fasted); or 3) a 6-hour fast followed by 15 minutes of access to a full point source feeder (refed). The 2 former groups were provided with an empty point source feeder as a control. All subjects (n = 12 subjects per vein per treatment) were captured and bled from either the brachial vein (with heparinized capillary tubes) or the jugular vein (with a 1-cc heparinized insulin syringe). For each subject, only a single blood sample was collected from only one vein. Steroid levels in plasma from the brachial vein reflect systemic levels, whereas steroid levels in plasma from the jugular vein indirectly reflect the neural steroidal milieu, including the synthesis and metabolism of steroids within the brain (24). Thus steroid profiles may differ between brachial plasma and jugular plasma, providing information on systemic vs central steroid levels. Bleed times averaged 4.7 ± 1.6 minutes, and blood was stored on ice until plasma was separated via centrifugation. Plasma was stored at −80°C.

Experiment 3: effects of an acute fast on tissue steroid levels

This experiment tested the hypothesis that acute fasting modulates local steroid levels within peripheral and central tissues. We repeated the above study (Experiment 2) in adult male finches with the same 3 treatment groups. Because handling stress can alter steroid concentrations, only one subject from each pair was used for this study, and the other subject was only a "companion" to avoid isolation stress (n = 8 subjects per treatment). After treatment, 1 subject from each cage was captured and euthanized via rapid decapitation within 1.4 ± 0.3 minutes of entering the testing room. Trunk blood was collected into heparinized capillary tubes. The brain was rapidly removed and separated into 4 parts: cerebellum (CB), diencephalon, rostral telencephalon, and caudal telencephalon (40). Peripheral tissues were collected in the following order: pectoralis muscle, furcular fat, liver, pancreas, small intestine, testes, and adrenal glands. All tissues were snap frozen on dry ice and time of collection was recorded. Trunk blood was centrifuged and plasma was collected. All samples were stored at −80°C.

Experiment 4: effects of an acute fast on E_2 levels in microdissected brain regions

This experiment tested the hypothesis that acute fasting increases neural E_2 levels within the social behavior network, using the Palkovits punch technique to sample tissue from specific regions (41–43). We repeated the above study (Experiment 3) in adult male zebra finches, and after treatment, 1 subject per cage was euthanized, with blood and other tissues collected as above, but here whole brains were snap frozen on powdered dry ice. Time of collection was again recorded and all samples were stored at −80°C.

Brains were coronally sectioned at 300 μm using a cryostat set at −10°C in a rostral to caudal direction and oriented to match the plane of sectioning in the zebra finch brain atlas (44). Brain regions were located relative to prominent neuroanatomical landmarks and punched using stainless steel cannulae (Brain Punch Set, catalog no. 57401, Stoelting Co). Punches for each brain region were taken from a single section and bilateral punches were pooled for subsequent analysis. Punches were either 1 or 2 mm in diameter (Table 1), depending on the region of interest, and were collected into microcentrifuge tubes and stored at −80°C. The brain regions sampled are area X (X), the magnocellular nucleus of anterior nidopallium, hippocampus (HP; 3 subregions: rostral, rHP; intermediate, iHP; caudal, cHP), preoptic area (3 subregions: rostral, rPOA; intermediate, iPOA; caudal, cPOA), nucleus rotundus, BST, LS, ventromedial nucleus of the hypothalamus (VMH), optic tectum, ventral tegmental area (VTA), periaqueductal gray (PAG), nucleus taeniae of the amygdala, caudomedial nidopallium, robust nucleus of the arcopallium, CB, locus coeruleus, and the nucleus of the solitary tract. These regions were selected because they are involved in the regulation of social behavior, energy balance, or the stress response, or because they served as negative controls (ie, little change in steroid levels was expected in optic tectum, CB, and nucleus rotundus).

Steroid extraction

For Experiment 2, 5 μL of plasma diluted 1:50 with assay buffer were used to measure CORT with a double-antibody ^{125}I RIA (MP Biomedicals; catalog no. 07120103; see below for assay details). Remaining plasma and all tissue samples had steroids first extracted using solid-phase extraction with C-18 columns (Varian, Bond-Elut; Catalog no. 12113045) as previously described (40, 41, 45). For soft tissues, samples were first homogenized in a bead homogenizer (settings: 8 m/s for 30 seconds × 2, Omni Bead Ruptor 24, Omni) in ice-cold 80% HPLC-grade methanol (MeOH) and left overnight at 4°C. Samples were centrifuged the following day and the supernatant was collected (up to 1 mL) to which was added 10 mL of dH_2O, prior to loading on C-18 columns. Columns had been primed with 3 mL 100% MeOH and then equilibrated with 10 mL dH_2O. Samples were then loaded on the column. This was followed by an interference elution with 10 mL 40% MeOH; this elution aimed to remove interfering lipids and glucuronidated and sulfated steroids. Finally, steroids were eluted with 5 mL 90% MeOH. Samples were then dried at 40°C in a vacuum centrifuge (Thermo Electron SPD111V Speedvac; Thermo Scientific) and stored at −20°C.

RIAs

Dried extracts from Experiment 2 were resuspended with 6 μL of absolute ethanol and 1194 μL of phosphate buffer containing sodium chloride, gelatin, and sodium azide (PBSG). This provided sufficient volume to measure T and DHEA in duplicate and E_2 in singleton. For E_2, samples were run as singletons to maximize the chance of detecting E_2 (41). Thus for Experiment

Table 1. Concentrations of E_2 (in pg/g) in Brain Regions of Adult Male Zebra Finches Exposed to ad Libitum Food (Control), a 6 Hour Fast (Fasted), or a 6-Hour Fast Followed by 15 Minutes of Access to a Point Source Feeder (Refed)

Brain Region	Punch Number and Size	Control	Fasted	Refed
Social Behavior Network				
POA				
Rostral	2 × 2 mm	434.3 ± 15.5 (100)	456.9 ± 18.3 (100)	466.1 ± 11.7 (100)
Intermediate	2 × 2 mm	425.4 ± 21.2 (100)	435.0 ± 25.5 (100)	393.6 ± 17.2 (100)
Caudal	2 × 2 mm	545.4 ± 29.8 (100)	503.7 ± 48.4 (100)	536.3 ± 56.7 (100)
BST	2 × 1 mm	ND	202.6 ± 39.1 (38)	ND
LS	1 × 2 mm	390.9 ± 71.5 (88)	509.9 ± 75.8 (86)	466.6 ± 81.5 (88)
PAG	1 × 2 mm	198.2 ± 81.5 (29)	612.6 ± 119.2 (80)	363.6 ± 108.6 (63)
VTA	2 × 1 mm	1083 ± 57.7 (100)	1388.5 ± 64.4 (100)	1189.6 ± 70.9 (88)
VMH	2 × 1 mm	583.6 ± 58.3 (100)	847.2 ± 78.2 (100)	782 ± 84.2 (100)
Nucleus taeniae of the amygdala	2 × 1 mm	72.3 (14)	ND	ND
Other brain areas				
Area X	2 × 2 mm	289 ± 69.6 (38)	19.0 (14)	300.8 ± 58.8 (38)
Magnocellular nucleus of the anterior nidopallium	2 × 2 mm	113.5 ± 44.3 (25)	86.1 ± 86.1 (13)	68.8 ± 68.8 (25)
Robust nucleus of the arcopallium	2 × 1 mm	1462.5 ± 186.1 (100)	1418 ± 164.0 (100)	1475.2 ± 178.7 (100)
Caudomedial nidopallium	2 × 2 mm	3251.4 ± 241.8 (100)	3451.7 ± 308.1 (100)	3027.9 ± 116.4 (100)
Nucleus rotundus	2 × 1 mm	231.5 ± 231.5 (13)	56.9 ± 56.9 (13)	ND
Nucleus tractus solitarius	1 × 2 mm	235.1 ± 9.5 (100)	ND	102.8 ± 28.6 (50)
Locus coeruleus	2 × 1 mm	ND	121.6 ± 21.6 (25)	187.6 ± 55.1 (40)
Rostral hippocampus	2 × 2 mm	381.7 ± 117.8 (100)	388.5 ± 140.4 (100)	464.1 ± 104.6 (100)
Intermediate hippocampus	2 × 2 mm	387.8 ± 132.6 (100)	367.8 ± 124.3 (100)	380.1 ± 126.7 (100)
Caudal hippocampus	2 × 2 mm	261.5 ± 20.1 (100)	290.5 ± 16.0 (100)	280.7 ± 7.5 (100)
Optic tectum	2 × 2 mm	ND	ND	ND
CB	1 × 2 mm	ND	ND	ND

All data are mean ± SEM and numbers in parentheses indicate percentage of detectable samples out of 8 birds within each group. ND, not detectable.

2, the T, DHEA, and E_2 assays used 28%, 36%, and 36% of the original sample, respectively. For Experiment 3, samples were resuspended using 4.5 μL of absolute ethanol and 645.5 μL PBSG. Assays for CORT, T, DHEA, and E_2 were all run in singleton to maximize the chance of detecting the analytes and used 8%, 28%, 16%, and 46% of the original sample, respectively. For Experiment 4, the small size of punches necessitated the entire sample be used to measure E_2 in singleton.

We used specific double-antibody ^{125}I RIAs to measure 4 steroids: CORT (see above), T (MP Biomedicals; catalog no. 07189102), DHEA (Beckman-Coulter; Immunotech DSL 8900), and 17β-E_2 (Beckman-Coulter; Immunotech DSL 4800). These were run according to manufacturer's recommendations; however, assays were modified to improve sensitivity and were previously validated for songbird samples (24, 40, 46). The detectable ranges for the assays were as follows: CORT = 1.5 to 1000 pg/tube; DHEA = 2–600 pg/tube; T = 0.3125 to 500 pg/tube; and E_2 = 0.192 to 19.2 pg/tube. Additional validations presented here include demonstrating parallelism between standard curves and serially diluted plasma and tissue (Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) and estimates of recovery of known steroid quantities within several tissues, prior to solid-phase extraction and subsequent comparison with "unspiked" samples (Supplemental Table 1). All data were corrected for recovery. Intrassay and interassay variation are reported in Supplemental Table 2.

Statistical analysis

All data were tested for normality of distribution (Kolmogorov-Smirnov test), homoscedasticity (Levene's test of equal variance), and for the presence of influential outlying data (Chauvenet's test). When required, data were arcsine square root or log transformed. Outliers were removed if they altered the results of the data analysis. We used repeated measures ANOVA to assess individual changes in body mass and fat and muscle scores.

To assess changes in behaviors with treatment (Experiment 1), we used a mixed model ANOVA approach. The repeated measure was the behavior before, immediately after, and 15 minutes after introduction of the point source feeder (ie, pre, post, 15-minutes post). To account for variation at the group level, we tested individual behaviors nested within groups (ie, cage identification). This enabled us to detect changes in behavior with food introduction, while controlling for the effects of specific groupings of subjects. Treatment was the between-subjects factor, and initial body mass, and the change in body mass were included as random factors in the model. Two behaviors (the occurrence of chase events and bill fencing) had a Poisson distribution and thus were analyzed using a repeated-measures logistic regression providing a Wald's χ^2 statistic, with treatment as the independent variable and food introduction (pre, post, 15 minutes post) as the repeated measure. To assess whether body mass or change in body mass influenced the order in which the point source feeder was accessed (ordinal data), we used a nonparametric Kruskal-Wallis one-way ANOVA (H test statistic) coupled to Tamhane's T_2 post hoc tests for unequal variances.

Changes in plasma steroid levels in response to treatment (Experiment 2) were assessed using multivariate analysis of covariance with treatment, steroid, vein where blood was sampled (brachial vs. jugular), and their interactions as the between-subjects factors, and with change in body mass as a covariate. Tissue steroid levels (Experiment 3) were analyzed using multivariate analysis of covariance with treatment and tissue type as between-subjects factors, and initial body mass as a covariate. Levels of E_2 in brain punches (Experiment 4) were analyzed using ANOVA with treatment and brain region as between- and within-subjects factors, respectively. Due to large number of brain regions sampled in this study (n = 21) that reduces statistical power, we split our analyses into those regions that make up the social behavior network (PAG, POA, BST, LS, VMH, VTA, and nucleus taeniae of the amygdala) (39, 47) and the other regions. Relevant interactions between independent variables were also included, and differences between tissues (Experiment 3) or brain regions (Experiment 4) were assessed using Tukey's honestly significant difference post hoc tests. The critical α level was set at $P \leq .05$ and statistics were performed using Windows versions of Statistica 9.1 (StatSoft Inc), SPSS 13.0 (SPSS), and GraphPad Prism 5 (GraphPad).

Results

Experiment 1: effects of fasting on morphology and aggressive behavior

Fasted subjects exhibited significant decreases in body mass ($F_{1,59} = 19.631$, $P \leq .001$; Figure 1A) and muscle score ($F_{1,59} = 6.893$, $P = .002$; Figure 1B), but not fat score ($F_{1,59} = 0.205$, $P = .653$; Figure 1C) compared with controls.

Prior to presentation of a point source feeder, control and fasted subjects behaved similarly and showed little social interaction. Both immediately after (fasted group) and 15 minutes after (refed group) the presentation of a point source feeder, subjects spent more time feeding ($F_{1,54} = 9.271$, $P \leq .001$; Figure 2A) and displayed more point source feeder displacements ($F_{1,54} = 10.365$, $P \leq .001$; Figure 2B) than controls, but the refed subjects displayed fewer overall behavioral acts than fasted subjects. Fasted and refed subjects initiated ($F_{1,54} = 5.201$, $P \leq .001$; Figure 2C) and received ($F_{1,54} = 3.770$, $P = .008$; Figure 2C) more behavioral acts than controls, but in both cases the trend toward fewer behavioral acts in the refed subjects was not significant from the fasted subjects.

Along with point source feeder-centered behaviors, other social behaviors were also more frequent in fasted compared with control and/or refed subjects. The occurrence of chases was more frequent in fasted compared with refed or control subjects (Wald's $\chi^2 = 6.937$, $df = 1$, $P = .048$; Figure 2D). Displacements from a perch were more frequent in fasted and refed subjects compared with controls ($F_{1,54} = 4.002$, $P = .016$; Figure 2E). However, no difference in the occurrence of bill-fencing behavior was observed among groups (Wald's $\chi^2 = 0.844$, $df = 1$, $P = .441$; Figure 2F).

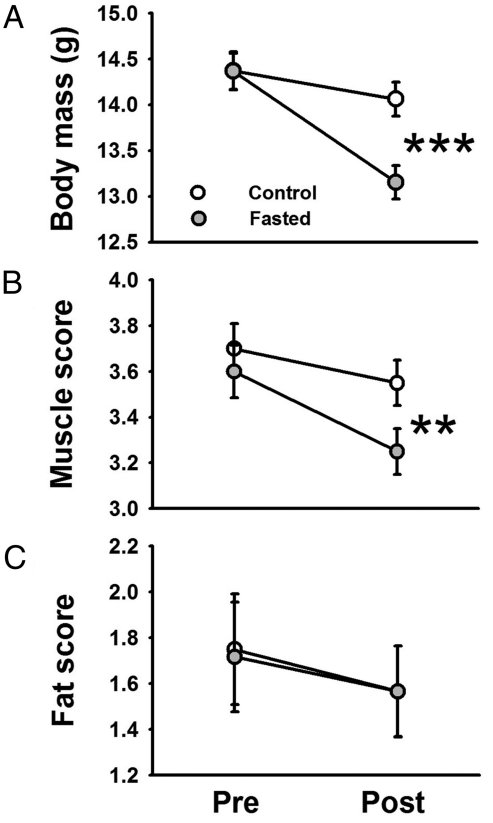

Figure 1. Effects of an acute fast (6 hour) on (A) body mass, (B) pectoralis muscle score, and (C) furcular fat score in adult male zebra finches (n = 60 per treatment). All data are mean ± SEM. *, $P \leq .05$; **, $P \leq .01$; and ***, $P \leq .001$.

Both initial body mass and the change in body mass influenced the order in which fasted subjects accessed the point source feeder, with heavier subjects accessing the feeder prior to lighter subjects (Kruskal-Wallis $H = 4.731$, $df = 1$, $P = .032$). A stronger predictor of access was the proportion of body mass lost during the fast, with subjects that lost more mass accessing the point source feeder prior to those that lost less mass (Kruskal-Wallis $H = 5.922$, $df = 1$, $P = .016$; Figure 3).

Experiment 2: effects of fasting on circulating steroid levels

Overall, plasma steroid concentrations were affected by treatment but not the vein sampled; however, the treatment × vein interaction was often significant (Supplemental Table 3). The change in body mass also affected plasma steroid levels (Supplemental Table 3).

Fasted subjects had significantly higher brachial and jugular CORT concentrations than control and refed subjects (Figure 4A and Supplemental Table 3). Plasma CORT levels also tended to be higher in subjects that lost

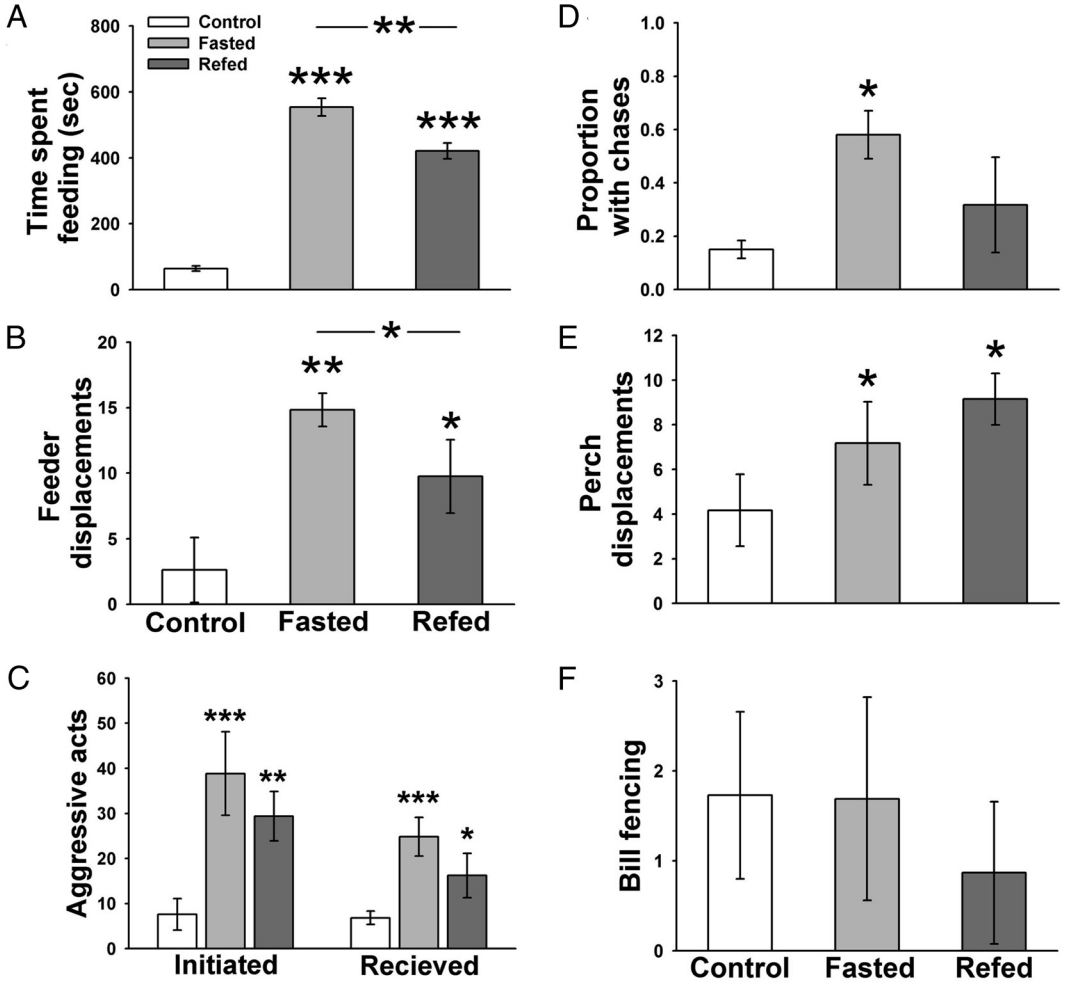

Figure 2. Aggressive behaviors within zebra finch groups exposed to ad libitum food (Control), a 6-hour fast (Fasted), or a 6-hour fast followed by 15 minutes of access to a point source feeder (Refed). A, The total time group spent at the point source feeder. B, The number of displacements of subjects from a point source feeder. C, The number of behavioral acts initiated by and received by an individual. D, The proportion of behavioral trials with an observed chase. E, The number of displacements of subjects from perches. F, The occurrence of bill fencing. n = 15 groups per treatment. All data are mean ± SEM. *, $P \leq .05$; **, $P \leq .01$; and ***, $P \leq .001$.

more body mass with fasting; however, this correlation was not significant (Pearson's $r = 0.274$; $P = 0.091$).

Plasma DHEA concentrations showed a significant treatment × vein interaction. In the brachial plasma, DHEA concentrations were significantly higher in fasted subjects than either control or refed subjects (Figure 4B and Supplemental Table 3). In stark contrast, in the jugular plasma, fasting did not affect DHEA concentrations, although DHEA concentrations in refed subjects were significantly lower than controls (Figure 4B).

Plasma T concentrations also showed a significant treatment × vein interaction (Supplemental Table 3). Fasted subjects had lower T levels in the brachial plasma, but not in the jugular plasma, compared with controls (Figure 4C). Refeeding increased T levels in the jugular plasma, but not in the brachial plasma, compared with fasted subjects (Figure 4C).

Plasma E_2 concentrations were not significantly affected by treatment, the vein sampled, or change in body mass (Supplemental Table 3). However, as expected, E_2 concentrations tended to be lower in brachial than jugular plasma for control subjects (Figure 4D).

Experiment 3: effects of fasting on tissue steroid levels

Overall, tissue steroid concentrations were significantly affected by treatment, tissue type, and often their interaction, but not by body mass (Supplemental Table 4).

CORT was detectable in all tissues and was above the limit of detection in all samples. Fasting increased CORT concentrations in all tissues, except furcular fat (Figure 5A). Notably, tissue CORT concentrations did not differ between controls and refed subjects (Figure 5A). As expected, CORT concentrations were highest in the adrenal gland (Figure 5A).

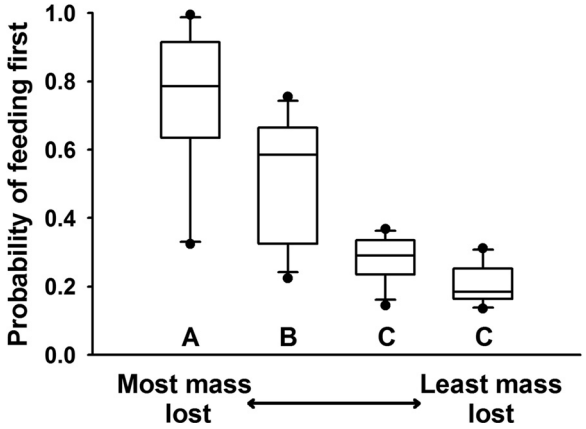

Figure 3. Box plot exhibiting the order of access to a point source feeder by groups of 4 adult male zebra finches in relation to the degree of body mass lost during a 6-hour fast. Different letters indicate significant differences at $P \leq .05$.

DHEA was detectable in all tissues, except for 3 samples (muscle-Refed: n = 1; rostral telencephalon-Fasted: n = 2). Treatment influenced DHEA concentrations in a tissue-specific manner (Supplemental Table 4). Fasting increased DHEA concentrations in the trunk plasma, liver, adrenal glands, caudal telencephalon, and diencephalon only (Figure 5B).

T was detectable in all tissues, but was below the limit of detection in 5 samples (muscle-Control: n = 1; muscle-Refed: n = 1; Intestine-Fasted: n = 1; diencephalon-Fasted: n = 2). Tissue T levels were not significantly affected by treatment but did vary across tissues (Supplemental Table 4). As expected, T concentrations were highest in the testes, which exhibited a nonsignificant trend for lower T levels in fasted subjects (Figure 5C).

Levels of E_2 in peripheral tissues were not assessed because no significant effect of treatment was observed on circulating E_2 concentrations.

Experiment 4: effects of fasting on E_2 levels in microdissected brain regions

In microdissected brain punches (≤1 mg), levels of E_2 were detectable (ie, within the range of the standard curve) in 19 of the 21 brain regions examined (Table 1). However, for some regions, only a small proportion of samples had detectable amounts of E_2, even though we used an ultrasensitive assay that can measure as little as 0.2 pg of E_2 per tube. To avoid testing small sample sizes, only regions with >25% of samples detectable in each treatment group were considered for further analysis (Table 1).

For both the HP and preoptic area (POA), we did not observe differences in E_2 concentrations among rostral, intermediate, and caudal levels (HP: $F_{2,272} = 1.427$, $P = .242$; POA: $F_{2,272} = 1.427$, $P = .242$; Table 1). Thus, a mean E_2 concentration was calculated for the HP and POA for subsequent analysis.

In the social behavior network, there was a significant treatment × brain region interaction (Supplemental Table 5). In contrast, in the other regions, there was an effect of brain region only, with no effect of treatment and no treatment × brain region interaction (Supplemental Table 5). In the social behavior network, post hoc comparisons revealed that 3 regions significantly responded to fasting (Figure 6). Fasted subjects exhibited significantly higher E_2 levels than controls within the PAG, VTA, and VMH (Figure 6). In addition, refeeding significantly reduced E_2 levels in 2 of these regions: PAG and VTA (Figure 6).

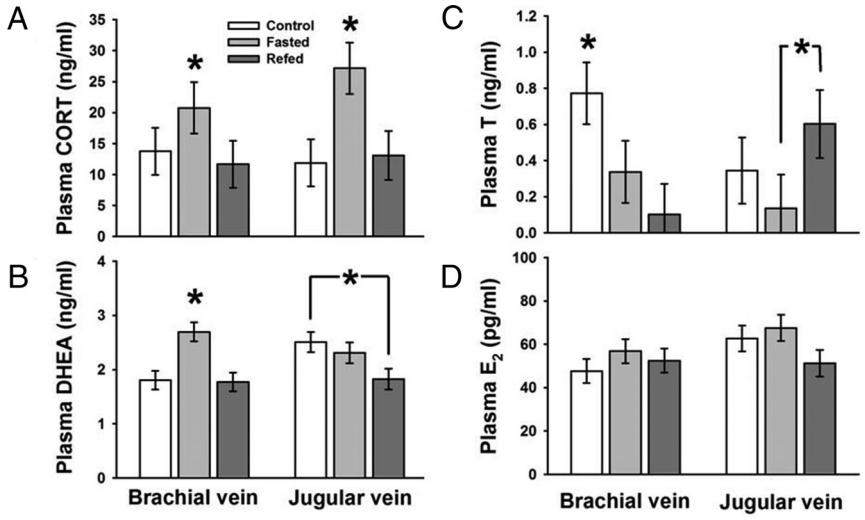

Figure 4. Plasma concentrations of (A) CORT, (B) DHEA, (C) T, and (D) E_2 in the brachial and jugular veins of adult male zebra finches exposed to ad libitum food (Control), a 6-hour fast (Fasted), or a 6-hour fast followed by 15 minutes of access to a point source feeder (Refed). n = 12 subjects per vein per treatment. All data are mean ± SEM. *, $P \leq .05$.

Discussion

Here, we propose a novel mechanism for regulating aggression over access to food resources, involving adrenal and hepatic DHEA secretion and subsequent local conversion of DHEA to E_2 within specific brain regions. An acute fast increased several aggressive behaviors immediately after food presentation, and these behaviors decreased (or trended toward a decrease) after 15 minutes of access to food presentation. Fasted subjects had reduced systemic T levels compared with controls. Thus systemic T levels cannot account for the aggressive competition over

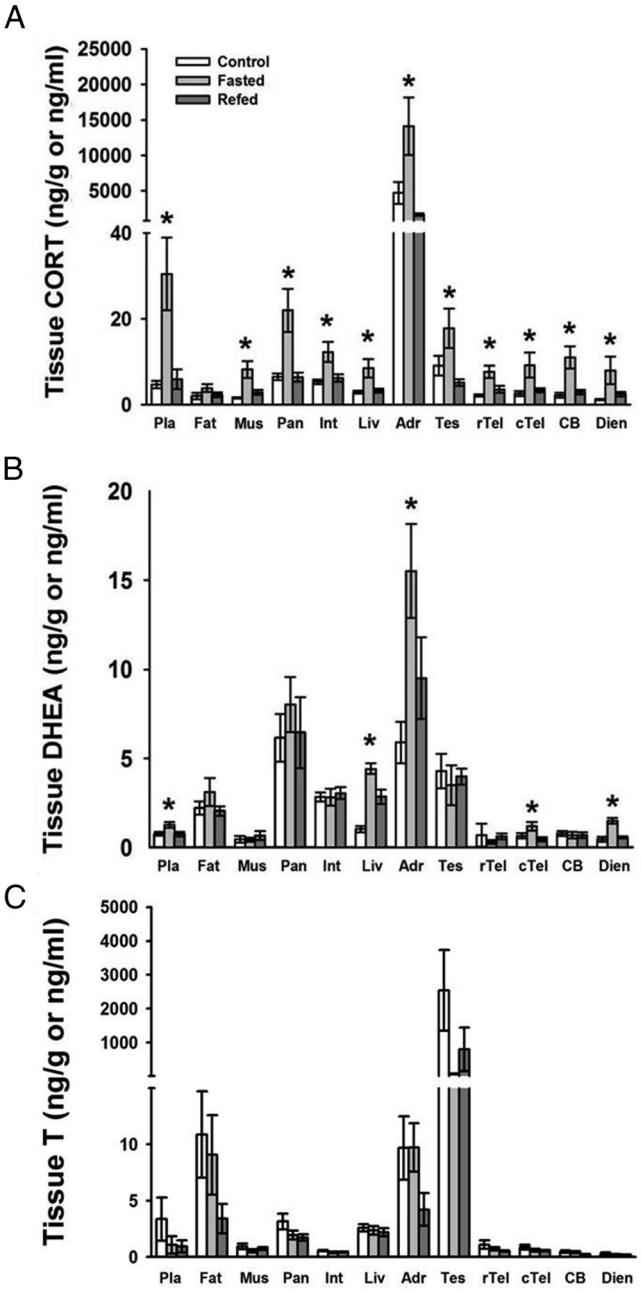

Figure 5. Concentrations of (A) CORT, (B) DHEA, and (C) T in trunk plasma and tissues of adult male zebra finches exposed to ad libitum food (Control), a 6-hour fast (Fasted), or a 6-hour fast followed by 15 minutes of access to a point source feeder (Refed) (n = 8 subjects per treatment). All data are mean ± SEM. *, $P \leq .05$.

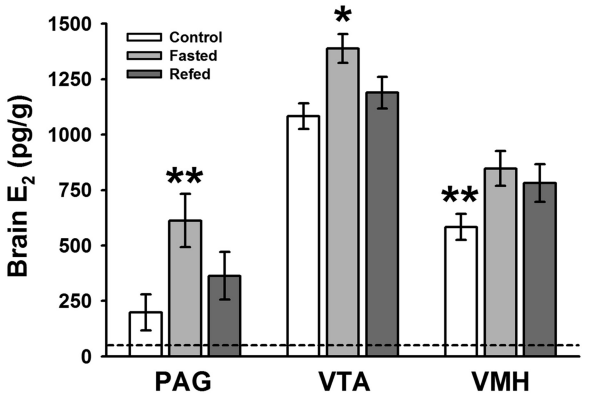

Figure 6. Concentration of E_2 within 3 brain regions in the social behavior network in adult male zebra finches exposed to ad libitum food (Control), a 6-hour fast (Fasted), or a 6-hour fast followed by 15 minutes of access to a point source feeder (Refed). Dotted line indicates the mean E_2 concentration in trunk plasma. n ranges from 3 to 8 subjects per treatment; percentage detectability of samples is detailed in Table 1. All data are mean ± SEM. *, $P \leq .05$; **, $P \leq .01$.

food. Fasting, however, increased plasma levels of 2 adrenal steroids, CORT (in both brachial and jugular veins) and DHEA (in brachial vein only). The CORT increase was widespread among tissues, consistent with its role in energy mobilization. In contrast, the adrenal glands and liver were the only peripheral tissues that exhibited increases in DHEA content with fasting. Both tissues had higher DHEA levels than plasma, suggesting these organs are the major sites of DHEA secretion during fasting. DHEA can be converted to active sex steroids (T and E_2) within the brain to modulate social behavior. Consistent with this idea, we observed localized increases in E_2 concentrations with fasting in three areas (PAG, VTA, and VMH) of the social behavior network. Surprisingly, providing access to food for only 15 minutes after the fast was often sufficient to normalize steroid concentrations in plasma, peripheral tissues, and the brain to control levels, which was consistent with the changes in behavior. Taken together, these data support a novel role for steroids as mediators of food-related aggression in male zebra finches, which may involve peripheral DHEA secretion in response to an energetic challenge and subsequent neural conversion of DHEA to E_2 to influence aggression.

An acute fast has dramatic effects on body mass, muscle, and adrenal steroids

In this study, zebra finches exhibited robust responses to a 6-hour fast, including an 8.5% decline in body mass and a decrease in pectoralis muscle score. These data support other songbird studies, indicating that even short-term food limitation constitutes a major energetic challenge (30, 48). The reduced muscle score likely reflects increased protein catabolism to meet the energetic demands of fasting. One likely mediator of this effect is CORT, which increased with fasting in plasma and across nearly all tissues investigated. During fasting, CORT maintains glucose levels (16) by promoting gluconeogenesis using substrates derived from muscle and fat catabolism (12, 30, 49). Furthermore, CORT encourages locomotor activity in captive birds (30, 31, 50), and this may contribute to the decreases in body mass and muscle score. Although fasting elevated CORT levels in most tissues, it did not significantly increase CORT levels in furcular fat.

We also did not observe a decrease in fat score with fasting, which collectively suggests that CORT does not mobilize energy from furcular fat under the current paradigm.

Refeeding lowered CORT concentrations to control levels, suggesting the rapid (≤15 minutes) metabolism or clearance of CORT once food becomes available. Many tissues express 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which inactivates CORT to dehydrocorticosterone (51, 52). Earlier studies have reported a half-life for CORT in plasma of 10–25 minutes under baseline conditions (53, 54), but more recent studies suggest shorter times of 5–10 minutes in rats (55–57). The higher metabolic rate of songbirds, compared with similarly-sized mammals (28), suggests an equivalent or faster rate of clearance of CORT in circulation. However, white-crowned sparrows (*Zonotrichia leucophrys*) that ingested mealworms injected with CORT showed a peak in circulating CORT after 7 minutes, and baseline levels were restored at about 1 hour (58). Ingested CORT, however, may be released slowly during digestion, in contrast to an injection of CORT. Fasting and subsequent refeeding may rapidly increase steroid clearance by up-regulating 11β-HSD2 activity. Fasting increases 11β-HSD2 in the rat kidney (59), and restraint stress elevates 11β-HSD2 activity in the rat placenta to buffer the fetus from high maternal CORT levels (60); however, no studies have investigated other tissues or the effects of refeeding.

Fasting also increased brachial, but not jugular, DHEA concentrations, and refed subjects had plasma DHEA levels that were equivalent to those of controls. This pattern was also observed in the adrenal glands and liver, where DHEA levels were higher than in trunk plasma. Previous studies in songbirds have demonstrated that both restraint and social stress were incapable of altering brachial DHEA levels, but could alter jugular and hepatic DHEA levels (23). The effects of fasting on brachial DHEA levels may involve increased hepatic DHEA secretion, potentially allowing metabolic processes in the liver to affect the brain and social behavior. The decline in plasma, adrenal, and hepatic DHEA levels within 15 minutes of refeeding is consistent with human studies, in which insulin (which increases in response to feeding) increases the metabolic clearance rate for DHEA (61, 62). Early studies suggest a much faster metabolic clearance rate for DHEA in rodents and rabbits as compared to primates (63, 64), with half-lives in circulation ranging from 15–40 minutes (65–67), but studies in birds have not been done.

An acute fast increases aggressive interactions but not systemic sex steroid levels

Fasted subjects displayed more agonistic behaviors than controls, which is consistent with prior studies (8, 68). As expected, this increase in aggression was observed in behaviors aimed at accessing the point source feeder, but also in interactions not directly related to the food, such as chases and perch displacements. Furthermore, these behaviors subsided after 15 minutes of refeeding concurrent with the decreases in circulating and brain steroid profiles. Another interesting observation was the relationship between the change in body mass and the likelihood of accessing the point source feeder first. One possible interpretation is that subjects that lost more body mass (ie, had a lower energy balance) were more motivated to feed, raising the possibility of individual variation in aggression and competitiveness driven by individual differences in energy balance.

CORT increases locomotor activity (30, 31) but is unlikely to solely contribute to fasting effects on aggressive interactions, because fasted and control subjects did not differ in behaviors prior to introduction of the point source feeder. Several lines of evidence suggest that systemic but not neural T levels were reduced by fasting: 1) fasting decreased T levels in brachial plasma but not jugular plasma; 2) refeeding increased T levels in jugular plasma but not brachial plasma; and 3) fasting tended to decrease T levels in the testes but not the brain. Taken together, these data suggest that systemic T is an unlikely candidate for regulating the heightened aggression during food-related contests.

Circulating E_2 levels tended to be higher in jugular plasma than brachial plasma in controls, although this was not significant. Our analysis limited univariate comparisons, but the lack of overlap in error bars (Figure 2D) suggests that, in controls, E_2 levels could be higher in the jugular vein than brachial vein. These data are consistent with previous findings that the brain is the predominant source of circulating E_2 in male zebra finches (69). However, neither brachial nor jugular plasma E_2 concentrations changed in response to fasting or subsequent refeeding. Thus circulating E_2 is also an unlikely regulator of aggression in a food-related context.

Neurosteroids, fasting, and aggression

The brain can synthesize active steroids (ie, neurosteroids) de novo from cholesterol as well as from circulating inactive precursors, such as DHEA (70, 71). Both E_2 and DHEA concentrations are typically higher in brain than plasma (69, 72) and are maintained even after castration, adrenalectomy, or gonadal regression (73). However, the factors that regulate neurosteroid production remain elusive.

Neural E_2 synthesis maintains aggression when systemic sex steroid levels are low, such as during the nonbreeding season (17, 37). Here, E_2 was present throughout

the male zebra finch brain, as previously reported (41), with highest levels in the caudomedial nidopallium, an aromatase-rich forebrain area involved in auditory processing of song (74). Statistical considerations limited our analyses to regions with reliably detectable levels of E_2. Interestingly, fasting specifically increased E_2 levels within 3 regions of the social behavior network: the PAG, VTA, and VMH. Furthermore, in the PAG and VTA, E_2 concentrations were rapidly reduced to baseline levels by refeeding, suggesting a time course consistent with a rapid nongenomic effect of E_2 on aggression.

The PAG regulates multiple behaviors, including vocalization, mating, fight or flight, and aggressive behaviors (75). The VTA is part of the mesolimbic dopaminergic system involved in motivation and reproductive behavior (38), and the VTA innervates the PAG and other social behavior regions (76). Aromatase is present in cell bodies in VTA and in fibers in PAG (77, 78), and aromatase activity is modulated by dopamine (79). In zebra finches, dopaminergic cells of the VTA respond to conspecific interactions (80), and in rats, VTA neurons are involved in the anticipation of feeding (81). Both the VTA and PAG project to regions regulating aggressive behavior, including the BST and LS (79, 82). The VMH is involved in satiety (83, 84), and although E_2 can suppress food intake (85), this action does not appear to involve the VMH (86). In song sparrows (*M. melodia*), E_2 treatment decreases immunoreactivity for phosphorylated tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis in the VMH (87). Together, these observations suggest higher E_2 levels within the PAG, VTA, and VMH may alter dopaminergic signaling to efferent regions that regulate aggression. Future research manipulating local E_2 levels in these regions is vital to elucidate the role of E_2 in mediating food-related aggression.

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