

Tonic Activation of Brain GnRH Immunoreactivity despite Reduction of Peripheral Reproductive Parameters in Opportunistically Breeding Zebra Finches

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Key Words

GnRH · Zebra finch · *Taeniopygia guttata* · Water restriction · LH · Testis volume · Reproductive timing · Birds

Abstract

Opportunistically breeding species offer the unique opportunity to understand mechanisms in reproductive physiology that allow for extreme flexibility in the regulation of reproduction. We studied a well-known opportunistic breeder, the zebra finch (*Taeniopygia guttata*) to test the hypothesis that the reproductive axis of opportunists is in a constant state of 'near-readiness'. In wild zebra finches, reproduction is highly correlated with rainfall, and in the laboratory, water availability and humidity are the strongest cues to affect reproductive activation. We therefore subjected individuals to water restriction for eleven weeks followed by a two week period of ad libitum access to water. The control group had water freely available for the entire experiment. We measured the state of activation of the hypothalamo-pituitary gonad (HPG) axis at three levels: in the hypothalamus by measuring immunoreactive (ir) cGnRH-I and cGnRH-II; in the anterior pituitary gland by measuring plasma luteinizing hormone (LH); and in the gonads by measuring gonadal volume and function. We found that water re-

striction caused a reduction in circulating LH concentrations and that testis volume was more likely to decrease in water restricted than in control birds. Subsequent short-term return to ad libitum water availability caused LH to return to baseline in water restricted birds. These changes occurred without significant changes in ir-cGnRH-I, ir-cGnRH-II, or in testis function. These data suggest that in these opportunistic breeders, an inhibition of parts of the reproductive axis is not necessarily correlated with full inactivation of reproductive potential. GnRH-ir cells in the hypothalamus appear to remain active and able to respond to subsequent stimulation.

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Introduction

To optimize reproductive success, organisms breeding in fluctuating environments use particular strategies to time breeding. In habitats in which resource abundance changes unpredictably, animals often use an opportunistic breeding strategy to exploit favorable conditions whenever they occur. Opportunistic breeders rely to a great degree on various environmental cues such as temperature, rainfall and food availability for the fast initiation of breeding. These cues have predictive value about habitat conditions in the short-term but are not tied to spe-

cific times of year for reproductive timing [Wingfield and Farner, 1993]. The opportunistic reproductive strategy contrasts with that of seasonal breeders in more temporally predictable habitats that reproduce at similar times each year and primarily rely on long-term cues such as photoperiod [Dawson et al., 2001].

Activation of reproductive physiology and behavior is primarily controlled by the hypothalamo-pituitary gonadal (HPG) axis. Peripheral sensory organs integrate environmental cues that culminate in a hypothalamic signal causing a hormonal cascade. The cascade starts with the induction of gonadotropin-releasing hormone (GnRH) production, processing and secretion [Hadley, 1996]. Increased GnRH secretion is considered the major switch between reproductive inactivity and activation, and elicits gonadotropin secretion (luteinizing hormone, LH, and follicle stimulating hormone, FSH) from the anterior pituitary gland. These hormones are secreted into the peripheral circulation, and lead to reproductive development and reproductive behavior. Therefore, reproductive activation can be measured at any of the following levels in this cascade: GnRH protein in the hypothalamus; circulating gonadotropins in the plasma; size and function of the gonads; and the expression of reproductive behaviors.

Farner and Serventy [1960; see also Farner, 1967] proposed over forty years ago that opportunistic breeders should maintain a tonically activated hypothalamo-pituitary axis year round. In this way, they achieve maximal reproductive flexibility and can take advantage of favorable breeding conditions regardless of the time of year. Zebra finches (*Taeniopygia guttata*) have long been considered the archetype of the opportunistic breeder and breed in arid and semi-arid regions of Australia [Immelmann, 1971; Zann et al., 1995; Hahn et al., 1997]. Adults forage mainly on grass seeds and feed freshly sprouted seeds to their young; therefore, reproduction in the wild is closely tied to aperiodic rainfall [Frith and Tilt, 1959; Davies, 1977; Zann et al., 1995; Zann, 1999]. Water availability and humidity per se are effective environmental cues to alter gonadal volume in the laboratory [Priedkalns et al., 1984; Vleck and Priedkalns, 1985], but changes in reproductive activity at the level of the hypothalamus have not been investigated in this species. Because previous work has shown that environmental factors such as water availability can modify reproductive activity at the level of the gonads and, to some extent, LH secretion [Priedkalns et al., 1984; Vleck and Priedkalns, 1985; Bentley et al., 2000], we used this paradigm to test the Farner and Serventy hypothesis specifically at the lev-

el of the hypothalamus in this well-known opportunistically breeding species.

In each class of vertebrates, more than one form of GnRH is produced, but typically one form is primarily responsible for eliciting gonadotropin secretion [Schally et al., 1971; King and Millar, 1995; Muske, 1997]. In seasonally breeding birds, immunoreactive (ir) chicken (c) GnRH-I in the hypothalamus, measured either by immunoassays for content or by immunocytochemistry (ICC) of cell number, area and/or fiber density, changes dramatically in response to changes in photoperiod [Foster et al., 1987; Goldsmith et al., 1989; Hahn and Ball, 1995; Dawson et al., 2001]. cGnRH-I is thought to be the primary neurohormone stimulating gonadotropin secretion [Mikami et al., 1988; Millam et al., 1993; van Gils et al., 1993; but see Clerens et al., 2003]. After photostimulation, circulating LH concentration can increase within 18 h of exposure to the first long day [20L:4D; e.g., Meddle and Follett, 1995]. Other environmental cues, such as temperature and food availability, can also have effects at all points along the HPG axis [Bruggeman et al., 1998; Kriegsfeld et al., 2000, 2001], but their neuroendocrine transduction has been less well-studied.

Another form of GnRH, cGnRH-II, can act as a neurotransmitter/neuromodulator to influence reproductive behavior directly [Riskind and Moss, 1983; Maney et al., 1997; Kauffman and Rissman, 2004]. This peptide is highly conserved across all vertebrates classes and there is some indication that it can transduce information about energy balance in female mammals [Temple et al., 2003; Kauffman and Rissman, 2004]. Cells containing ir-cGnRH-II are found in brain areas that are distinct from cGnRH-I [Muske, 1993; Bentley et al., 2003; Millam et al., 2003], and cGnRH-II cell numbers and fiber density have been shown to increase in response to food restriction [Temple et al., 2003]. Although cGnRH-I appears to be the primary regulator of gonadotropin secretion, cGnRH-II might respond to other environmental cues and indirectly influence the reproductive system via the direct activation of reproductive behavior. Because rapid stimulation of courting behavior and nest building have been reported in wild zebra finches in response to rainfall [Immelmann, 1971], we were interested in testing whether cGnRH-II is associated with rapid activation of the reproductive axis in zebra finches.

In order to manipulate reproductive activity, we restricted water availability in captive male zebra finches for 11 weeks and subsequently restored ad libitum access. We took measurements at several levels of the HPG axis to test the hypothesis that the hypothalamus and pituitary

are tonically activated in opportunists. If all levels of the HPG axis are regulated in parallel, we expected that changes in the periphery (i.e., in gonads or plasma LH concentration) in response to water restriction would be accompanied by changes in hypothalamic GnRH (i.e., the HPG axis could be temporarily deactivated by water restriction). Conversely, if activation of the hypothalamus occurs independently of changes in the periphery, we expected that changes in the periphery would not be accompanied by measurable changes in GnRH (i.e., the GnRH system remains in a state of 'readiness' regardless of environmental conditions). Finally, because it is well-known that HPG axis activation can be inhibited by chronically elevated glucocorticoids [Harvey et al., 1984], and specifically corticosterone in birds [Holmes and Phillips, 1976], we tested whether water restriction increased corticosterone secretion in this species.

Materials and Methods

Housing and Maintenance

We purchased zebra finches from the Canary Bird Farm, Old Bridge, New Jersey and initially housed them in indoor aviaries at Princeton University. After two weeks in group aviaries, birds were moved to smaller cages (55 × 25 × 25 cm). Each cage housed two males that could see and hear each other, but could not make physical contact because of a thin plastic mesh dividing the cage into two halves. Groups of 8 birds each were housed separately in sound proof environmental chambers. Because Bentley et al. [2000] showed that zebra finches have the ability to respond to photoperiod, we housed birds on short days (8 h light:16 h dark), so that any effects we measured were the result of water availability cues and not photostimulation. Temperature inside the chambers averaged 21 °C and fans continuously circulated the air. Birds were given ad libitum finch seed (Kaytee Products, Chilton, WI) and water depending on treatment conditions (see below). The research reported here was performed under guidelines established by the Princeton University Institutional Animal Care and Use Committee.

Experimental Protocol

Birds were assigned to control (n = 11) or treatment groups (n = 12) in order to balance initial mean testis volumes between groups. Control birds had free access to ad libitum water for the entire experiment. The amount of water available to the water restricted (WR) group was slowly decreased from 5 ml/bird/week (which is greater than their average water consumption under ad libitum conditions) [Oksche et al., 1963] to 1 ml/bird/week over nine weeks. The WR group then continued to receive 1 ml/bird/week for two weeks. At the start of week 12, the WR group was given free access to ad libitum water and fresh green wheat grass for two weeks. We chose wheat grass to simulate new green growth that would follow rainfall in the wild and because it grows easily in the lab. The control group was given empty plastic containers to control for a new object in the cage.

We collected pre-treatment measures of testis volume, mass, fat, wing length. Blood was collected to measure plasma LH before the beginning of the experimental treatment (baseline). Additional measures of testis volume, mass, fat and plasma LH were collected at the end of week 7 (mid water restriction) and the end of week 11 of water restriction (end of water restriction), and two weeks after ad libitum water was returned to the WR group (ad lib water). Baseline plasma corticosterone measurements were collected at the end of water restriction.

ICC and Testis Histology

Brain tissue was collected to measure GnRH immunoreactivity using immunocytochemistry (ICC) before treatment (n = 5), at the end of water restriction (n = 6), and after ad libitum water was returned to the restricted group (n = 5 controls, n = 6 WR group). Birds were killed by first being deeply anaesthetized and then perfused intracardially with heparinized saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). Brains were removed, postfixed for 24 h, then cyroprotected in 30% sucrose overnight. Both brain and gonadal tissue were flash frozen on dry ice and stored at -80 °C. Brain tissue was cut serially into 50-µm sections in the coronal plane using a cryostat and alternate sections were collected. In order to balance variation in staining across ICC runs, 1–2 brains from each group were processed together in each run. Free-floating sections were washed in 0.3% hydrogen peroxide, and then in 0.1 M PBS. Sections were incubated in 2% normal goat serum in 0.2% PBS containing 0.3% Triton X-100 (PBS-T) for 1 h at room temperature, and then overnight at 4 °C. Sections were then incubated in primary antibody (diluted 1:10,000) for 72 h at 4 °C, and washed in 0.1% PBS-T. The primary antibody was donated by H. Urbanski and was a polyclonal rabbit anti-GnRH antiserum which has been validated for use in birds [Cho et al., 1998; MacDougall-Shackleton et al., 2001]. Sections were then incubated in biotinylated secondary antibody (goat anti-rabbit IgG diluted 1:250 in 0.1% PBS-T) for 1 h, and washed in 0.1% PBS-T. The antibody-antigen complex was localized using the avidin biotin horseradish peroxidase method (Vectastain ABC Elite Kit; 1:200 in 0.1% PBS-T), followed by two washes in 0.1% PBS-T and one wash in 0.1 M PBS. Sections were visualized with diaminobenzidine tetra-hydrochloride (DAB, Sigma). The reaction was terminated with 5 washes in 0.1 M PBS, sections were mounted on gelatin coated slides, and left to dry overnight. Slides were then dehydrated and cover slipped.

Testis tissue was fixed during perfusion, cryoprotected and frozen in the same way as brain tissue. To characterize spermatogenesis, testes were cut serially into 25 µm sections in the coronal plane using a cryostat and directly mounted onto slides (Fisher Superfrost® Plus). We collected approximately 30 sections from the widest section of both testes. Slides were immersed in 0.25% cresyl violet acetate (Aldrich) for 5 min followed by an acetic acid rinse (0.01%) and serial dehydration in alcohol. Background was cleared using two 2-min washes in xylene (Fisher). Slides were coverslipped with Permount.

Measurements

To assess reproductive development, we measured the length and width of the left testis to the nearest millimeter by unilateral laparotomy under light general anesthesia (Isoflurane, Abbott Laboratories, Chicago, IL). Volume of the testis was calculated from the formula for an ovoid sphere: $V = 4/3 \pi a^2 b$, where V is volume,

a is the radius of the testis at its widest point and b is half the long axis. To gauge whether water restriction influenced testis function, we measured the proportion of individuals with spermatozoa lining the seminiferous tubules, the proportion with measurable lumen within the tubules, and the proportion with phagocytes in the interstitial spaces between tubules, suggesting a degradation of Leydig cells [Murton and Westwood, 1977, their fig. 3.5a]. We also ranked the numbers of sperm, degree of lumen space and numbers of interstitial phagocytes on a scale of 0 to 3, with 0 having none of these structures present and 3 having several.

To measure circulating concentrations of LH and corticosterone, blood was collected into micro-capillary tubes after puncturing the wing vein with a 26-gauge needle. For measurements of LH, approximately 150 μ l of whole blood were collected from each individual at each sampling time. We calculated hematocrit as the percentage of packed red blood cells within each micro-capillary tube. For corticosterone, 75 μ l of whole blood were collected within 3 min of opening the chamber door. Samples for LH and corticosterone were never collected on the same day. Plasma was separated by centrifugation, collected with a Hamilton syringe and stored at -20°C until assay. Plasma LH was measured using a micromodification of the radioimmunoassay originally devised by Follett and colleagues [1975]. Plasma samples were measured in a single assay in 15 μ l duplicates to eliminate interassay variation. Intra-assay variation was 4.9% and the detection limit was 0.039 ng/ml. The radioimmunoassay protocol used to measure corticosterone is described by Wingfield et al. [1992]. Samples were measured in a single assay and average recovery values were 80%. Intra-assay variation was 23% and the lower detection limit was 1.38 ng/ml.

Mass was measured to the nearest 0.1 g on a Pesola spring scale. Fat score was quantified in the furculum and abdomen areas using a five-point scale [Wingfield and Farner, 1978], in which 0 = no visible fat and 5 = gross bulging fat. Total wing length, tarsus length and head-bill length were measured using calipers to the nearest 0.1 mm.

Data Analysis

In order to measure ir-GnRH, we collected brain tissue before water restriction (baseline), after water restriction (water restriction) and after water was returned to ad libitum conditions (ad lib water and control). Therefore, sample sizes decreased as the experiment progressed. In order to test for differences in condition, testis volume and LH between groups over time, we used only birds that remained at the end of the experiment ($n = 5$ controls and $n = 6$ WR). We calculated averages for all measurements taken during week 7 and week 11 of water restriction which are shown as the 'water restriction' time point.

Body Measures. We used repeated measures analysis of variance (ANOVA) to test for differences in mass, fat score and hematocrit between groups and across time. Because absolute testis volume could potentially vary with body size, we tested for differences in body size between groups by calculating a size index using Principal Component Analysis. We included tarsus length, head-bill length and the average mass over all sampling times, and the first factor was used as an index of structural size. The first factor explained 82.9% of the variance and the loading for each measurement was above 0.78. Because there were no differences in body size index between WR and control groups ($t(9) = 0.28$, $p > 0.70$), we did not consider body size in further analyses.

Reproductive Morphology and Hormones. Because the variation was unequal across groups we log-transformed both testis volume and LH, and used repeated measures ANOVA with treatment group as a between subjects variable and time as a within subjects variable. When we found significant main effects or interactions, we conducted post hoc tests. Because our sample sizes were small, instead of testing between group differences at each time point, we calculated two difference scores using log-transformed data: the change in each variable during water restriction (water restriction minus baseline) and during renewed access to ad libitum water (ad lib water minus baseline) and performed post hoc independent t tests with a Bonferroni correction. The laparotomy of one bird was not successful during the baseline measure, so we substituted the mean for the group at that time point. To test whether our water restriction protocol activated secretion of corticosterone, we compared baseline circulating corticosterone concentrations after water restriction between groups using an independent t test. The intra-assay variation (variation between replicate plasma samples) was above 15% (see above), but our group sizes were too small to omit data points with especially high variation. Instead, we tested data resulting from average replicates and from randomly selected replicates. Both analyses yielded similar results, so the former are reported. To test for differences in testis function, we used nonparametric statistics for measures of proportion (Fisher's exact test) and categorical ranks (Kruskal-Wallis test), and when assumptions of normality and equal variance were met, parametric statistics for tubule area (ANOVA). Similarly, we tested for associations between testis volume and tubule area using Pearson correlation, and between testis volume and categorical ranks using Spearman's rho.

GnRH Measures. The neuroanatomical distribution of GnRH containing neurons is largely conserved across vertebrate groups [Muske, 1993; Millam et al., 2003]. cGnRH-I and cGnRH-II are found in discrete areas; therefore, we used location and morphology to distinguish cGnRH-I and cGnRH-II. cGnRH-I cell bodies were found in the preoptic area and cGnRH-II in the midbrain. Slides were examined using bright-field light microscopy. Total numbers of cells were counted and cell area calculated in all sections from the tractus septomesencephalicus (TSM) caudally through the midbrain. The counter was blind to the treatment group of all images. We also used Image-J to calculate the density of fibers in the median eminence relative to background. We cannot distinguish between GnRH-I and GnRH-II fibers in this area, but it is likely that most of the fibers terminating in the median eminence project from GnRH-I cell bodies [Mikami et al., 1988; Millam et al., 1993; van Gils et al., 1993; but see Clerens et al., 2003]. We tested for differences among groups using one-way ANOVA.

We graphed all data as means \pm one standard error of the mean, and differences were considered significant when $p < 0.05$.

Results

Body Measures, Hematocrit, and Corticosterone

Both groups decreased in mass over time (fig. 1a; $F_{(2, 18)} = 10.39$, $p = 0.001$, time \times group, $p > 0.20$), and mass was similar between control and water restricted groups at all time points ($F_{(1, 9)} = 0.79$, $p = 0.40$). Fat score did not change significantly over time (fig. 1b; $F_{(2, 18)} =$

0.86, $p = 0.44$, time \times group, $p = 0.60$) and there were no differences between groups at any time point ($F_{(1,9)} = 0.01$, $p = 0.92$).

Hematocrit increased significantly over time (fig. 1c; $F_{(2,20)} = 17.21$, $p < 0.001$, time \times group, $p = 0.50$) and there were no differences between groups at any time point ($F_{(1,10)} = 0.02$, $p = 0.90$). Specifically, hematocrit increased (i.e., the proportion of plasma decreased) from the initial measurement and remained elevated through the end of the experiment (Tukey's post hoc tests, $p < 0.01$). After water restriction, baseline corticosterone concentrations were low and similar between groups (2.83 ± 1.20 ng/ml in controls and 2.31 ± 1.53 ng/ml in water restricted birds; $t(7) = 0.51$, $p = 0.63$, $n = 3$ and 6 , respectively).

Testis Volume and Plasma LH

Although there was a trend for WR birds to decrease testis volume from baseline while control birds remained unchanged (table 1 and fig. 2a; time \times group interaction $F_{(2,18)} = 3.12$, $p = 0.07$; effect of group, $p > 0.10$), these differences were not significant. Because we were attempting to reduce testis volume with our treatment, the

Fig. 1. Body condition measures before (baseline), during water restriction (water restriction) and after two weeks of restored ad libitum water (ad lib water). Body mass decreased during the study but was similar between water restricted (WR, open symbols, $n = 6$) and control groups (a, filled symbols, $n = 5$). Fat score did not change in either group during the study (b). Hematocrit increased during the study but was similar between WR and control groups (c). All data are mean \pm standard error of the mean.

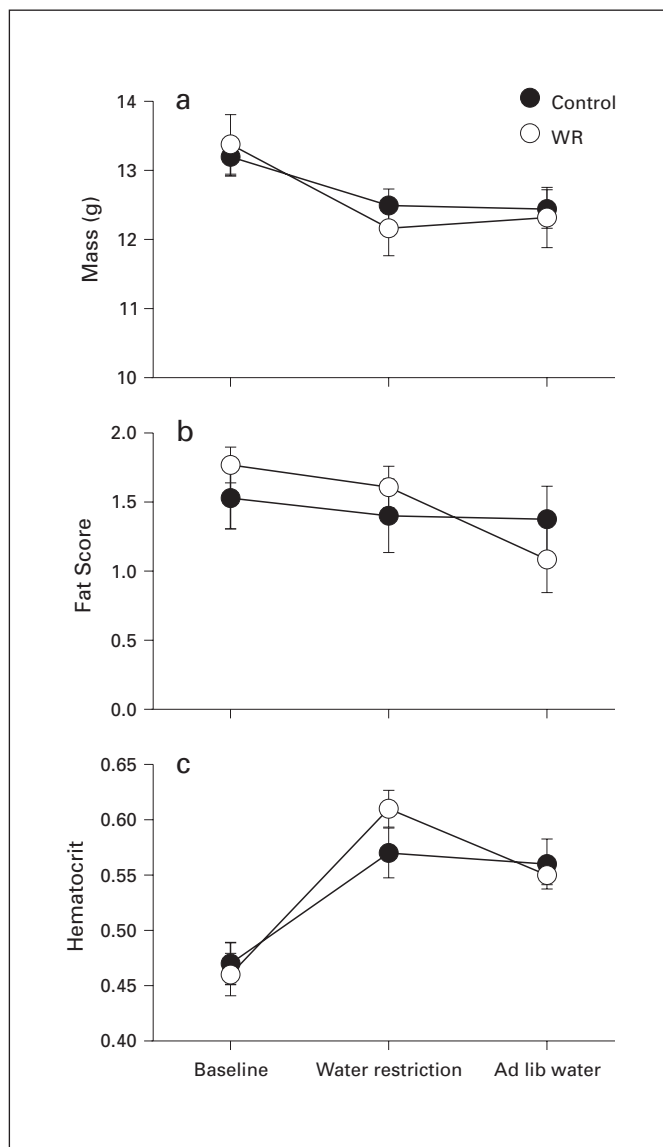


Table 1. Average testis volume and LH concentration (\pm SEM)

	Testis volume (mm^3)		LH concentration (ng/ml)	
	Controls (n = 5)	Water restricted birds (n = 6)	Controls (n = 5)	Water restricted birds (n = 6)
Baseline	12.91 \pm 4.06	15.19 \pm 5.09	0.81 \pm 0.37	0.81 \pm 0.37
Water restriction	16.48 \pm 1.40	9.96 \pm 5.67	1.36 \pm 0.86	0.39 \pm 0.25
Ad lib water	16.27 \pm 2.34	8.27 \pm 2.91	1.13 \pm 0.61	1.22 \pm 0.40

Before water restriction, during restriction and following return to ad lib conditions.

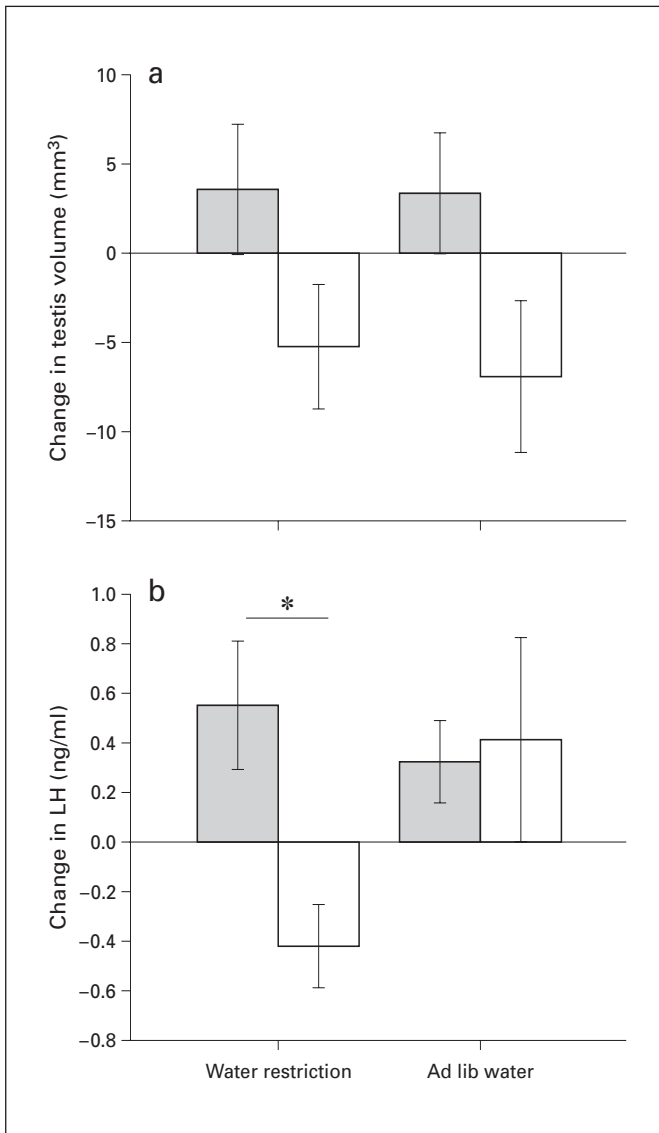


Fig. 2. Changes from baseline in testis volume and plasma LH concentration during water restriction (water restriction) and after two weeks of restored ad libitum water (ad lib water). Testis volume (**a**) tended to decrease in the WR group (white bars, $n = 6$) both during water restriction and after ad libitum water was restored, whereas there was no change in control birds (gray bars, $n = 5$). Plasma LH concentration (**b**) decreased with water restriction and returned to baseline with two weeks of restored ad libitum water in the WR group. All data are mean \pm standard error of the mean. * = Statistically significant.

initial testis volume of each individual was important as birds with low initial testis volumes are unlikely to reduce testis size further. Data from individual birds in each treatment (including birds sacrificed for GnRH analyses) are shown in figure 3. Although control birds showed both

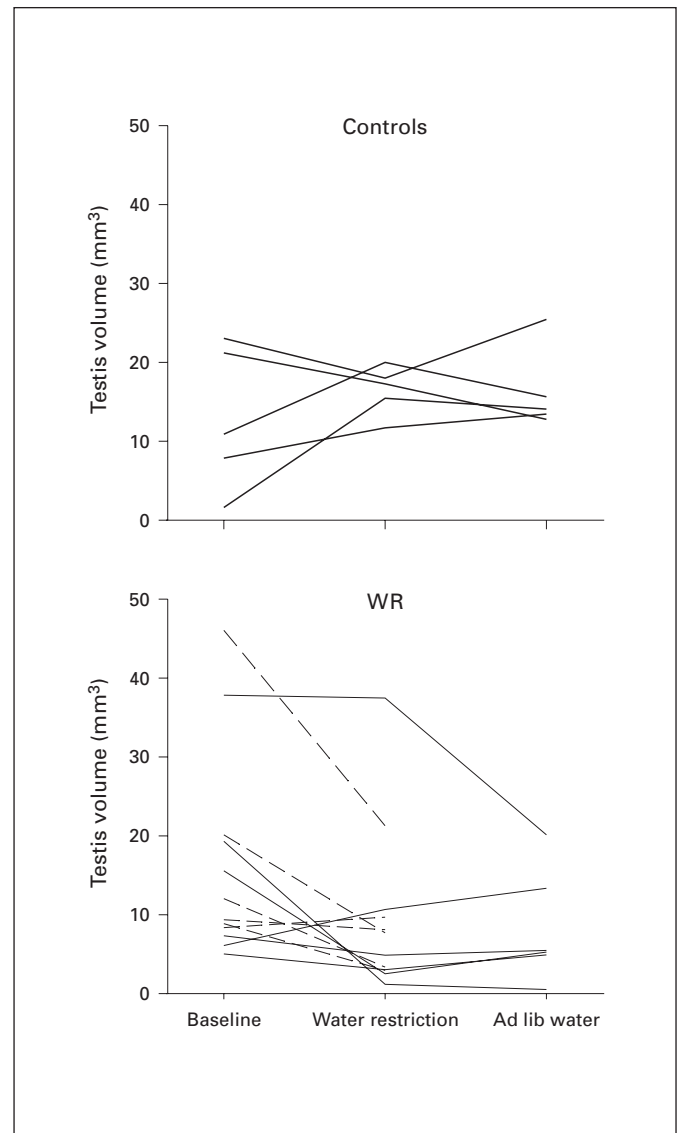


Fig. 3. Individual changes in testis volume in controls (top panel, $n = 5$) and in WR birds (bottom panel, includes birds sacrificed for GnRH analysis at the end of water restriction, broken lines $n = 6$, and WR group, solid lines, $n = 6$).

increases and slight decreases in testis volume over time, all but one of the WR birds decreased or maintained similar testis volume during water restriction (sign test, $p = 0.04$). Birds with the smallest testis volumes were likely to maintain small gonads in the WR group.

Circulating LH concentrations changed differently over time in WR birds and in controls (table 1 and fig. 2b; time \times group interaction $F_{(2, 18)} = 5.63$, $p = 0.01$; time effect $F_{(2, 18)} = 6.17$, $p = 0.01$ and group effect $p > 0.50$).

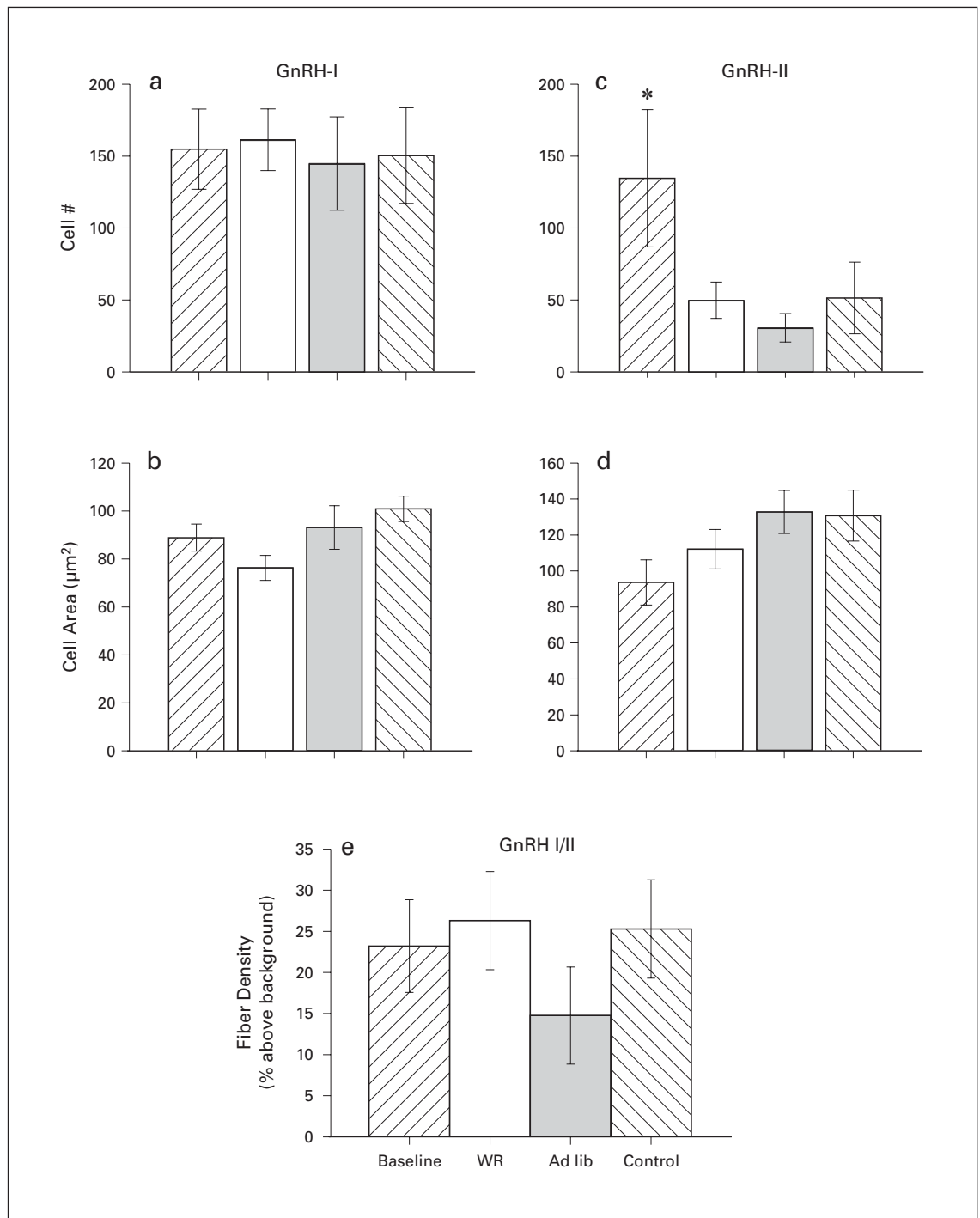


Fig. 4. Immunoreactive cGnRH-I (left) and cGnRH-II (right) measures before (baseline) and after water restriction (WR) and after two weeks of restored ad libitum water (ad lib and control). The number (**a**) and area (**b**) of ir-GnRH-I cells did not change with water restriction. The number (**c**) of ir-cGnRH-II cells was reduced with water restriction, but cell numbers also decreased in controls. The area (**d**) of ir-cGnRH-II was not affected by water restriction. Density of ir-GnRH fibers in the median eminence (**e**) did not change with water restriction. All data are mean \pm standard error of the mean. * = Statistically significant.

Table 2. Measures of testis function before, during and after water restriction

	Tubule area	Sperm, %	Lumen, %	Phagocytes, %
Baseline	15.83 ± 1.44	100 (2/2)	100 (2/2)	100 (2/2)
Water restricted	17.37 ± 3.93	66 (4/6)	66 (4/6)	100 (6/6)
Ad lib water	24.43 ± 5.88	83 (5/6)	83 (5/6)	83 (5/6)
Control	18.61 ± 3.95	80 (4/5)	80 (4/5)	100 (5/5)

Numbers in parentheses represent the number of birds with each parameter present out of the total for that group.

Plasma LH increased slightly from baseline at both time points in controls, whereas it decreased in WR birds with water restriction and then returned to levels similar to baseline after two weeks of ad libitum water. The change in LH concentrations was significantly different between the two groups after water restriction ($t(9) = 3.06$, $p = 0.01$).

ir-GnRH

Water restriction did not affect *ir-GnRH-I* cell number (fig. 4a; $F_{(3, 21)} = 0.06$, $p = 0.98$) or cell area (fig. 4b; $F_{(3, 21)} = 2.38$, $p = 0.10$). There was a significant decrease in the number of *ir-GnRH-II* cells in all time points measured after baseline (fig. 4c; $F_{(3, 21)} = 3.07$, $p = 0.05$), but cell area did not change significantly (fig. 4d; $F_{(3, 19)} = 2.06$, $p = 0.15$). At the median eminence, fiber density was similar between groups as well (fig. 4e; $F_{(3, 19)} = 0.78$, $p = 0.52$).

Testis Histology

Using our measures to estimate testis function (tubule volume, presence of sperm flagella, tubule lumen and phagocyte cells in the interstitium), there were no differences among treatments (table 2). Furthermore, testis volume was not correlated with any of the testis function measures (tubule area $\rho = 0.36$, $p = 0.13$, all spermatocyte, lumen and phagocyte scores $p > 0.20$).

Discussion

This experiment attempted to test directly the hypothesis that opportunistically breeding zebra finches maintain a tonically active hypothalamo-pituitary axis regardless of environmental conditions [Farner and Serventy, 1960; Farner, 1967]. Our data confirm that some peripheral components of the HPG axis responded to water availability, such as LH secretion and to a lesser extent

testis volume. However, despite a tendency for reduction in testis volume, spermatogenesis was maintained. Furthermore, in support of the Farner and Serventy hypothesis, there were no changes in *cGnRH-I-ir*. Interestingly however, we did observe changes in *cGnRH-II*, but we could attribute these to either water restriction or other factors (see below).

Effects on the Reproductive Axis without Effects of Stress

Complete regression, along with a seasonal loss of testis function, can occur within a few weeks in seasonal breeders [Bentley et al., 1997, 1998]. We feel confident that the combined effects of the water restriction treatment, exposure to short day lengths and isolation from female conspecifics were sufficient to cause complete regression within 11 weeks, if it was going to occur at all. By very slowly restricting water availability over nine weeks, we were able to influence parts of the reproductive axis without activating increased secretion of glucocorticoids, one measure of the perception of stressful stimuli [Wingfield and Romero, 2001]. This makes us confident that our results are not due to stress, but rather to the action of an environmental cue (low water availability) that is commonly experienced in the arid and semi-arid habitats of Australia where zebra finches breed [Immelmann, 1971; Zann et al., 1995].

Zebra finches possess physiological adaptations to cope with drought, and indeed in the lab are able to survive for at least 250 days after complete withdrawal of drinking water [Cade et al., 1965; Lee and Schmidt-Nielsen, 1971; Sossinka, 1974]. Cain and Lien [1985] found increased corticosterone secretion in bobwhite quail (*Colinus virginianus*) after nine days of abrupt water restriction. Perhaps rapidly withdrawing water is more likely to activate the stress axis and corticosterone secretion as this is a much more drastic treatment representing a less realistic scenario in free living animals. Our mea-

tures of body condition were also not affected by water restriction. Water restricted birds had increased hematocrit, but no more than control birds with free access to water. It is therefore likely that these data are the result of some other common experimental parameter and not water restriction per se. Similarly, both groups lost mass during the experiment which is likely due to the transition from flight aviaries to smaller cages and/or to repeated laparotomies over time [Bentley et al., 2000]. These data suggest that we were measuring reproductive adjustments to a relevant environmental cue that was not mediating its effect through a long-term activation of the stress axis.

Water Restriction and Peripheral Measures of Reproduction

Zebra finches responded to restricted water availability by reducing gonadal volume by approximately 46%. However, changes in organ size did not result in consistent changes in testis function (table 2). In early studies, Oksche and colleagues [1963] and Sossinka [1974] found no effects of dehydration on testis volume, but these studies did not follow individuals over time, but compared independent groups. Vleck and Priedkalns [1985] withdrew water over 12 days (to 1 ml/bird/week) and found that testis length decreased with dehydration and increased with return to ad libitum water alone or with water plus humidity or green grass. We withdrew water at a slower rate than Vleck and Priedkalns (63 days in the present study), but the data of the two studies are nevertheless consistent. If we consider only testis length in the present study, both studies showed a reduction of testis length by approximately 20%. With two weeks of abrupt water restriction (1 ml/bird/week) followed by a return to ad libitum water availability (alone or together with increased humidity or green grass), Priedkalns and colleagues [1984] found an increase in testis length with water alone or together with increased humidity or green grass.

In contrast to previous studies, we did not find a significant increase in testis volume following return to ad libitum conditions. When significant increases have been found [Priedkalns et al., 1984; Vleck and Priedkalns, 1985], males were housed together with females, whereas in the present study males were isolated from females. It is unlikely that we would have found an increase in testis volumes if we had waited one more week because there was no trend for an increase nor an increase in variability after two weeks. Numerous studies have shown that social cues can enhance effects of stimulatory environ-

mental cues on gonadotropin secretion, reproductive development and reproductive behaviors [reviewed by Wingfield and Farner, 1993; Wingfield et al., 1997; Tramontin et al., 1999], and it is likely that social interactions in previous studies augmented the rate of testis development after free access to water was restored.

Functional and Behavioral Consequences

During drought conditions or during periods of low food availability, zebra finches are thought to travel over large areas [Serventy, 1971; Zann and Straw, 1984; Rozman et al., 2003]. The ability to reduce testis volume without compromising sperm production may be part of a suite of physiological responses to drought that help to reduce body mass and redirect energy reserves during irruptive movements, although this remains to be tested. Our finding that some sperm production was maintained regardless of water availability (table 2) is consistent with previous studies in zebra finches [Priedkalns et al., 1984; Vleck and Priedkalns, 1985], as well as in the bobwhite quail. Cain and Lien [1985] experimentally elevated corticosterone levels in bobwhite quail to those found in individuals under water restriction. Despite significantly reduced testis weight, sperm production continued. However, this scenario might not be true for all opportunistic breeders. Hau et al. [2004] suggested that island-dwelling Darwin's finches (*Geospiza fuliginosa*) may in fact completely shut down the reproductive axis during long periods of drought as the opportunity to search for more favorable habitat is severely restricted on these oceanic islands. The ability to maintain functional gonads during drought conditions might be a common characteristic of many opportunistic breeders that have the ability to be nomadic.

One common assumption is that opportunistic breeders should be able to perceive and respond rapidly to changing environmental conditions. Anecdotal reports of stimulation of courting behavior and nest building in wild zebra finches following rainfall initially supported this idea [Keast and Marshall, 1954; Immelmann, 1971]. However, more recent long-term studies show that there is more commonly a lag of 2–3 months (depending on time of year) between significant rains and the initiation of breeding [Zann et al., 1995]. It is unclear in the present study how quickly males detected increased water availability. Increased gonadotropin concentration in the plasma has often been used as one measure of reproductive activation. The perception of long day lengths in highly photoperiodic species can be detected by an increase in plasma LH after a single long day [e.g., Meddle and Fol-

lett, 1995]. Similarly, restoration of ad libitum food availability following food restriction in mammals causes a rebound of LH pulse frequency within hours [Sisk and Bronson, 1986]. A rapid behavioral response to a non-photic cue, food supplementation, has been found in birds. Live insect prey increases singing behavior within a few days of supplementation [Hau et al., 2000]. In the present study, plasma LH concentrations in water restricted birds decreased with water restriction and returned to baseline levels following access to ad libitum water and green grass for two weeks. These findings contrast with previous studies that did not find correlations between water availability and LH concentrations [Priedkalns et al., 1984; Vleck and Priedkalns, 1985]. We suggest that in previous studies when birds were housed as pairs, social interactions may have maintained elevated LH concentrations.

Tonic Activation of ir-GnRH

Even though we found a significant reduction in circulating LH after water restriction, ir-cGnRH-I was not significantly altered in the brain according to our measurements using ICC. We found no change in either ir-cGnRH-I neuron cell number, area or fiber density of GnRH neurons in the median eminence. This suggests that the reproductive axis was not deactivated at the level of the hypothalamus and supports the Farner and Serventy hypothesis. It is important to keep in mind that measurements of ir-GnRH by ICC do not give direct information about changes in production or secretion of the protein. We did not measure the GnRH precursor (pre-pro-GnRH) to get a closer approximation of GnRH processing and or secretion [Parry et al., 1997]. It is possible that pre-pro-GnRH production or processing was decreased together with decreased release, resulting in no net change in ir-GnRH peptide. In north temperate species of highly flexible breeders such as the male white-winged crossbill (*Loxia leucoptera*), GnRH-I-ir also remains unchanged over a seasonal cycle despite peripheral changes in gonadal volume [MacDougall-Shackleton et al., 2001]. In females of this species there are some seasonal changes in ir-GnRH-I, but the magnitude of change in cell number or fiber area is much less dramatic when compared to seasonal breeders. In sharp contrast, ir-GnRH cell numbers and fiber density [Foster et al., 1987; Goldsmith et al., 1989; Hahn and Ball, 1995], and most importantly, GnRH content in the hypothalamus [Dawson et al., 2001] decreases dramatically in seasonal breeders during the time of year when the gonads are regressed. An alternative hypothesis for our findings is that the

changes in the periphery that we measured were the result of a decrease in sensitivity of the anterior pituitary to GnRH stimulation, so an unchanged cGnRH-I signal elicited decreased LH secretion with water restriction. This seems unlikely given that pituitary sensitivity to exogenous cGnRH-I remains unchanged over a seasonal cycle in strongly photoperiodic species [Wingfield et al., 1979]. Injections of exogenous GnRH in water restricted zebra finches would answer this question.

There have only been few documented examples of environmental cues influencing cGnRH-II immunoreactivity [Rastogi et al., 1997; Bluhm et al., 2000; Temple et al., 2003] and the role of cGnRH-II in reproductive physiology remains unclear. In the present study, ir-cGnRH-II cell numbers decreased after our baseline sample in all groups. This finding is interesting because it has been that proposed cGnRH-II is involved in reproductive behaviors in female mammals in response to food cues [Temple et al., 2003; Kauffman and Rissman, 2004] and cGnRH-II administration can enhance reproductive behavior in female birds [Maney et al., 1997]. Although there are several potential explanations for this effect, birds collected at these sampling times had all been housed in single sex groups for at least 11 weeks. Thus, a hypothesis worth testing would be that cGnRH-II plays a role in rapid modulation of reproductive behavior in male zebra finches.

Conclusions

Opportunistic breeding may be accompanied by adaptations in reproductive physiology that allow considerable flexibility in the regulation of reproduction. Although zebra finches are able to respond to changes in photoperiod [Bentley et al., 2000], they are particularly sensitive to environmental cues that give more immediate information about environmental conditions. Water availability appears to be one such cue that can cause changes in the periphery of the reproductive axis (i.e., at the level of the pituitary and gonads). However, as hypothesized by Farner and Serventy [1960] it appears that the hypothalamus remains prepared to respond to favorable conditions even when cues should be inhibitory. Reduction of testis volume during drought might be an adaptation to help reduce mass and conserve energy for nomadic migrations, but it appears that zebra finches are able to maintain spermatogenic activity even under such severe environmental conditions.

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