Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2012) **81**, 1327–1339
doi:10.1111/j.1095-8649.2012.03411.x, available online at wileyonlinelibrary.com

Measuring water-borne cortisol in *Poecilia latipinna:* is the process stressful, can stress be minimized and is cortisol correlated with sex steroid release rates?

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(Received 14 April 2012, Accepted 22 June 2012)

The stress of water-borne hormone collection process was examined in sailfin mollies *Poecilia latipinna*. Baseline release rates of the stress hormone cortisol were measured and minimum confinement time for water sampling was evaluated for a standard 60 min *v*. a 30 min protocol. A 30 min hormone collection period reflects release rates over 60 min. Potential stress response to confinement in the beaker for the water-borne collection process was tested over 4 days. There was no evidence of stress due to the collection methods, as cortisol release rates did not differ significantly across four sequential days of handling for *P. latipinna*. Males and females did not differ significantly in baseline cortisol release rates. Baseline cortisol release rates from fish immediately after being collected in the field were also not significantly different than those in the 4 day confinement experiment. After exposure to a novel environment, however, *P. latipinna* mounted a stress response. Stress may also affect sex steroids and behaviour but cortisol release rates were not significantly correlated with sex steroids [11-ketotestosterone (KT), testosterone, or oestradiol], or mating attempts. The correlation between water-borne release rates and plasma steroid levels was validated for both cortisol and KT. Finally, normalizing cortisol release rates using standard length *in lieu* of mass is viable and accurate. Water-borne hormone assays are a valuable tool for investigating questions concerning the role of hormones in mediating stress responses and reproductive behaviours in *P. latipinna* and other livebearing fishes.

Key words: hormone; live bearing fish; sailfin mollies; validation.

INTRODUCTION

Glucocorticoids are stress hormones released in response to stressors such as the threat of predation, social conflicts and confinement. Repetitive exposures to such stimuli can lead to decreased growth, immune responses and reproductive behaviours (Sapolsky *et al*., 2000). Glucocorticoids can be released within the first 10 min of a stressor, but generally actions are not exerted for *c*. 1 h after the onset of the stressor (Sapolsky *et al*., 2000). In birds and reptiles, however, glucocorticoids can have rapid effects on behaviour (from seconds to minutes) (Orchinik *et al*., 1991; Moore & Orchinik, 1994). For example, within a few minutes stress responses such as

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diversion of energy to muscles, inhibition of reproductive physiology and decreased feeding are observed (Sapolsky *et al*., 2000).

In fishes, the energetic costs of stress have been found to affect reproductive processes (Schreck, 2010). Part of the reason is because glucocorticoids have the potential to alter the production of other hormones (Scott *et al*., 2008; Milla *et al*., 2009). Downstream effects of increased glucocorticoid release can include decrease rates of the production of sex steroids. Milla *et al*. (2009), however, found glucocorticoids to have either deleterious or positive effects on fish reproduction. This is because it is important to distinguish between acute and chronic levels of stress (Sapolsky *et al*., 2000). For example, mild stress can enhance reproductive performance (Schreck *et al*., 1997; Schreck, 2010) while severe, frequent or prolonged stressors can have negative effects on reproductive processes (Milla *et al*., 2009; Schreck, 2010). It is not always clear where the bounds of high and low stress lie. As such it is important to establish the baseline glucocorticoid levels for each organism and examine how this changes with repeated sampling (Wong *et al*., 2008). It is also important to explore the relationship between glucocorticoid levels and sex steroid levels for each species being studied. If stress alters the production of other sex steroids, then it is important to evaluate possible stressors presented during handling and hormone sampling.

Drawing hormones from plasma can be a major stressor upon a fish or require killing a small fish. Though less invasive and possibly less stressful, water-borne hormone collection techniques could also cause levels of cortisol (the primary glucocorticoid in fishes) to increase (Scott *et al*., 2008; Wong *et al*., 2008). The less invasive water-borne technique has been an accepted method of quantifying the circulating hormones released in response to a stimulus (Scott & Ellis, 2007) and in fishes, a positive correlation has been found for plasma cortisol levels and for cortisol collected from the water (Sebire *et al*., 2007; Scott *et al*., 2008; Wong *et al.,* 2008). Nonetheless, Wong *et al*. (2008) found that initial confinement to a beaker to measure individual water-borne hormone levels in convict cichlids *Amatitlania nigrofasciata* (Günther 1867) is stressful, but the *A. nigrofasciata* could be habituated by exposing them repeatedly to the water-borne hormone collection process.

The water-borne hormone collection method was used to assess whether this method poses a stressor or handling effect on a small live-bearing fish, the sailfin molly *Poecilia latipinna* (LeSueur 1821). The water-borne hormone collection method has been used previously with *P. latipinna*. Gabor & Grober (2010) found that male and female *P. latipinna* increased 11-ketotestosterone (KT) production when they mated but not when they were paired together without mating. They did not find such a response with testosterone (T) or oestradiol (E2). Gabor & Grober (2010), however, did not examine if there is a correlation between blood plasma KT and water-borne KT release rates nor whether cortisol levels affected sex steroid levels and male mating behaviour.

To decrease the potential stress of sampling, the minimum confinement time to obtain cortisol concentrations that accurately reflect circulating levels in response to a stressor was determined for a 60 min *v*. a 30 min protocol. Next, the potential stress response to confinement in the beaker over 4 days was examined for the water-borne collection process. Third, to determine the effect of baseline cortisol release rates on other sex steroids the correlation between cortisol and T, E2 and KT was examined. For the fourth study, the correlation between free plasma and

free water-borne KT and cortisol release rates was validated. For the fifth study, the potential stress response to exploring a new environment was examined. Finally, cortisol was measured from field-collected *P. latipinna* and then cortisol release rates across the studies were compared.

MATERIALS AND METHODS

Poecilia latipinna were collected from Spring Lake, Hays County, Texas (29° 53′ 24″ N; 97° 49′ 12″ W) in April to May 2011 for experiment 1 and studies 2 and 4. *Poecilia latipinna* from experiment 1 and studies 2–5 were maintained on 14 L:10 D cycle using UV lighting to simulate daylight and fed Purina Aqua Max 200 (www.purina.com) twice a day and live brine shrimp *Artemia* sp. once per day. *Poecilia latipinna* in studies 2, 4 and 5 were fed Aqua Max 1 h before the respective study while those for experiment 1 were fed 15 min before testing. *Poecilia latipinna* from all studies were fed Aqua Max and *Artemia* sp in the evening between 1600 and 1730 hours. *Poecilia latipinna* from experiment 1 and studies 2–5 were housed in single sex groups for 30 days prior to testing in 38 l aquaria. Individual *P. latipinna* were isolated in 19 l aquaria for 20 h prior to each experiment (1–5). Hormones were collected from *P. latipinna* in experiment 1 and studies 2–5 from 0900 to 1100 hours to control for diurnal hormonal fluctuations (Lorenzi *et al*., 2008).

EXPERIMENT 1: 30 MIN *v*. 60 MIN HORMONE COLLECTION

Male and female *P. latipinna* were individually confined ($n = 8$ per sex) in 250 ml hormone collection beakers with 100 ml of dechlorinated water (hormone collection set-up) for 30 or 60 min on day 1. A cut Nalgene bottle (high-density polyethylene; HDPE) with holes was placed at the bottom of each beaker (Fig. 1). Each beaker was set within a box to reduce visual stimuli. Following each treatment, the Nalgene cup was lifted out leaving the water behind and the *P. latipinna* was returned to the isolation tanks and then tested in the opposite treatment on day 2. These data were used to examine whether cortisol release rates doubled from the 30 to the 60 min collection period. This study was performed in May 2011.

STUDY 2: FOUR DAY REPEATED HORMONE COLLECTIONS

Male ($n = 16$) and female ($n = 8$) *P. latipinna* were individually confined in the hormone collection set-up for 30 min. Each beaker was set within a box to reduce visual stimuli. Following each trial, *P. latipinna* were returned to their isolation tanks and trials were repeated with the same individual the following day for 4 days to examine whether cortisol release rates changed across the 4 days. This study was performed in June 2011.

STUDY 3: CORRELATION BETWEEN CORTISOL, SEX STEROIDS AND BEHAVIOUR

For this study, hormone samples were obtained and analysed from samples previously collected by Gabor & Grober (2010) who had examined KT, E2 and T from male *P. latipinna*. Gabor & Grober (2010) collected *P. latipinna* in Tamaulipas, Mexico (25° 06′ 36″ N; 97° 33′ 36″ W). The hormone samples originated from male *P. latipinna* ($n = 14$) that had been placed in the same hormone collection set-up for 1 h prior to testing for mate choice (baseline release rates). Following this, Gabor & Grober (2010) allowed males to mate (or not) with a female and recorded the total number of times males attempted to mate (thrusted their gonopodium toward females). This study was performed in September–October 2008 (all samples were stored at −20° C). For the current study, samples from Gabor & Grober (2010) were used to obtain cortisol data and to examine the correlation between baseline cortisol and previously attained values on baseline sex steroid release rates (KT, T and E2) and subsequent mating behaviour.

FIG. 1. Water-borne hormone collection set-up with a male *Poecilia latipinna* in a Nalgene cup with holes in the bottom set within a 250 ml beaker. Use of the cup allowed for easy removal of the *P. latipinna* from the water sample (gloves were worn for the experiment).

STUDY 4: CORRELATION BETWEEN WATER-BORNE HORMONE RELEASE RATES AND PLASMA HORMONE LEVELS FOR CORTISOL AND KT PLUS CORRELATION BETWEEN CORTISOL AND KT

Female *P. latipinna* ($n = 9$) were confined in the hormone collection set-up for 30 min. *Poecilia latipinna* were euthanized with an overdose of MS-222 and blood was drawn *via* a dorsal aorta puncture with a 26× g syringe, prepared with 4% sodium citrate as an anti-coagulant. Samples were centrifuged at 3000 *g* for 10 min to separate blood from plasma. Twenty microlitres of plasma were transferred into a new microcentrifuge tube and stored at −80° C until ready for processing free-hormones. The correlation between water-borne hormone release rates and plasma hormone levels was examined for cortisol and KT. The correlation between cortisol and KT release rates was also examined. This study was performed in July 2011.

STUDY 5: DO STRESSED *P. LATIPINNA* MOUNT A CORTISOL RESPONSE?

Male ($n = 4$) and female ($n = 5$) *P. latipinna* were tested in a novel environment to examine whether the novel environment resulted in *P. latipinna* mounting a cortisol response. Males were collected from Spring Lake, TX, and females were collected from McAllen, TX (26° 09′ 36″ N; 98° 13′ 48″ W). Each *P. latipinna* was confined in the hormone collection set-up for 1 h to get baseline (pre-trial) cortisol release rates the day before the experimental trial. The next day, the individuals were placed into an opaque, acclimation chamber (15 cm in diameter) for 5 min within an experimental arena (70 × 40 × 10 cm). After acclimation, a door located on the exterior of the acclimation chamber was remotely opened to permit the

P. latipinna to explore the tank. The *P. latipinna* were then placed in the hormone collection set-up for another hour (post-trial). The post-trial hormone collection was taken within 15 min of the time the pre-trial hormone collection was taken the prior day to control for diurnal hormone fluctuations. This study was performed in May 2011.

STUDY 6: COMPARISON OF BASELINE CORTISOL IN *P. LATIPINNA* ACROSS LABORATORY STUDIES AND FIELD DATA

To compare baseline release rates of cortisol in the laboratory *v.* the field, males ($n = 10$) and females ($n = 11$) were collected by dip net from Spring Lake, TX, and then immediately individually confined to the hormone collection set-up for 30 min. These samples were obtained in September 2011.

HORMONE ASSAYS

All water samples were either immediately processed for extraction of hormones or stored at -20° C for later processing. Water-borne hormones were extracted using Sep-Pak Plus C18 columns (www.waters.com) with a vacuum manifold. Total (free + conjugated) hormones were collected by eluting with methanol following the methods outlined in Gabor & Grober (2010). Residues were resuspended in 5% ethanol: 95% EIA buffer totalling 350 µl resuspension volume.

To obtain free hormones from plasma and water-borne hormones in study 4, the methods of Wong *et al*. (2008) were followed. The water-borne hormone residues were resuspended in 5% ethanol: 95% EIA buffer totaling 350 µl resuspension volume (1:1 dilution) and then further diluted to 1:10, 1:14 or 1:16 depending on the study. The plasma residues (from 20 µl plasma) were re-suspended in 5% ethanol: 95% EIA buffer totaling 2 ml of solution (1:100 dilution). All values reported are dilution adjusted.

The use of KT EIA kits (Cayman Chemical; www.caymanchem.com) for *P. latipinna* was previously validated (Gabor & Grober, 2010). To validate cortisol EIA kits (Cayman Chemical) for *P. latipinna* water samples were obtained from 10 non-experimental *P. latipinna* using collection and extraction methods similar to those described above. The re-suspended samples were combined in a concentrated pool. The pool was diluted to 1:2 for the serial dilutions and 1:10 for quantitative recovery.

Parallelism of the serial dilution curve (run in duplicate) was assessed using the pooled control for *P. latipinna*. The $\log_{10}$–logit transformed dilution curve was constructed using average per cent maximum binding and pg ml^{-1} concentrations for five dilution samples (from 1:2 to 1:32 dilution). The dilution curve was parallel to the standard curve (comparison of slopes, $t = 0{\cdot}16$, d.f.= 8, $P > 0{\cdot}05$).

The quantitative recovery of the water-extracted hormones was determined by spiking the pooled control for *P. latipinna* with each of the eight standards and running an unmanipulated pooled control sample. Expected recovery concentrations were based on the known amount of cortisol in control samples. Minimum observed recovery for *P. latipinna* was 103%. The slope of the observed *v.* expected curve was 0·89, indicating a linear relationship between observed and expected ($F_{1,7} = 223{\cdot}357$, $r^2 = 0{\cdot}97$; $P < 0{\cdot}001$).

STATISTICAL ANALYSES

The hormone data were normalized by dividing by the standard length (L_S) of the *P. latipinna*. In *P. latipinna*, L_S and mass (M) are strongly correlated (linear regression; $r^2 = 0{\cdot}97$, $n = 15$, $P < 0{\cdot}001$). To assess the difference between normalizing using L_S *v.* M, the mass of each *P. latipinna* was approximated using this linear regression for L_S and M. It was found that in each experiment there was a strong positive correlation between the release rates normalized using L_S *v.* approximate M (Table I). The L_S normalized hormone data met the assumptions of parametric analyses when ln transformed. Male and female cortisol values were compared using an unpaired t-test in experiment 1 and studies 2 and 5. To

TABLE I. Cortisol release rates across studies and populations normalized using standard length (L_S) or the approximate mass (M) of the *Poecilia latipinna*. The cortisol release rates using these two methods were significantly correlated

Study	n	Population	Mean ± S.E. L_S (mm)	Mean ± S.E. cortisol (pg L_S^{-1} h^{-1})	Mean ± S.E. cortisol (ng approximate M^{-1} h^{-1})	Pearson correlation (P value)
1. 60 min	16	Spring lake, TX, U.S.A.	37·23 ± 1·65	26·54 ± 3·09	0·89 ± 0·19	0·76 (<0·001)
2. First habituation	24	Spring lake, TX	38·17 ± 1·29	75·37 ± 12·70	2·32 ± 0·50	0·90 (<0·001)
3. Sex steroid	14	Tamaulipas, Mexico	33·29 ± 0·94	68·66 ± 23·46	2·44 ± 0·87	0·96 (<0·001)
4. Free water-borne	9	Spring lake, TX	57·89 ± 2·34	47·44 ± 16·75	0·493 ± 0·18	*1·0 (<0·001)
5a. Pre-exploratory	9	Spring lake–McAllen, TX	44·84 ± 2·00	75·99 ± 13·53	1·46 ± 0·19	*0·71 (<0·05)
5b. Post-exploratory	9	Spring lake–McAllen, TX		169·28 ± 36·89		
6. Field collected	18	Spring lake, TX	31·63 ± 0·86	53·43 ± 4·07	2·11 ± 0·46	0·80 (<0·001)

*Pearson correlation adjusted for small sample sizes.

evaluate whether the cortisol release rate at 30 reflected those at 60 min, the 30 min cortisol values were doubled and compared to the 60 min values and the 60 min values were halved and compared to the 30 min values using a paired t-test. Repeated measures ANOVA was used to evaluate changes in cortisol release rates over 4 days. Pearson's correlation was used to look at the correlation between baseline cortisol and sex steroids. A Kendall's τ was used to examine the correlation between baseline cortisol and gonopodial thrusting (mating attempts) because thrusting did not meet the assumptions of parametric analyses. Pearson's correlation adjusted for small samples sizes (Sokal & Rohlf, 1995) was used to examine the relationship between free KT and cortisol in water and the respective levels in plasma. A Kruskal–Wallis test was used to compare cortisol levels between studies followed by a Wilcoxon test because the data did not meet the assumption of parametric analyses. The baseline cortisol release rates from study 2 were compared with the baseline release rates in part 6 (t-test). To examine whether the novel environment elicited an increase in cortisol the pre-trial cortisol release rates were compared with the post-trial cortisol release rates with a t-test. Data were analysed with JMP v9.0.2 (SAS Institute; www.jmp.com/software/jmp10). Unless noted, the data met the assumptions of parametric analyses ($\alpha = 0{\cdot}05$).

RESULTS

EXPERIMENT 1:30 MIN *v.* 60 MIN HORMONE COLLECTION

Male (mean ± s.e. = 25·50 ± 5·52 pg L_S^{-1} h^{-1}) and female (27·58 ± 3·16 pg L_S^{-1} h^{-1}) cortisol release rates did not differ significantly for 30 min trials (unpaired t-test, $t = -1{\cdot}43$, d.f. = 14; $P > 0{\cdot}05$) or 60 min trials (unpaired t-test, $t = -0{\cdot}79$, d.f. = 14; $P > 0{\cdot}05$). When cortisol values were doubled for 30 min and halved for 60 min these expected values did not differ significantly from the observed values of 60 and 30 min, respectively (paired t-test, $n = 16$, 30 min: $t = 1{\cdot}42$, $P > 0{\cdot}05$; 60 min: $t = -1{\cdot}42$, $P > 0{\cdot}05$; Fig. 2). Given these results, water-borne hormone collections from fish in the subsequent studies (2, 4 and 5) were taken over 30 min.

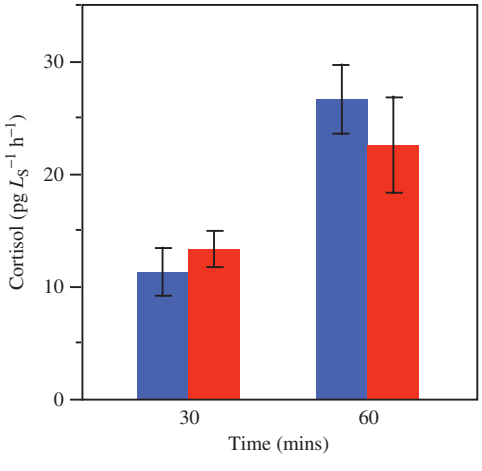

FIG. 2. Mean ± s.e. cortisol release rates at 30 and 60 min (■) as compared to expected release rates (■) of 30 min cortisol level doubled and 60 min cortisol release rate halved in *Poecilia latipinna* (L_S, standard length).

STUDY 2: FOUR DAY REPEATED HORMONE COLLECTIONS

There was no significant difference in cortisol values across the 4 days of confinement (RM ANOVA, $F_{(3,21)} = 0{\cdot}053$, $P > 0{\cdot}05$; Fig. 3). Male (mean ± s.e. = 69·99 ± 29·56 pg L_S^{-1} h^{-1}) and female (78·05 ± 12·87 pg L_S^{-1} h^{-1}) cortisol release rates also did not differ significantly (unpaired t-test, $t = -0{\cdot}85$, d.f. = 22, $P > 0{\cdot}05$).

STUDY 3: CORRELATION BETWEEN CORTISOL, SEX STEROIDS AND BEHAVIOUR

Baseline release rates of cortisol in male *P. latipinna* were not significantly correlated with premating KT release rates (Pearson's correlation: $n = 14$, $r = -0{\cdot}05$, $P > 0{\cdot}05$), E2 ($n = 14$, $r = 0{\cdot}32$, $P > 0{\cdot}05$) or T release rates (Pearson correlation adjusted for small samples; $n = 9$, $r^* = 0{\cdot}13$, $P > 0{\cdot}05$). Cortisol release rates were also not correlated significantly with the number of times males attempted to mate (Kendall's $\tau = 0{\cdot}198$, $n = 14$, $P > 0{\cdot}05$). Cortisol release rates did not differ significantly whether males mated or not (unpaired t-test, $t = 1{\cdot}21$, d.f. = 12, $P > 0{\cdot}05$).

STUDY 4: CORRELATION BETWEEN WATER-BORNE HORMONE RELEASE RATES AND PLASMA HORMONE LEVELS FOR CORTISOL AND KT PLUS CORRELATION BETWEEN CORTISOL AND KT

There was a significant positive correlation between free water-borne cortisol and free plasma cortisol [Pearson correlation adjusted for small samples sizes: $n = 9$, $r* = 0{\cdot}87$, $P < 0{\cdot}01$; Fig. 4(a)]. There was also a significant positive correlation between water-borne KT and plasma KT [Pearson correlation adjusted for small samples sizes: $n = 9$, $r* = 0{\cdot}81$, $P < 0{\cdot}05$; Fig. 4(b)]. There was no significant correlation between cortisol and KT release rates (Pearson correlation adjusted for small samples sizes: $n = 9$, $r* = 0{\cdot}69$, $P > 0{\cdot}05$).

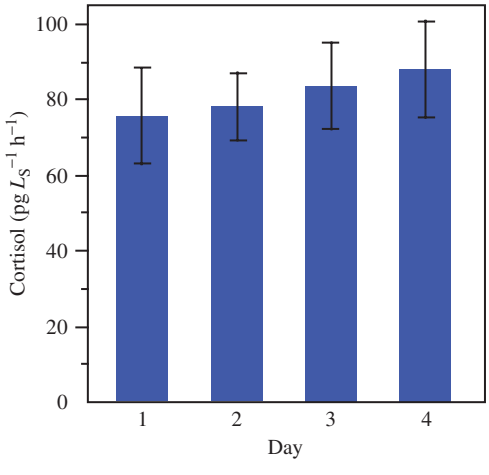

FIG. 3. Mean ± s.e. change in cortisol release rates across four successive days of repeated beaker confinement in *Poecilia latipinna* (L_S, standard length).

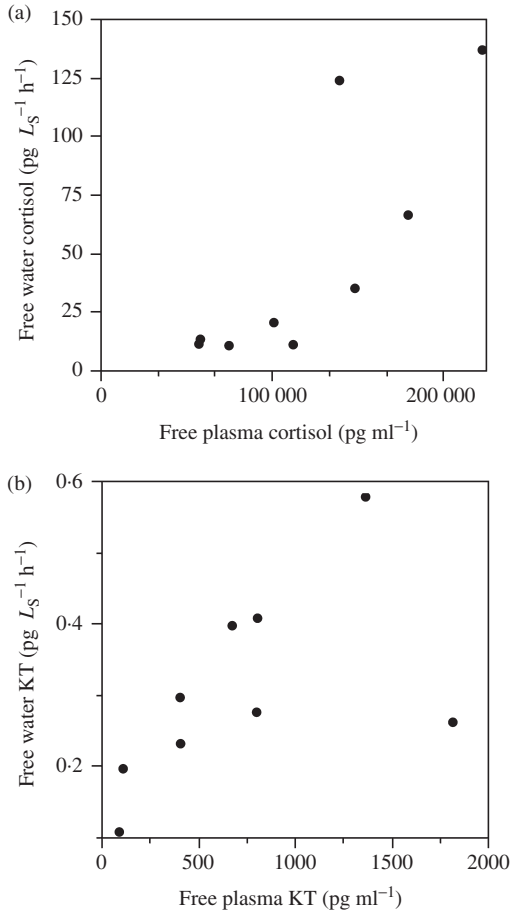

FIG. 4. Significant positive correlation in female *Poecilia latipinna* ($n = 9$) between (a) free plasma and free cortisol release rates ($r^* = 0{\cdot}87$, $P < 0{\cdot}01$) and (b) free plasma and free 11-Ketotestosterone (KT) release rates ($r^* = 0{\cdot}81$, $P < 0{\cdot}05$). *Pearson correlation adjusted for small samples sizes (L_S, standard length).

STUDY 5: DO STRESSED *P. LATIPINNA* MOUNT A CORTISOL RESPONSE?

Male (mean ± S.E. = 58·70 ± 6·94 pg L_S^{-1} h^{-1}) and female (mean ± S.E. = 58·70 ± 6·94 pg L_S^{-1} h^{-1}) cortisol release rates did not differ significantly (unpaired t-test, $t = 0{\cdot}63$, d.f. = 7, $P > 0{\cdot}05$). After exposure to a novel environment *P. latipinna* had significantly higher cortisol release rates (paired t-test, $t = -2{\cdot}68$, d.f. = 8, $P < 0{\cdot}05$; Table I).

STUDY 6: COMPARISON OF BASELINE CORTISOL IN *P. LATIPINNA* ACROSS LABORATORY STUDIES AND FIELD DATA

Male (mean ± S.E. = 50·17 ± 4·69 pg L_S^{-1} h^{-1}) and female (55·51 ± 6·04 pg L_S^{-1} h^{-1}) cortisol release rates also did not differ significantly for samples collected

from *P. latipinna* dip netted from the field (unpaired t-test, $t = -0{\cdot}48$, d.f. $= 16$, $P > 0{\cdot}05$). There was a significant difference in cortisol release rates across the six studies (Kruskal–Wallis test, $\chi^2 = 19{\cdot}18$, d.f. $= 5$, $P < 0{\cdot}05$; Table I). Cortisol release rates in experiment 1 were significantly lower than cortisol in study 2 (habituation: Wilcoxon, $P < 0{\cdot}01$), study 5 (exploratory: $P < 0{\cdot}001$) and part 6 (field: $P < 0{\cdot}01$), no other comparisons were significantly different.

DISCUSSION

One of the benefits of the water-borne hormone collection technique is that it is possible to get repeated hormone measurements from small fishes because the technique does not require sacrificing the organism for plasma collection. It is important, however, to determine whether the water-borne technique is stressful and how long the fish need to be sampled to accurately reflect circulating levels of hormones. The results from the present study indicate that 30 min release rates reflect the 60 min release rates of cortisol in *P. latipinna*. These results suggest that decreasing confinement times of *P. latipinna* to 30 min can minimize stress yet provide an accurate assessment of circulating cortisol levels. Using male three-spined sticklebacks *Gasterosteus aculeatus* L. 1758 Sebire *et al*. (2007) placed the same *G. aculeatus* in beakers for four separate 30 min trials. They found, similar to the present study, that the release rate at the first and second 30 min trial were similar and that *G. aculeatus* mounted a cortisol response on the third 30 min trial and then returned to baseline in the fourth trial. Male and female *P. latipinna* do not differ in the release rates of cortisol at 30 or 60 min indicating that they do not differentially respond to the collection process. Males and females also did not differ in cortisol release rates across the other studies in this paper indicating that the stress response is not sex dependent. In a future study, it is important to examine cortisol release rates in shorter intervals or with more severe stressors to determine how long it takes to mount a cortisol response.

It is also important to determine whether *P. latipinna* show a stress response to beaker confinement. The results from the present study demonstrate that *P. latipinna* do not mount a stress response during beaker confinement, as their cortisol values did not change over four sequential days of testing. These results are in contrast to the findings of Wong *et al*. (2008) as they found that *A. nigrofasciata* were initially stressed by the beaker collection method but eventually habituated on the last sampling day (3 days for females, 4 days for males). One hypothesis for why *P. latipinna* may not have mounted a response to beaker confinement is that they are quite small relative (less than half the L_S) to *A. nigrofasciata* and maybe less confined by the collection beakers. In summary, the results from the present study suggest that the handling effects associated with water-borne hormones collection from *P. latipinna* are minimal and that *P. latipinna* can be measured repeatedly without additional stress.

Stress has been found to differentially affect the release of sex steroids in different species of fishes (Milla *et al*., 2009). Baseline cortisol release rates were not significantly correlated with baseline KT, T or E2 release rates in male *P. latipinna* using data from the mating trials of Gabor & Grober (2010). Baseline cortisol and KT release rates were also not correlated in the samples used for plasma validations.

One hypothesis is that the lack of correlation is because cortisol can take up to 1 h to affect sex steroids (Sapolsky *et al*., 2000). There was also no significant correlation, however, between baseline cortisol release rates and the number of times male *P. latipinna* attempted to mate and whether they attempted to mate at all. Moreover, mild stress may even enhance reproductive performance in some fishes (Schreck *et al*., 1997; Schreck, 2010). Taken together, these results suggest that males are not stressed by the hormone collection technique because they did not behave differently based on their baseline cortisol release rates. This relationship might not hold true when males mount a cortisol response, as seen when males are placed in a novel environment such as in study 5, or when they are exposed to prolonged stressful events (Milla *et al*., 2009; Schreck, 2010). In a future study, it would be interesting to expose male *P. latipinna* to short and long-term stressors and then see how it affects their mating behaviour and their sex steroid levels.

There was a significant positive correlation between plasma cortisol concentrations and water-borne cortisol release rates, indicating that free steroids released from the gills (Ellis *et al*. 2004) of *P. latipinna* reflect circulating concentrations. A positive relationship has been validated in several other species of fishes (Sebire *et al*., 2007; Wong *et al*., 2008). There was also a significant positive correlation between plasma KT concentrations and water-borne KT release rates further validating that free steroids released from the gills in *P. latipinna* are indicative of circulating concentrations. One prior study with *G. aculeatus* also validated this method for KT (Sebire *et al*., 2007).

Overall, the release rates of free cortisol from plasma are within the range seen by others using similarly sized fishes [*G. aculeatus*: Sebire *et al*., 2007; *Brachyrhaphis episcopi* (Steindachner 1878): Archard *et al*., 2012]. The mean total cortisol release rates of studies 2, 3, 5 and 6 were also in the range found by Wong *et al*. (2008) on the first 2 days of beaker confinement study. The water-borne cortisol release rates in experiment 1, however, were significantly lower than in studies 2, 5 and 6 but all of the other cortisol release rate combinations did not significantly differ (Table I). Cortisol release rates in study 3 and part of 5 were from a different population yet they did not differ from most of the other studies. Additionally, cortisol release rates from the field (part 6) did not differ from three of the other laboratory cortisol release rates suggesting that laboratory maintenance does not cause additional stress. Cortisol release rates in experiment 1 may have differed because *P. latipinna* were on a different feeding schedule than studies 2, 4 and 5 but *P. latipinna* in the field were not on a feeding schedule. *Poecilia latipinna* in studies 2, 4 and 5 were fed 1 h before the respective study but for experiment 1, they were fed 15 min before testing. Cortisol levels usually spike an hour before feeding so the *P. latipinna* in all but experiment 1 might have been caught during their cortisol feeding spike. While the cortisol release rates were higher in experiment 1, the results from study 5 indicate that the *P. latipinna* were not so stressed that they could not mount a further cortisol response. It was found that *P. latipinna* exposed to a new environment can still mount a cortisol response significantly above their baseline release rates. In future studies, feeding will be performed in the evening before trials to minimize this effect.

In this study, the M of each *P. latipinna* was not measured. Instead, release rates were normalized using the highly correlated variable, L_S. Using the data generated on the relationship between L_S and M, approximate M was back-correlated (Table I).

This was performed for each data set and it was found that the cortisol release rates of pg L_S^{-1} h^{-1} *v.* pg M^{-1} h^{-1} were significantly positively correlated (Table I) indicating that for *P. latipinna*, it is reasonable to use L_S *in lieu* of M. In fact, it is argued, that in the current system where wet M can be variable and the variance in the M of the fish are relatively small, it is preferable to normalize release rates with L_S over M.

In summary, the results from the present study suggest that water-borne hormone collection methods are not maximally stressful to *P. latipinna*. Moreover the baseline cortisol release rates are not correlated with other sex steroids (KT, T or E2) or male mating behaviour for *P. latipinna*. Additionally, the results from the current study indicate that normalizing cortisol release rates using L_S *in lieu* of M is viable and accurate. Finally, the correlation between plasma and water-borne release rates of both cortisol and KT has been validated. The results of this study provide an important basis for future studies using the water-borne hormone collection method in *P. latipinna* and other livebearing or small fishes.

Thanks to A. Aspbury and two anonymous reviewers for insightful comments that greatly improved the manuscript. Thanks to G. Aron for loaning equipment and space needed for this study. Thanks to L. Alberici da Barbiano for collecting the field samples and the GASP laboratory for fish maintenance. This research was partially funded by a National Science Foundation IOS grant # 1021873 (to CRG) and by a Francis Rose Excellence in Undergraduate Research Award (to A.C.). Our protocols were reviewed and approved by Texas State University Institutional Animal Care and Use Committee (protocol # 1105_0505_06).

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