Measuring multiple hormones from a single water sample using enzyme immunoassays

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ABSTRACT

Many aquatic species, such as teleosts, release into the water and detect multiple bioactive substances to assist in schooling, migration, alarm reactions, and to stimulate behavioral and physiological responses during reproduction and in parent–offspring interactions. Understanding the complex relationship between hormones, behavior and their function in communication requires the simultaneous examination of multiple circulating hormones. However, repeated blood sampling within a short time period is not possible in smaller animals without impacting the very behaviors under investigation. The non-invasive technique of collecting and measuring hormone values in holding water using either radioimmuno-assay (RIA) or enzyme immunoassay (EIA) is becoming widely used in teleost research. Commercial assay kits in particular enable rapid and reliable data generation, yet their assay buffers are often specific and potentially incompatible with each other, which can hinder measuring multiple hormones from the same sample. We present here the validation and application of a "nested" elution technique we developed that allows for repeated sampling of multiple reproductive hormones – testosterone (T), 17β-estradiol (E2), progesterone (P), prostaglandin F$_{2\alpha}$ (PGF) and 11-ketotestosterone (11KT) – from individual samples of animal holding water by using commercial EIA systems. Our results show that when using appropriate controls to account for possible technical and biological confounds, this technique provides a powerful new tool for research in aquatic endocrinology and physiology.

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1. Introduction

Aquatic organisms commonly release bioactive compounds such as neurochemicals, hormones and pheromones into the water (Lafont and Mathieu, 2007; Janer and Porte, 2007; Stacey and Sorensen, 2002), which can act as chemical signals between conspecific and heterospecific individuals in a variety of contexts, including reproduction (Mathis and Smith, 1992; Chivers and Smith, 1998; Sisler and Sorensen, 2008; Cole and Stacey, 2006; Frade et al., 2002; Pinillos et al., 2002; Murphy et al., 2001; Stacey and Cardwell, 1995; Sorensen et al., 1988). While the hormonal control of teleost reproduction has been examined in a variety of teleost species (e.g., goldfish: Poling et al., 2001; Nile tilapia: de Souza et al., 1998; arctic charr: Sveinsson and Hara, 1995), detailed time course studies across reproductive cycles have generally been hampered by the low temporal resolution of hormonal profiles, with few studies performing repeated measurements on the same

individuals, as it is often necessary to sacrifice the animal to obtain the measurement (but see Haddy and Pankhurst, 2000; Morehead et al., 1998; Clearwater and Pankhurst, 1997). In order to better understand the relationship between hormones and reproductive behaviors, it is necessary to simultaneously measure multiple hormones from individual fish. Survival blood drawing from relatively small fish is possible but can compromise the subject's well-being and behavior and often results in increased mortality (Scott and El-lis, 2007; Dzieweczynski et al., 2006; H.A.H., unpublished observations). Furthermore, the volume of plasma recovered from small fishes inherently limits the number of hormones that can be measured. To enable frequent repeated collection of hormone measures and to minimize the impact on behavior, it is necessary to switch to a less invasive and traumatic means of assaying hormone levels.

Until fairly recently, steroid hormones and their metabolites were predominantly measured by RIA. However, most of the free steroids can now be measured with commercial EIAs, which are as sensitive as RIA (Table 1). While the cost per sample is higher for EIAs compared with RIA (E2 and T: ∼20%; P: ∼26%; PGF: 12%, according to our estimates), initial instrument costs are

Table 1
Sensitivities of several commercial RIA and EIA systems according to the manufacturers' specifications.

Assay	E2	T	P	PGF	11KT
RIA MP Biomedicals	10–3000 pg/ml	0–100 pg/ml	0.15–80 ng/ml	N/A	N/A
RIA Coat-a-count	20–3600 pg/mL	0–50 pg/ml	0–40 ng/ml	N/A	N/A
RIA Inst. of Isotopes	N/A	0–10.15 ng/ml	0–37.7 ng/ml	1.2–300 pg/ml	N/A
EIA Assay Designs	29.3–30,000 pg/ml	7.81–2000 pg/ml	15.62–500 pg/ml	3.05–50,000 pg/ml	N/A
EIA Cayman Chemical	6.6–4000 pg/ml	3.9–500 pg/ml	7.8–1000 pg/ml	3.9–500 pg/ml	0.78–100 pg/ml

considerably higher for RIA, not to mention the issues associated with the handling and disposal of radioisotopes. Other advantages of commercial EIAs include ease of use, time savings, reliability and consistency. Although there are currently no commercial EIAs available to specifically measure the sulfated or glucuronidated steroids, several companies offer a custom assay-design service, which could be used to manufacture assays for measuring these conjugated steroids.

Measuring waterborne hormones has become more common for teleost fishes in recent years (Scott and Ellis, 2007). Teleosts release sulfonated steroids via urine and glucuronidated steroids (collected in bile) via feces (Vermeirssen and Scott, 1996), but there is a considerable delay between the release of these hormones into the bloodstream and their excretion in conjugated form due to the transit time through the excretory organs (Scott and Sorensen, 1994; Stacey et al., 1989). In contrast, free steroids, released passively through the gills have been shown to most closely mirror plasma steroid levels (Ellis et al., 2005; Sorensen et al., 2000; Vermeirssen and Scott, 1996). However, very few studies have made direct comparisons between hormone concentrations in fish holding water and those in plasma. In the few studies which did make direct comparisons (using only custom-made hormone assays), a tight correlation between plasma and fish holding water values was recovered for free steroids (cortisol, 11KT, AD: Sebire et al., 2007; cortisol: Ellis et al., 2004; E2, 17,20β-dihydroxy-4-pregnen-3-one, and 17,20β,21-trihydroxy-4-pregnen-3-one: Greenwood et al., 2001; 17α,20β: Stacey et al., 1989).

Commercial EIAs have been used for measuring T, 11KT, E2 or P in a broad taxonomic range of species such as molluscs (Gauthier-Clerc et al., 2006a; Siah et al., 2002), amphibians (Lynch and Wilczynski, 2006), mammals (Dhali et al., 2006; Wommack et al., 2004; Ortiz et al., 2003), birds (Jensen and Durrant, 2006; Lynn et al., 2007), and reptiles (Radder et al., 2001). In teleosts, commercial EIAs have been used in multiple species to measure a variety of hormones either in plasma (Parikh et al., 2006b; Hoffmann et al., 2006; Nichols et al., 2001; Wang et al., 2001; Kelly and Woo, 1999) or holding water (Rodgers et al., 2006; Dzieweczynski et al., 2006; however, see Hong et al., 2006 for water and tissue measurements). None of these studies have demonstrated that hormone measures taken from holding water correlate to hormone levels circulating in blood plasma.

A common EIA validation strategy (called "parallelism") involves comparing samples spiked with known amounts of steroids to a standard curve in order to determine the appropriate sample dilution (Plikaytis et al., 1994). However, this approach does not establish whether waterborne hormone levels correlate with plasma levels. Thorough validation thus requires that plasma and water measures obtained from the same individuals at the same time show a positive and significant correlation. To our knowledge, no study using commercial RIAs or EIAs has directly determined for any hormone whether plasma levels and waterborne correlate.

Blood plasma contains steroids in both free form and bound to hormone binding globulins (SHBG). While free steroids readily penetrate cell membranes (e.g., gill epithelial cells), the steroid–SHBG complex does not (Norris, 1997). In many teleosts, steroids exhibit differential binding to steroid binding proteins (Scott and

Ellis, 2007; Scott et al., 2005; Chang and Lee, 1992; Chang and Chen, 1990; Pasmanik and Callard, 1986). Since free steroids have to pass out of the blood and through cell membranes before being released into the environment, the amount of free steroid present in water samples may be affected by this differential binding. As a consequence, the amount of free relative to bound steroids may vary considerably between species, reproductive condition and between hormones. Thus, in order to reliably estimate plasma hormone levels from water samples it is necessary to determine the relationship between waterborne and plasma levels for each hormone in each species and experimental condition.

In the present study, we approach these questions in an African cichlid fish, *Astatotilapia burtoni*. This species has become a major model system for the analysis of the molecular, endocrine and neural mechanisms underlying socially regulated plasticity in brain and behavior (Fernald, 2002; Hofmann, 2003). Our study aims to (a) develop a novel procedure that allows the measurement of multiple sex steroid hormones (17β-estradiol, testosterone, 11-ketotestosterone and progesterone) as well as the fatty acid derived hormone prostaglandin $F_{2\alpha}$ from a single concentrated water sample using commercial EIAs; (b) determine whether the waterborne levels of these hormones are positively correlated with plasma levels; (c) demonstrate that careful validation and control of potential confounds are of fundamental importance when applying this technique to novel organisms and hormones.

2. Methods

2.1. Study organism

Forty adult female *A. burtoni* ranging in size from 4.5 to 5.5 cm standard length from an inbred laboratory population descended from wild-caught stock from Lake Tanganyika, East Africa were used to assay T, E2, P, and PGF. Thirty-six adult male *A. burtoni* ranging in size from 5.85 to 6.0 cm standard length from the same inbred laboratory population were used to assay 11KT and T. In a separate experiment, we used 23 adult (3.8–5.5 cm SL) female convict cichlids, *Amatitlania nigrofasciata*, to assess the effects of reproductive condition on PGF levels in water relative to plasma. Fish were housed in 100 l aquaria with conditions similar to their natural environment (26–28 °C, pH 8.0–8.4, 12L:12D with 10 min of each dawn and dusk, gravel substrate). All fish were fed flake food once daily in the morning and handled according to the regulations of the Institutional Animal Care and Use Committee at The University of Texas.

2.2. Sample collection

All samples were collected between 10:00 and 11:00 A.M. Glassware was rinsed prior to use with 100% ethanol (EtOH), rinsed with reverse-osmosis (RO) water and the beakers were then filled with 300 ml of clean (unused) aquarium water (CAW). Fish were removed from their aquaria, placed in individual beakers for 1 h and at the end of the water sample collection, fish were removed from the beaker and blood was drawn from the dorsal aorta using

a heparinized infusion set (SURFLO Winged Infusion Sets, #SV-25BLK). Blood was transferred to a microcentrifuge tube containing ~5 µl of heparin, placed on ice and centrifuged at 4000 rpm for 15 min. Plasma was transferred to a new microcentrifuge tube and stored at −80 °C until the EIA was performed. The water sample was filtered to remove particulate matter and the hormones were extracted (see Section 2.4 below).

2.3. Controls

An inter-assay positive control (E2/T $n = 4$, P/PGF $n = 4$) was made by collecting four water hormone samples (as described above) from females at various reproductive states, which were later pooled (see eluting samples section for details). An inter-assay negative control (E2/T $n = 4$, P/PGF $n = 4$) was made by collecting four CAW samples, which were later pooled (see eluting samples section for details). This inter-assay negative control was performed to confirm there are little to no hormones in our CAW. An intra-assay control sample was made by taking five aliquots of the same water sample mixing it them well, filtering and then dividing into five 300 ml aliquots. These samples were processed as described above, assayed on one EIA plate and used to determine the intra-assay variability for each hormone.

2.4. Extraction procedure

We employed the following extraction procedure to recover hormones from water samples (Supplementary Fig. 1). Briefly, hormones from each water sample were collected using Saint-Gobain Tygon tubing (#AER00003) with the end shaved to fit into a Sep-Pack Plus C18 (C18) cartridge (Waters #WAT020515). This C18 cartridge was attached to a 12-port vacuum manifold (Alltech #210351) and a flow rate of 10 ml/min maintained for all liquids processed through the cartridge. Each C18 cartridge was primed by flushing 6 ml of EtOH and then 6 ml of Millipore ultrapure water (Synergy #SYNSV00US) through the cartridge. Hormones were then collected by processing each sample of fish holding water through separate cartridges, followed by 6 ml of Millipore water. The cartridge was then removed from vacuum manifold, the ends were covered with Parafilm (American National Can #ACA PM996) and it was stored at −20 °C until eluted.

2.5. Eluting samples and inter-assay controls

Cartridges were removed from the freezer and allowed to reach room temperature for half an hour. Next, the cartridges were reattached to the vacuum manifold, eluted with 4 ml of 100% EtOH into 13×100 mm glass tubes. To assess elution efficiency, we performed repeated elutions from the same cartridge (using 4 ml ethanol in each case) and found a significant decrease in, e.g., PGF levels between the 1st and 2nd elution (ANOVA: $F_{1,14} = 5.752$, $p = 0.031$). Also, PGF levels in the 2nd ethanol elution were not significantly different from our "no fish" samples (ANOVA $F_{1,10} = 0.162$, $p = 0.696$), thus confirming very high elution efficiency overall. In order to determine the (as of yet unknown) efficiency of ethanol elution for the fatty acid derived hormone PGF, we tested different elution solvents (4 ml ethanol, 4 ml ethyl acetate, as well as a three-step process with a prewash with 4 ml 15% EtOH followed by 4 ml hexane prior to eluting using 4 ml of ethyl acetate) and found no appreciable difference in the efficiency between them. Assay buffers for diluting samples often vary considerably between different hormones in commercial EIAs, even if produced by the same manufacturer. This was also the case in our study, where most of the assay kits (T, E2, P and PGF) were obtained from Assay Designs Inc. The 11KT EIA was obtained from Cayman Chemical. The buffers provided for the E2 and T EIA kits

are similar yet very different from the buffers contained in the P and PGF EIA kits, which again are similar to each other. Since our goal was to measure multiple hormones from a single sample, we prepared two aliquots from the eluted sample, with one aliquot being used for the E2 and T assays and the other for the P and PGF assays. The eluted samples were then completely dried under nitrogen gas at room temperature using the Evap-o-rac (Cole-Parmer #EW-01610–15). The aliquots with the dried pellets were stored at −20 °C until the EIAs were performed. For the male A. burtoni samples, we only assayed two hormones, T and 11KT, so one aliquot was used for the T assay (using the "universal buffer", details below) and the other aliquot was used for the 11KT assay (using the EIA buffer from the Cayman 11KT kit).

To facilitate the simultaneous measurements of multiple waterborne hormones from the same sample, we developed two novel "universal" assay buffers. For the E2 and T EIAs the buffer consisted of 0.5 M Tris pH 7.0 (Ambion #9850G), 0.15 M NaCl (Ambion #9759), 10% BSA (Sigma A7906), DEPC H_2O (Ambion #9924); for the P and PGF assays, the "universal" buffer contained 0.5 M Tris pH 7.0, 1.5 M NaCl, 1% BSA, DEPC H_2O. Either (160 µl) the E2/T, P/PGF or 11KT EIA kit buffer were added to the two aliquots. Next, the samples were vortexed and left to sit for 5 min, which was repeated two more times. The samples were then transferred into microcentrifuge tubes as 50 µl aliquots. The elution for the inter-assay negative (four C18 cartridges) and inter-assay positive (four different C18 cartridges) control samples was slightly different, in that the eluted samples were eluted with 4 ml EtOH but were not divided into two equal aliquots. Instead, the 4 ml sample was dried as described above and 320 µl of assay buffer (either E2/T or P/PGF) was added to the tube. Then the four inter-assay negative control (E2/T or P/PGF) samples and the four inter-assay positive control (E2/T or P/PGF) samples were combined, mixed well, and aliquots of 50 µl were stored at −20 °C until the EIA was performed. Due to space considerations, no negative control was assayed for the 11KT assay and aliquots of a large sample of A. burtoni male plasma were measured on three different plates and used as the inter-assay positive control for the 11KT assay. This same male A. burtoni plasma sample was assayed four times on the same plate and used to determine the intra-assay variability.

2.6. Hormone assays

Four of the assay systems used in this study were purchased from Assay Designs Inc., namely T (#900-065), E2 (#900-008), PGF (#900-069) and P (#900-011). The 11KT assay system was purchased from Cayman Chemical (#582751.1). Sensitivities for each assay kit are shown in Table 1 and the cross-reactivities for each assay are shown in the Supplementary Table. The subsequent description of the EIA protocol is identical for all four Assay Designs assays and the manufacturer's instructions were followed unless stated otherwise. For the 11KT kit, the manufacturer's instructions were followed as written. Previous studies in A. burtoni using the Assay Designs Inc. testosterone EIA kit found that a 1:30 dilution was optimal for plasma samples (Parikh et al., 2006b; Clement et al., 2005). Therefore in our study, all plasma aliquots were diluted 1:30 with the respective assay buffers. The water samples, negative, and positive controls were diluted 1:4 with the assay buffer solution from the individual EIA kit. Plates were read at 405 nm using a Beckman Coulter DTX 880 Multimode Detector.

2.7. Testing for potentially confounding biological factors

EIA manufacturers often do not test assay cross-reactivity with hormones or metabolites of potential importance to fish physiology and communication, if they pose little or no relevance to

biomedical considerations. This was of particular concern in the case of P since a major bioactive fish progestin is 17α,20β, a derivative of P (Nagahama and Yamashita, 2008). We also determined whether another fish specific hormone (11KT), which is important in male reproductive physiology, would cross-react react with the T assay. We determined the cross-reactivity of the commercial P assay by spiking 10 pg of 17α,20β (Sigma cat #P6285) into two CAW samples. The cross-reactivity reactivity of the commercial T assay was determined by spiking 500 pg/ml of 11KT (Standard from Cayman 11KT kit) into an eluted CAW sample.

It is conceivable that biologically important variation in physiological state (e.g., reproductive condition) may affect the strength or nature of the relationship between circulating and waterborne hormone levels. An essential reproductive hormone, PGF, is produced in mature reproductive tissues of all female vertebrates and is a potent releaser of ovulation and sexual behavior (Murdoch et al., 1993). PGF levels can vary dramatically over short time scales, which could result in a breakdown of the correlation between waterborne and plasma hormone levels. This is particularly important, since PGF acts as a potent reproductive pheromone in a wide range of species (Kobayashi and Stacey, 1993; Liley and Tan, 1985; Villars et al., 1985), including pair-bonding cichlids (Cole

and Stacey, 1984). Since PGF levels can vary dramatically over short time scales, we wanted to examine whether female reproductive status affected the water versus plasma correlations for this hormone. We measured PGF levels in water and plasma of female convict cichlids, *A. nigrofasciata*, of known reproductive state which were pair-bonded with a male. Hormone collection, extraction and quantification were performed as described above, except that water and plasma samples were collected at three discrete time points: Early Spawning (after the first row of eggs was deposited), After Spawning (1 h after the last egg was laid), and day 7 (7 days after Spawning).

2.8. Statistical analysis

We calculated the Pearson product moment correlation coefficient to examine the association of plasma and water values for each assay, followed by Bartlett's χ^2 Statistic to test for significance. To assess the intra-assay variation for each EIA, the coefficient of variation (CV) was calculated (using standard deviation as the numerator) for five replicates of the same water sample used to determine the recovery rate. We used the negative and positive control samples performed on each assay plate to assess inter-
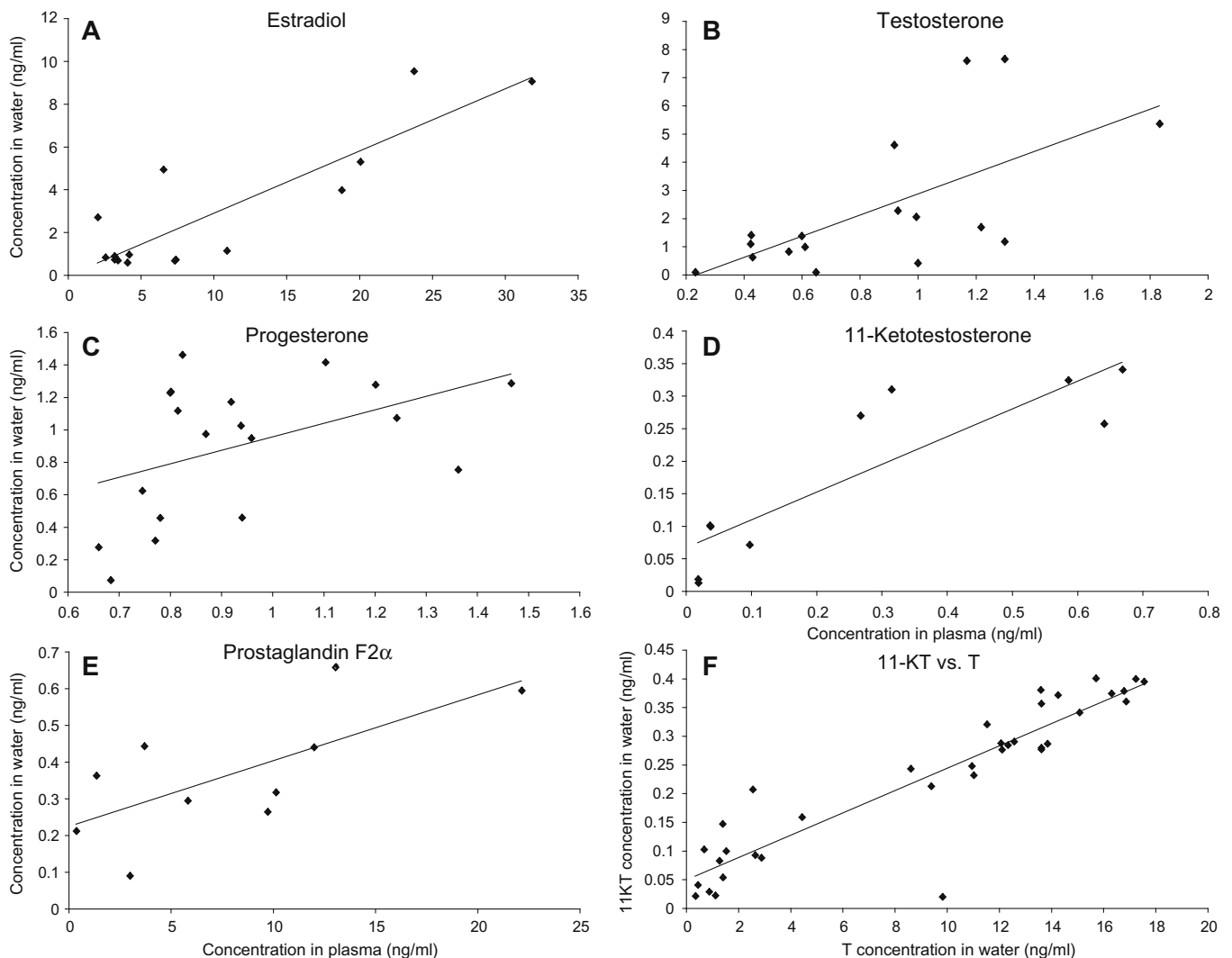
Fig. 1. Concentrations of E2, T, 11KT, PGF and P in plasma correlate with those in water samples. (A) For 17β-estradiol, the equation for y is $0.2906x - 0.0003$ with $r_{15} = 0.872$, $p < 0.001$. (B) For testosterone y is $3.3319x - 1.0018$ with $r_{17} = 0.632$, $p = 0.007$. (C) For progesterone y is $0.8315x + 0.1258$ with: $r_{19} = 0.456$, $p = 0.050$. (D) For 11-ketotestosterone y is $0.4281x + 0.0673$ with $r_{10} = 0.878$, $p = 0.001$. (E) For prostaglandin F$_{2α}$ y is $0.0179 + 0.2247$ with $r_{10} = 0.689$, $p = 0.028$. (F) There is a high correlation between 11KT and T: y is $0.0195x + 0.0497$ with $r_{36} = 0.918$, $p < 0.001$.

assay variability by calculating a z-score for each value to discover any outliers and calculating the coefficient of variation for the negative and positive control samples quantified on each hormone assay.

3. Results

### 3.1. Inter-assay and intra-assay variation

Measurement error is a major concern with all hormone assays and so we first examined the extent of measurement variation for any given sample measured in replicate on either the same assay plate or on different plates. For virtually all intra-assay and inter-assay comparisons the z-score was within one standard deviation (SD) of the mean. This indicates that there are no outliers in our data for any of the hormone assays. The inter-assay CVs of the negative controls were relatively high (between 14.20% and 25.69%), as expected for values at or near zero. The inter-assay CVs of the positive controls were considerably lower: 7.73%, 7.80%, 16.23%, 4.97% and 4.40% for the E2, T, P, PGF and 11KT, respectively. As expected, the intra-assay CVs were low: E2: 1.90%; T: 2.89%; P: 1.70%; PGF: 6.23%; 11KT: 9.99%.

3.2. Correlations between plasma and waterborne hormone levels

Next, we determined for all five hormones whether the concentrations of waterborne hormones corresponded to plasma levels obtained from the same individuals. Fig. 1 shows that waterborne and plasma concentrations of four hormones indeed correlated significantly in samples obtained from A. burtoni. (E2: Pearson's $r_{15} = 0.872$, $p < 0.001$; T: $r_{17} = 0.643$, $p = 0.005$; PGF: $r_{10} = 0.689$, $p = 0.028$; 11KT: $r_{10} = 0.878$, $p = 0.001$) and approached significance in the case of P ($r_{19} = 0.456$, $p = 0.050$). We also found that waterborne T and 11KT levels were highly correlated ($r_{36} = 0.918$, $p < 0.001$), with 11KT concentrations about one order of magnitude lower than those of T (Fig. 1F).

3.3. Tests for confounding factors

Both the 17α,20β samples were extracted and processed as described above. We found that the values for these 17α,20β samples were towards the upper end of the standard curve, well above the negative control, thus clearly demonstrating that 17α,20β strongly cross-reacted with the P assay (>100%). The value for the 11KT
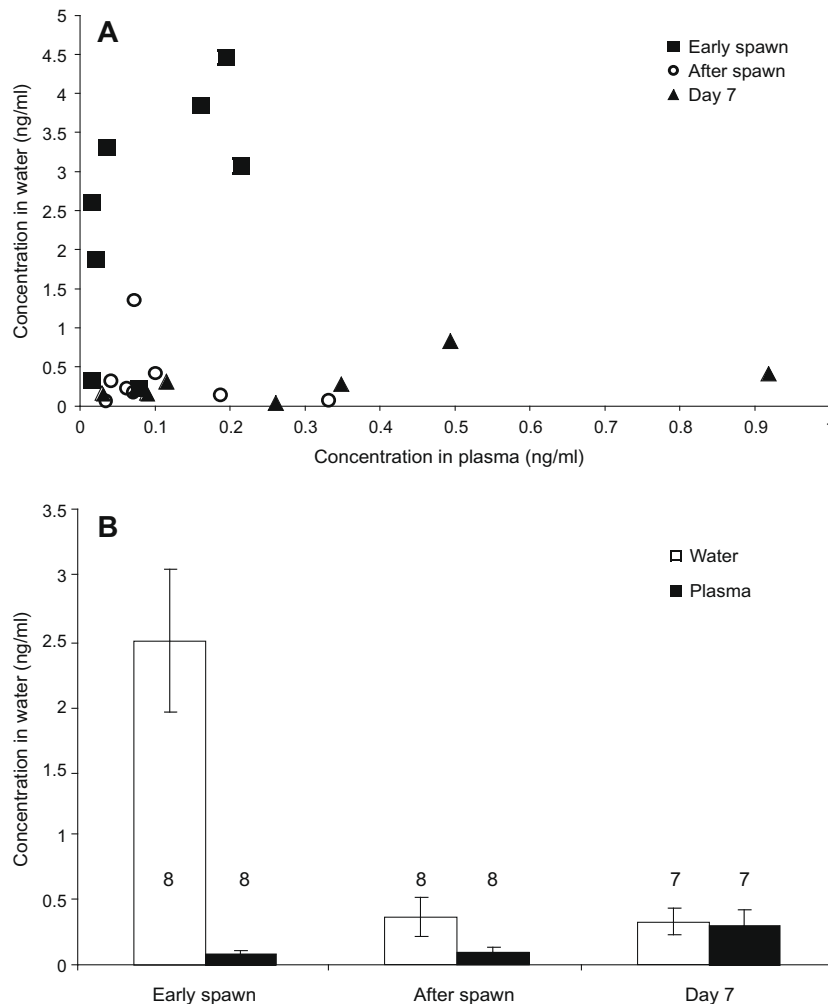
Fig. 2. Female reproductive stage can affect the correlation between hormone levels measured in plasma and water samples. (A) When samples from three reproductive time points are combined, PGF levels measured in water and plasma are not correlated ($r_{23} = -0.220$, $p = 0.314$). However, when samples from the Early Spawning time point are removed, there is a significant positive correlation ($r_{15} = 0.878$, $p = 0.001$). (B) The difference in correlation coefficients is due to the significant elevation (ANOVA, $F_{2,20} = 12.791$, $p = .0003$) of PGF in water samples taken during the Early Spawning time point.

spiked sample indicated that this hormone showed very low cross-reactivity with the T assay (<5%).

We examined female convict cichlids, *A. nigrofasciata*, at different stages throughout their reproductive cycle and found that reproductive status had a dramatic effect on the relationship between levels of PGF in water and plasma samples (Fig. 2). When samples from all time points are considered (Fig. 2A), PGF levels measured in water and blood do not correlate significantly ($r_{23} = -0.220$, $p = 0.314$). However, when samples from the Early Spawning time point are excluded, PGF levels between water and blood are strongly correlated ($r_{15} = 0.878$, $p = 0.001$). The difference in correlation coefficients is due to the significant elevation (ANOVA, $F_{2,20} = 12.791$, $p = 0.0003$) of PGF in water samples taken during the Early Spawning time point (Fig. 2B), possibly indicating an active release of this hormone during mating.

4. Discussion

We have adapted commercial EIAs and measured multiple important reproductive hormones in fish holding water from the same sample at the same time point. This technique for measuring multiple hormones from one concentrated sample generated measurements that are similar to values previously published for cichlids (Parikh et al., 2006a; Park et al., 2007; Francis et al., 1992; Trainor and Hofmann, 2006; Desjardins et al., 2005; Aubin-Horth et al., 2007; Cornish, 1998). Additionally, we show for the first time for commercially available EIA systems strong positive correlations between concentrations of waterborne hormones and their corresponding plasma levels. The technique described in this study has several implications for the study of reproductive physiology and behavior in smaller aquatic species.

The size of many organisms, like cichlids, restricts the frequency and amount of blood that can be collected. As a consequence, relatively large numbers of animals are generally needed to assess the levels of multiple hormones at consecutive time points. Our novel approach permits the examination of individual variation in hormone levels, reduces stress on animals after several measurements (Wong et al., 2008) and allows for repeated measurements in the same individuals at a high temporal resolution, which is impossible in small fish when using plasma samples. As we have shown, waterborne hormone levels correlate well with plasma hormone levels, thus our technique will considerably advance our understanding of the complex relationship between multiple hormones and their impacts on behavior.

Many hormones have been shown to change over the course of the reproductive cycle in fishes and while most of this time course work has been done using plasma samples (Dahle et al., 2003; Moore et al., 2000; Carolsfeld et al., 1996; Kobayashi et al., 1988; Smith and Haley, 1988; Koob et al., 1986), the increasing evidence that waterborne hormone levels can correlate with plasma hormone levels makes this a powerful technique to use in examining the complex relationship between multiple hormones and their impacts on behavior.

Another prospective use of this technique is to measure the secondary effects of environmental toxins (such as estrogenic and androgenic chemicals) on endogenous hormone levels in smaller individuals, such as juveniles or small species of fishes, than was previously possible using only plasma samples. Steroids, estrogenic chemicals and androgenic chemicals have been shown to have profound physiologic effects on aquatic invertebrates and aquatic vertebrates like the shifting of sex ratios (Wang and Croll, 2004; Moss, 1989; Koger et al., 2000; Katsiadaki et al., 2007) and reduction in immunocompetence (Gauthier-Clerc et al., 2006b; Canesi et al., 2004; Watanuki et al., 2002; Filby et al., 2007; Law et al., 2001). This technique allows for smaller species or smaller

individuals to be used, such as juveniles where more pronounced endocrine and/or developmental effects may be seen (Haeba et al., 2008; Maunder et al., 2007; Mukhi et al., 2007; Iguchi et al., 2006). Given the flexibility of commercial EIA kits, which have been used across such disparate classes as Bivalvia, Amphibia, Mammalia, Aves, Reptilia and Osteichthyes, it seems likely that this technique of measuring waterborne hormones could be expanded beyond teleosts and used in other aquatic organisms, especially aquatic invertebrates.

Using primarily radioimmunoassays or gas chromatography, several studies have shown that vertebrate steroids can be bioaccumulated in aquatic invertebrates (Lafont and Mathieu, 2007; Janer and Porte, 2007). In molluscs, commercial E2 (Le Curieux-Belfond et al., 2005; Gauthier-Clerc et al., 2006a), T and P (Siah et al., 2003) EIAs have been used to measure hormone levels in tissue homogenates. It has not yet been shown whether molluscs can also release steroids after accumulating them. Our technique for measuring multiple waterborne hormones mollusc holding water could shed some light on this question. While it is clear that commercial EIAs can work with invertebrate samples (Gauthier-Clerc et al., 2006a; Siah et al., 2003), careful validation of each assay system will be necessary as this technique is expanded to new species (Scott et al., 2008).

One potential issue when assessing EIA kit performance is consistency in measurements, both within and across assay plates. In this study, all of the negative control samples in each assay clustered around the minimum value of the standard curve (i.e., near the detection limit of the assay), as would be expected, and not surprisingly all had much higher coefficients of variation than the positive control samples. The inter-assay positive control CVs for the E2, T, PGF and 11KT were all below 16.23%. The intra-assay CVs for all EIA systems used in this study are below 9.96%, well in line with previously published studies. Overall, the measurement variation in our study is comparable with the CV variation published in other EIA and RIA studies (Rodgers et al., 2006; García-López et al., 2006; Ellis et al., 2004; Siah et al., 2002; Wang et al., 2001; Irwin et al., 1999; Francis et al., 1992; Smith and Haley, 1988).

Commercial EIA systems are commonly designed for hormone detection in mammalian samples and when used for assaying hormones in non-mammalian species, the potential for cross-reactivity to species-specific hormonal compounds or metabolites needs to be considered, especially if the assay manufacturer did not test the extent to which these substances would cross-react in a given assay. For example, 17α,20β, which is not found in mammals, is a potent trigger for reproductive behaviors and oocyte maturation in teleosts, yet information on its cross-reactivity with the P EIA used in this study was not available. When we tested samples spiked with 17α,20β, they clearly strongly cross-reacted with the P assay, thus confirming the utility of this EIA system for our model system. In some situations this cross-reactivity could be a major confounding factor and researchers need to be aware of this potential problem and take steps to address it.

In addition to these technical issues associated with detecting hormones in animal holding water, excreted hormones can act as potent chemical signals (pheromones) facilitating communication between individuals (Lafont and Mathieu, 2007; Janer and Porte, 2007; Stacey and Sorensen, 2002; Mathis and Smith, 1992). Also, the release rate of hormones into the water can be affected by the reproductive state of the animal (Sorensen et al., 2005; Scott and Sorensen, 1994; García-López et al., 2006). As a consequence, the positive correlation of hormone levels detected in plasma versus water could possibly break down during certain reproductive events. PGF in particular is thought to aid in the coordination of gamete release between males and females of many teleosts (Goetz and Cetta, 1983; Sorensen and Goetz, 1993) and the

administration of PGF to males and females of multiple species of teleosts has been shown to induce Spawning behaviors regardless of reproductive state (*Cichlasoma bimaculatum*, Cole and Stacey, 1984; *Carassius auratus*, Kobayashi and Stacey, 1993; *Clarias batrachus*, Tikare et al., 1983; six species, Stacey, 1987). Our finding that during Early Spawning females of the convict cichlid *A. nigrofasciata* (a pair-bonding biparental substrate spawner) appear to release into the water a disproportionate amount of PGF relative to their plasma levels is thus not surprising. As a consequence, the positive correlation between water and plasma concentrations of PGF breaks down at this reproductive time point. However, because teleost mating systems are very diverse (Breder and Rosen, 1966; Blumer, 1979), this kind of disassociation may occur at different points in the reproductive cycle for other species. We are currently conducting a detailed analysis of PGF in females *A. burtoni* (a polygynous maternal mouthbrooder) to account for this possible difference (M.R.K. and H.A.H., unpublished data). In conclusion, even if hormone values in water and plasma samples from the same individuals co-vary strongly at one time point during the reproductive cycle, extrapolating to other time points and/or species has to be done with caution and proper controls must be conducted first.

In this paper, we have described a novel approach that allows the efficient and robust analysis of multiple hormones from the same sample of animal holding water using commercial EIAs. We found that the levels of five hormones (E2, T, P, PGF, 11KT) show significant positive correlations between water and plasma samples collected from the same individuals. Further, we explored potential confounding factors, such as cross-reactivity with non-mammalian hormone metabolites and biologically relevant disassociation between waterborne and plasma hormone levels. Provided careful quality control procedures are followed, this technique will enable repeated measurements of multiple hormones from the same individuals, which will facilitate a more complete and detailed understanding of the complex interrelationship between reproductive physiology and behavior.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2009.07.008.

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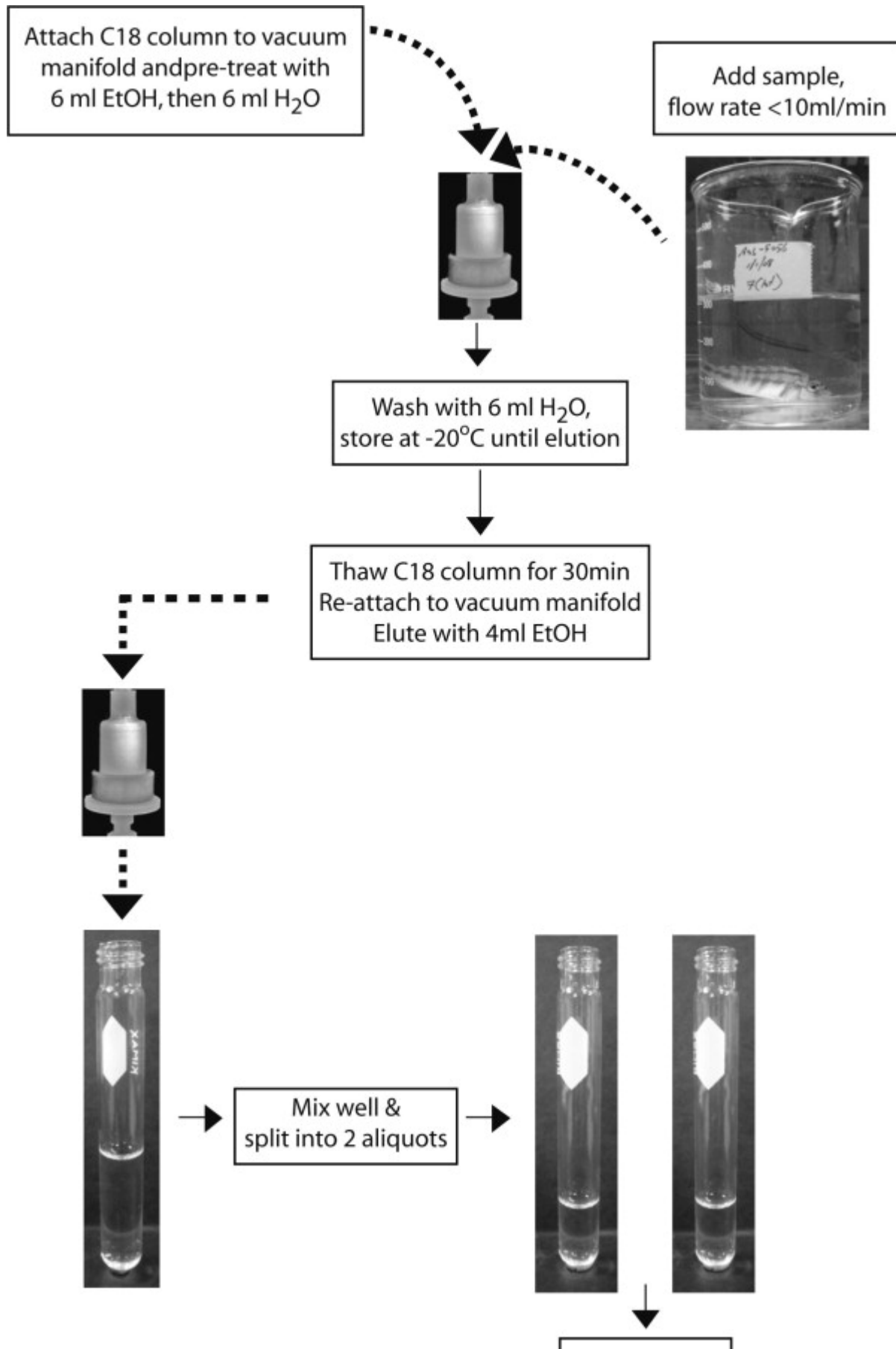
Supplementary Table

Hormone	Crossreactivity:
T	T = 100%; 19-hydroxytestosterone 14.64%; androstendione 7.2%; **11-ketotestosterone < 5%;** all else below 0.73%;
11KT	11KT = 100%, T = <0.01%, all else below 0.01%
E2	E2 = 100%, estrone 4.64%, all else below 0.54%
PGF$_{2\alpha}$	PGF$_{2\alpha}$ = 100%; PGF$_{1\alpha}$ 100%; PGD$_2$ 3.62%; 6-keto-PGF$_{1\alpha}$ 1.38%; PGI$_2$ 1.25%; all else below 0.78%
P	P = 100%; 5$_\alpha$-Pregnane-3,20-dione 100%; **17$_\alpha$,20β > 100%**; 17-OH-progesterone 3.46%; 5-pregnen-3β-o1-20-one 1.43%; all else below 0.78%

3

4   **Table 2**

5   Cross-reactivity values for non-target substances are provided for each EIA system.. All
6   values are according to manufacturer specifications except the ones for 11-KT in the T assay
7   and 17$_\alpha$,20β in the P assay (bolded), which were derived in the present study.

8