Noninvasive Measurement of Steroid Hormones in Zebrafish Holding-Water

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Abstract

Zebrafish (*Danio rerio*) has recently emerged as a new animal model in neuroendocrinology and behavior (e.g., stress physiology and ecotoxicology studies). In these areas, the concentrations of steroid hormones in the blood are often used to study the endocrinological status of individuals. However, due to the small body size of zebrafish, blood sampling is difficult to perform and the amount of plasma obtained per sample for assaying hormones is very small (ca. 1–5 μL), and therefore most studies have been using whole-body hormone concentrations, which implies sacrificing the individuals and hampers sequential sampling of the same individual. Here a noninvasive method to assay steroid hormones from zebrafish holding-water, based on the fact that steroids are released into the fish holding-water through the gills by passive diffusion, is validated. Cortisol and the androgen 11-ketotestosterone (KT) were measured in water samples and compared to plasma levels in the same individuals. Cortisol released to holding-water correlates positively with plasma concentrations, but there was a lack of correlation between KT water and circulating levels. However, KT levels showed a highly significant sex difference that can be used to noninvasively sex individuals. An ACTH challenge test demonstrated that an induced increase in circulating cortisol concentration can be reliably detected in holding-water levels, hence attesting the responsiveness of holding-water levels to fluctuations in circulating levels.

Introduction

IN RECENT YEARS, the use of zebrafish as a model organism in ecotoxicology and in endocrinology,[1–3] namely in stress research,[4] raised the need for measuring circulating levels of steroid hormones. However, due to its small size, blood collection is difficult in zebrafish and implies sacrificing the animals, and the amount of plasma obtained is small (i.e., below 5 μL). As an alternative, researchers have been using whole-body homogenates to assay steroid hormones,[5–9] but again, this method implies sacrificing the experimental animal, which imposes methodological constraints on these studies, such as making nonviable within-subject experimental designs where sequential sampling of the same individual is requested.

A noninvasive method for assaying steroids from fish holding-water, initially developed for studying pheromones, has since then been applied to measure steroid hormones in other contexts in a number of different species with promising results (see Scott and Ellis,[10] for an historic perspective of the development of the method). Studies using holding-water steroid measurements have been successfully accomplished not only in groups of fish but also in single

fish, and cover a wide range of research topics, from reproductive physiology,[11–15] to stress research in aquaculture, fish welfare, and conservation contexts,[16–22] to behavioural endocrinology.[13,23–30] This method is based on the principle that different steroid fractions exit fish through different routes: free steroids are released into the water through the gills by passive diffusion, whereas sulfated and glucuronidated steroids come mainly from the urine and the feces (via the bile), respectively.[31,32] Therefore, free steroids in the water must reflect circulating levels of the physiologically active fraction in the plasma.

In brief, the procedure for assaying holding-water steroids consists in extracting and concentrating the steroids present in the water sample using solid-phase extraction cartridges and organic solvents, such as diethyl ether or ethyl acetate. Steroids are then measured by specific immunoassays (e.g., RIA or ELISA). If levels from a single fish are to be obtained, that fish has to be housed individually in a small volume of water (e.g., 0.5 to 1 L, depending on size of the fish) for a given time period (e.g., 30 min to 1 h). Despite its simplicity, this method needs to be validated when it is first applied to a new species, because species differences in steroid metabolism may occur (e.g., variation in steroid biosynthetic pathways and release

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rates), and because different living conditions may also influence steroid release rates (e.g., cool vs. warm waters; freshwater vs. seawater; relatively clean vs. organically rich waters).[10,33] This is particularly critical in zebrafish since the expression of a steroid-binding globulin in its gills has been described,[34,35] which may interfere with the dynamics of steroid release.

In this article we aim to validate this method for assaying cortisol and the main teleost androgen, 11-ketotestosterone (KT). A number of validation procedures will be used, as proposed by a consensus article recently co-authored by majority of the active researchers in the field,[33] namely to check:

(1) if the water provided to the fish has no background activity;
(2) that recovery rates of known amounts of steroids added to the water are acceptable (i.e., above 80%) and consistent;
(3) that there is parallelism between extracts and standards;
(4) for correlations between plasma and holding-water steroid levels;
(5) if holding-water steroid levels respond in a biologically meaningful manner to exogenous physiological challenges [e.g., if holding-water cortisol levels increase in response to an adrenocorticotrophic hormone (ACTH) injection].

The validation of this method in zebrafish would add another useful tool to the research tool kit already available for this species that makes it an increasingly popular model organism.

Materials and Methods

Animals and housing

Adult wild-type zebrafish (*Danio rerio*) from the stock held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) were used. Fish were kept in a recirculating system (ZebraTec, Tecniplast), at 28°C with a 14L:10D photoperiod. Water was monitored for nitrites (<0.2 ppm), nitrates (<50 ppm), and ammonia (0.01–0.1 ppm), while pH and conductivity were maintained at 7 and 700 μSM, respectively. Fish were fed twice per day with *Artemia salina* and commercial flakes, except on the day of experiments.

Study 1: Correlation between plasma and holding-water steroid levels

Individuals [12 females and 27 males, body weight (average ± S.E.M.): 0.36 ± 0.11 g and 0.38 ± 0.07 g, respectively] were removed from their home-tank and placed individually in a 2 L beaker filled with 500 mL of clean water (i.e., filtered through a reverse osmosis system) for 1 h. While in the beaker, individuals had no visual contact with other fish or with the researcher. After this period, individuals were anesthetized with Tricaine solution (MS222, Pharmaq; 160 mg/L in water, buffered with Tris base 1 M to pH 7), weighed, and blood was collected from the caudal vein using a 30G heparinized syringe. Collected blood was kept in microcentrifuge tubes on ice until centrifugation (800 g, 10 min, 4°C). The pellet was discarded and plasma was transferred to a new microcentrifuge tube and stored at −20°C until further processing. Experiments were always conducted in the morning, and blood sampling always took place between 11:00 and 12:00 AM to avoid spurious effects of diurnal steroid variation. All glassware was washed with absolute ethanol and then distilled water to avoid possible contaminations. Water samples were processed immediately after sampling for 11-ketotestosterone and cortisol levels.

Study 2: ACTH challenge test

An ACTH challenge test, following the procedure described in Bshary and co-workers,[29] was used. Fourteen females and 8 males (weight: 0.84 ± 0.31 g and 0.67 ± 0.05 g, respectively) were used. Fish were removed from their home tanks and placed individually in a 2 L beaker with 500 mL of clean water for 1 h. Experiments always started at the same time to account for diurnal steroid variations, and all material was washed with absolute ethanol and distilled water to avoid possible contamination. None of the subjects had visual contact with other animal or with the investigator. After this period, subjects were collected from the sampling container, anesthetized with Tricaine solution as mentioned above and weighted. An intraperitoneal injection, using a syringe with a 30G heparinized needle, of either ACTH solution (adrenocorticotrophic hormone porcine pituitary; Sigma A-6303; physiological challenge treatment) or Ringer solution (control treatment) was given and individuals were placed in a new beaker with clean water. Two different concentrations of ACTH were tested (0.046 and 0.23 IU/ 50 μL/g body weight), and fish were transferred to a new beaker at 15 min, 30 min, 1 h, 2 h, 3 h, and 4 h after the i.p. injection, making six sampling points. Sample sizes were: $n = 11$ for the high-dose of ACTH, $n = 4$ for the low-dose of ACTH, and $n = 7$ for the Ringer treatment. All glassware was washed with absolute ethanol and then distilled water to avoid possible contamination. Water samples were immediately processed for cortisol analysis.

Determination of steroid extraction recoveries

Water samples were spiked with known quantities of steroids (5 ng/L of F, $n = 3$; and 0.5 ng/L of KT, $n = 2$). Samples were then processed, steroids quantified, and their respective rates of recovery determined.

Sexing

At the end of experiments 1 and 2, fish were sacrificed with an overdose of Tricaine solution (4 g/L in water, pH 7) and dissected in order to confirm sex, by direct inspection of the gonads under the microscope, using the aceto-carmine squash method.[36]

Extraction procedure

Water samples were filtered to remove particulates, and steroids were then extracted using a C18 solid-phase extraction cartridge (500 mg, #1.02023.0001, Merck), previously activated with 2×2 mL absolute ethanol followed by 2×2 mL of distilled water, with the help of a vacuum pump attached to a 12-port vacuum manifold. Columns were then stored at −20°C until further processing. Columns were later thawed at room temperature and steroids were eluted with 2×2 mL of ethanol, into glass tubes. Ethanol was then evaporated in a fume hood, eluent was resuspended in 100 μL of distilled water, 4 mL of diethyl ether were added, and the resuspension

was agitated for 20 min. Samples were the centrifuged (163 g, 5 min, 4°C) for phase separation and kept at -80°C for 115–20 min in order to freeze the water phase. The organic layer was decanted into a glass vial and ether treatment repeated to the aqueous phase with addition of 3 mL of this solvent, in order to obtain a higher extraction efficiency.[11] The final volume of ether was evaporated in a Speed-vac and the dried organic phase containing the free steroid fraction was reconstituted with 1 mL of the buffer solution supplied with the Enzyme Immunoassay (EIA) kit. Samples were stored at -20°C until analysis.

Plasma samples

Due to the reduced quantity of blood and consequently of plasma obtained for each subject (ca. 1–5 μL), plasma samples were not extracted. Instead, they were diluted in EIA buffer to 1:50–1:200 dilution. Samples were stored at -20°C until assayed. To confirm the reliability of nonextracted plasma samples, these were serially diluted and compared to a standard curve. Equality of slopes between the serial dilution and the standard curves indicates that there were no interferences of other immunoreactive substances in the nonextracted plasma samples (ANCOVA: cortisol, $F_{1,14} = 0.087$, $p = 0.773$; KT, $F_{1,8} = 2.326$, $p = 0,166$).

Hormone analysis

Cortisol and KT levels, both from water and from plasma, were assayed using enzyme immunassay (EIA) kits from Cayman Chemical Company (#500360 and #582751, respectively) following the manufacturer's instructions. For each individual, water samples were analyzed within the same assay, and it was used a 1:5 dilution for the analysis of cortisol and 1:5 and 1:20 dilutions for the analysis of KT in females and males, respectively. Both kits were validated for plasma and water samples by assessing parallelism of a serial dilution curve, run in duplicate, with the standard curve. The serial dilution curve was obtained, by diluting in EIA buffer, a pool of plasma or water samples from several individuals, to make the following dilutions: 1:2, 1:4, 1:8, 1:16; 1:32, and 1:64. Intraassay coefficients of variation were 6.49% for cortisol and 4.41% for KT; inter-assay coefficients of variation were 12.6% and 6.2% for cortisol and KT, respectively.

Statistical analysis

In the first experiment, parametric statistics were used, since data followed the assumptions of normality and homogeneity of variance. Correlations between hormone levels in plasma and water were obtained with Pearson correlation coefficients. A Student's t-test was used to assess sex differences in hormone values both in plasma and in water. In the ACTH challenge experiment, cortisol fold-change (i.e., final cortisol value – initial cortisol value) was used as dependent variable to account for variation between individuals in baseline values. The effect of the different injection treatments on cortisol fold-change was analyzed using a repeated measures ANOVA [repeated factor = sampling time; independent factor = treatment (ringer: low ACTH dose: high ACTH dose), followed post-hoc tests and by planned comparisons of least squares means between the control (Ringer) and each ACTH dosage for each sampling point. Even though data did not follow the assumptions of normality, this parametric test was still undertaken due to the lack of an equivalent nonparametric test and since the F-statistic is known to be remarkably robust to deviations of normality.[37] An ANCOVA was used to compare the slopes between the serial dilution and standard curves. A value of $p < 0.05$ was used for statistical significance in all tests. All statistical tests were run on the STATISTICA data analysis software system, version 10 (StatSoft, Inc. 2011, www.statsoft.com).

Ethical note

All experiments were conducted in compliance with the regulations on animal experimentation in Portugal.

Results

There is little background (i.e., water without fish) steroid immune-reactivity in the water (cortisol: $n = 6$, mean $\pm$ SEM $= 0.404 \pm 0.052$ ng/L; KT: $n = 2$, mean $\pm$ SEM $= 0.084 \pm 0.002$ ng/L) so that the presence of the fish is associated with a 16-fold increase in water cortisol and a 3-fold increase in water KT concentrations (Table 1).

Although there are no significant sex differences in circulating cortisol levels, males have higher cortisol release rates into the water than females (Table 1). There is a positive significant correlation between holding-water and plasma cortisol levels both for males and females (males: $n = 27$, $r = 0.53$, $P < 0.01$; females: $n = 12$, $r = 0.68$, $p < 0.05$, Fig. 1A). Both KT circulating levels and KT release rates are significantly higher in males than in females (Table 1). Water and plasma KT levels are not correlated in any of the sexes (males: $n = 19$, $r = 0.18$, $p = 0.46$; females: $n = 16$, $r = 0.33$, $p = 0.21$, Fig 1B).

In the ACTH challenge test, there was a main effect of treatment ($F_{2,19} = 5.06$, $p < 0.05$; post-hoc Tukey unequal N HSD test: ACTH high dose > saline = ACTH low dose, $p < 0.05$) and of time after injection ($F_{4,76} = 4.51$, $p < 0.01$; post-hoc Tukey unequal N HSD test: 15 min = 30 min = 1 h = 2 h > 4 h, $p < 0.05$

TABLE 1. SEX DIFFERENCES IN CORTISOL AND 11-KETOTESTOSTERONE (KT) PARAMETERS IN ZEBRAFISH

| Variable	Males Mean $\pm$ S.E.M. (N)	Females Mean $\pm$ S.E.M. (N)	T-test
Body mass (g)	0.38 $\pm$ 0.01 (41)	0.36 $\pm$ 0.02 (20)	T = 1.09, $p = 0.28$
Plasma cortisol (ng/mL)	57.47 $\pm$ 8.28 (30)	45.85 $\pm$ 8.04 (12)	T = 0.82, $p = 0.41$
Water cortisol (ng/L)	7.47 $\pm$ 0.42 (31)	4.35 $\pm$ 0.37 (12)	T = 4.37, $p < 0.0001$
Cortisol release rate (ng/g/h)	20.16 $\pm$ 1.28 (31)	13.17 $\pm$ 1.74 (12)	T = 3.00, $p < 0.01$
Plasma KT (ng/mL)	2.39 $\pm$ 0.28 (21)	0.15 $\pm$ 0.03 (16)	T = 6.92, $p < 0.0001$
Water KT (ng/L)	0.37 $\pm$ 0.03 (21)	0.09 $\pm$ 0.01 (17)	T = 8.39, $p < 0.0001$
KT release rate (ng/g/h)	0.97 $\pm$ 0.07 (21)	0.27 $\pm$ .03 (17)	T = 8.26, $p < 0.0001$
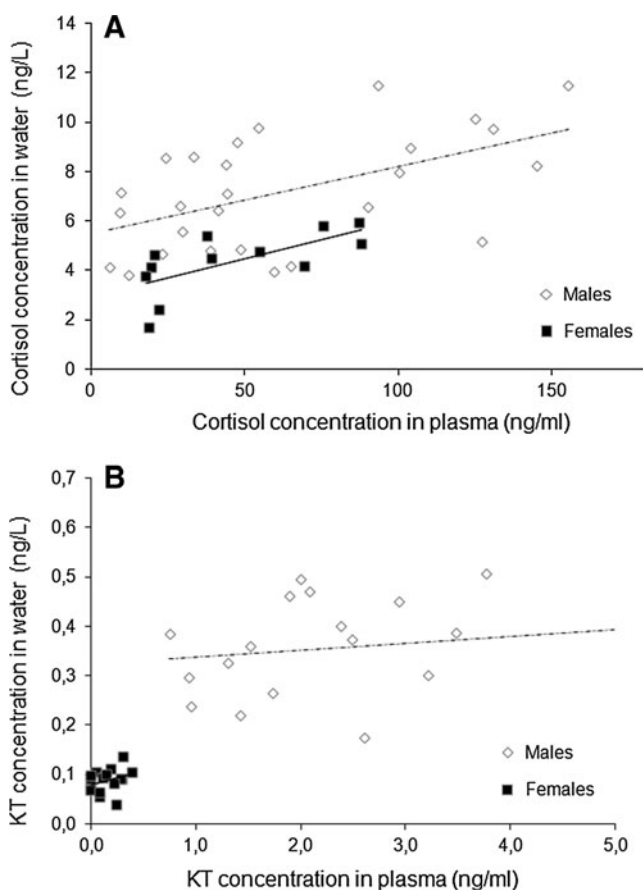
FIG. 1. Correlation between plasma and holding-water cortisol **(A)** and 11-ketotestosterone **(B)** concentrations for male and female zebrafish.

or higher), and here was no significant interaction between treatment and time post-injection ($F_{8,76} = 1.41$, $p = 0.21$), indicating a similar time course of the cortisol response to the 2 ACTH dosages. Planned comparisons between the saline treatment and each ACTH dosage for each sampling point revealed that cortisol levels elicited by the high ACTH dose are significantly higher than those of control treatment 15 min ($F_{1,19} = 10.41$, $p < 0.01$), 30 min ($F_{1,19} = 6.06$, $p < 0.05$), 1 h

($F_{1,19} = 5.67$, $p < 0.05$), and 2 h ($F_{1,19} = 8.03$, $p < 0.05$) after injection, but not at 4 h post-injection ($F_{1,19} = 0.69$, $p = 0.42$), whereas cortisol levels induced by the low ACTH dose were not significantly higher than control levels at any of the sampling points (all p values > 0.20, Fig. 2).

The dilution curves (slopes) for extracted cortisol and for extracted KT from holding-water did not differ from their respective standard curves (ANCOVA: cortisol, $F_{1,7} = 0.28$, $p = 0.61$; KT, $F_{1,8} = 0.01$, $p = 0.93$). Average steroid recoveries were 82.60 ± 0.03 % ($n = 3$) for cortisol and 82.50 ± 0.09 % ($n = 2$) for KT.

Discussion

The results presented here validate the use of holding-water as a noninvasive measure of circulating cortisol levels: there is residual background cortisol immunoreactivity, cortisol recovery is considerably high (82.6%) and within the scope of recovery values reported in other studies,[17,26] and the cortisol values for the extracts dilution curve parallels those of the standard curve, indicating an absence of interference by other immunoreactive substances in the medium. Furthermore, plasma and holding-water cortisol levels show positive significant correlations for both sexes despite males having higher cortisol baseline levels than females, and the ACTH challenge elicited a significant increase in holding-water cortisol levels. The response of cortisol immunoreactivity in the water to the high ACTH challenge was already detected in the first sampling point (15 min post-injection), reached a peak 30 min after the injection, and remained until 2 h post-injection. The saline control (Ringer injection) of this experiment produced similar cortisol values (4.81 ± 1.14 ng/L) to those obtained in Study 1 (4.99 ± 1.33 ng/L), suggesting that, despite being handled several times, fish in the control treatment exhibit baseline cortisol levels. When compared to other species, zebrafish cortisol release rates are one order of magnitude higher (13–20 vs. 0.13–2.5 ng g^{-1} h^{-1}).[17,19,20] This higher cortisol release rates in zebrafish have also been independently documented by another laboratory that also assayed cortisol from holding-water in zebrafish (ca. 10 ng g^{-1} h^{-1}, M. Pavlidis, personal communication, September 2011). It is not clear at the moment which factor contributes to this difference. It is known that temperature may influence cortisol
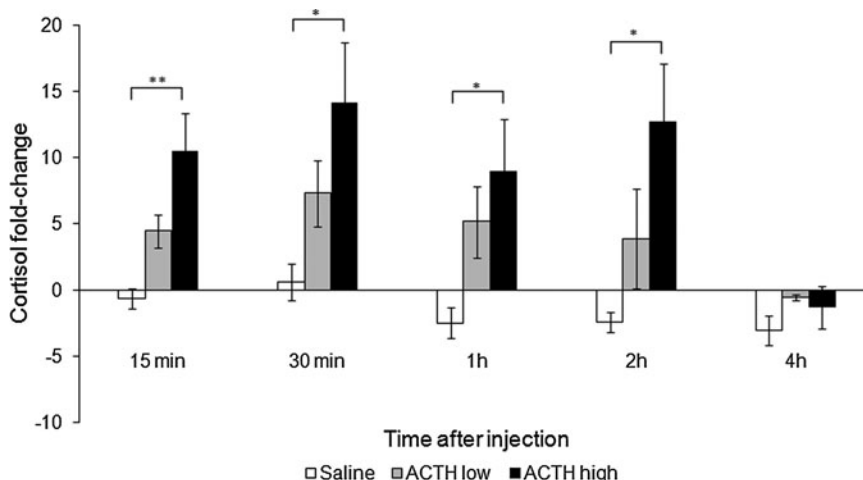

FIG. 2. Response of holding-water cortisol to an ACTH challenge. Bars = Mean + SEM; $*p < 0.05$; $**p < 0.01$.

release rates in the scope of one order of magnitude within the same species (e.g,. $0.13 \, ng \, g^{-1} \, h^{-1}$ at 25°C vs. $0.01 \, ng \, g^{-1} \, h^{-1}$ at 19°C in the dusky grouper, *Epinephelus marginatus*),[20] and in comparison to most other species for which cortisol release rates into the water have been reported, zebrafish was kept at much higher temperatures (28°C for zebrafish and 12–22°C for the other species.[17,19,20] However, other warm water species still have lower cortisol release rates than zebrafish (e.g., $0.150–300 \, pg \, g^{-1} \, h^{-1}$ at 26°C in the convict cichlid *Amatitlania nigrofasciata*[38]), suggesting also an effect of species differences. Finally, the fact that plasma levels of cortisol are similar between the sexes but males have higher cortisol release rates into the water than females may suggest a sex difference in the ratio of free to protein-bound (to a putative cortisol binding globulin) circulating cortisol in zebrafish. In other teleost species, it has been shown that females have a lower percentage of free cortisol than males (ca. 50% less).[39] If that is also the case in zebrafish, since the cortisol present in the holding-water in principle reflects only the free fraction and not the protein-bound, it would explain the higher cortisol holding-water levels for males.

The validation of the cortisol holding-water assay for zebrafish is a significant breakthrough for stress research, since zebrafish is emerging as a model organism for translational stress research both in biomedicine and in aquaculture, due to the similarity of its neuroendocrine stress axis to that of mammals,[40] and to the available genetic resources and logistic advantages when compared to commercial species.[41,42] Furthermore, this method presents several advantages such as: (1) the measured water concentrations represent steroid integration over time, reducing the problem of short-term fluctuations in hormone levels that may occur in plasma due to the pulsatile nature of their release; and (2) enables the measurement of sequential hormone levels, which cannot be achievable either by blood collection or by whole-body homogenates.

Although the results for KT also show little background immunoreactivity, a relatively high recovery (82.5%), there was a lack of correlations between KT levels in holding-water and in plasma for both sexes. This fact can be explained by the presence of a steroid hormone binding globulin (SHBG) in zebrafish gills, which has high binding for androgens and almost no affinity for cortisol.[43] Despite the lack of correlation between plasma and water concentrations, the KT sex difference detected in plasma is also detectable in holding-water. Males release higher concentrations of androgen KT to water than females, with the minimum concentration obtained for males being above the maximum value reported for females. Therefore, although KT water levels cannot be used to study changes in KT circulating levels, they can be used as a tool to sex zebrafish with high reliability. Since sex dimorphism is reduced in zebrafish, leading to common mistakes in sexing fish for experiments, using KT can prove to be a valuable research tool that can give researchers a priori sex identification using a noninvasive method.

In summary, the results presented here validate the use of zebrafish holding-water to measure circulating levels of cortisol and to noninvasively sex individuals with high reliability.

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Disclosure Statement

No competing financial interests exist.

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