Research paper

Validation of water-borne steroid hormones in a tropical frog (*Physalaemus pustulosus*)

Alexander T. Baugh [a],[*], Brandon Bastien [a], Meghan B. Still [b], Nicole Stowell [a]

[a] Department of Biology, Swarthmore College, 500 College Avenue, Swarthmore, PA 19081, USA
[b] Department of Integrative Biology, The University of Texas at Austin, 1 University Station, C0930, Austin, TX 78712, USA

ARTICLE INFO

ABSTRACT

Minimally invasive methods for estimating hormone concentrations in wild vertebrates offer the opportunity to repeatedly measure behavior and hormone concentrations within individuals while minimizing experimenter interference during sample collection. We examined three steroid hormones (corticosterone, CORT; 17-β estradiol, E_2; progesterone, PROG) in túngara frogs (*Physalaemus pustulosus*) using non-invasive water-borne methods. Using solid-phase extraction of water samples and liquid extraction of plasma and homogenate samples, coupled with enzyme immunoassays, we complimented the conventional validation approaches (parallelism, recovery determination) with dose-response assays that incorporated pharmacological challenges with adrenocorticotropic hormone (ACTH) and human chorionic gonadotropin (HCG). We also compared steroid concentrations in water to those observed in plasma and whole body homogenates. Lastly, we identified the constituent steroids in each sample type with a panel targeting 30 steroid species using high performance liquid chromatography-mass spectrometry (HPLC-MS). We found that a 60-min water-bath captures physiologically relevant changes in concentrations of CORT, E_2 and PROG. Peak levels of water-borne CORT were found at approximately 2 h after ACTH injection. Water-borne CORT and E_2 concentrations were positively correlated with their plasma and homogenate equivalents, while water-borne PROG was uncorrelated with homogenate PROG concentrations but negatively correlated with homogenate E_2 concentrations. Together, our findings indicate that sampling water-borne hormones presents a non-invasive and biologically informative approach that will be useful for behavioral endocrinologists and conservation physiologists.

© 2018 Elsevier Inc. All rights reserved.

1. Introduction

As habitat destruction increases globally, a large number of vertebrate species are experiencing the sublethal and lethal effects of anthropogenic environmental degradation. Some of these threats—such as endocrine disruptors—are known to chronically dysregulate gonadal and adrenal endocrine systems (reviewed in Hayes et al., 2006; McMahon et al., 2011; León-Olea et al., 2014; McMahon et al., 2017). Many others, such as the spread of diseases such as chytrid fungus (reviewed in Van Rooij et al., 2015) and noise pollution (Blickley et al., 2012; Tennessen et al., 2014), can acutely interfere with reproductive and stress endocrinology. Population declines may arise due to the sublethal impacts of anthropogenic stressors, such as pesticides, on adult breeders or developing animals (Hayes et al., 2002), and these may in turn be mediated by major endocrine systems including the hypothalamic-pituitaryadrenal axis (or -interrenal in amphibians; HPA and HPI) and hypothalamic-pituitary-gonadal axis (HPG). The emerging field of conservation physiology focuses in part on developing and deploying non-destructive methods for detecting and measuring perturbations in reproductive and stress endocrinology.

The health of animal populations hinges on successful reproduction and coping with stressors. Although acute activation of the endocrine stress response represents a critical adaptation for coping with unpredictable events (noxious and non-noxious) encountered during normal life (Breuner and Hahn, 2003; Cockrem and Silverin, 2002; Thaker et al., 2009; Koolhaas et al., 2011), the impacts of chronically elevated stress hormones (glucocorticoids; CORT) can have a variety of deleterious effects, including suppression of both the immune system (Sapolsky et al., 2000) and reproduction through interactions with the HPG axis (McEwen and Wingfield, 2003), decreased growth rates, protracted periods of development and diminished sensitivity to endocrine secretagogues (Glennemeier and Denver, 2002).

The act of collecting tissue samples for hormone measurement can directly impact subsequent secretion patterns and behavior (Romero and Reed, 2005; Baugh et al. 2013, 2017a), and avoiding these impacts in experimental studies often requires the use of unbalanced experimental designs that position hormone sampling as the final step and limit our understanding of bi-directionality in hormone–behavior relationships. Sample collection methods that minimize the influence of the experimenter on the animal's experience have the potential to improve our ability to draw inferences about bi-directionality and causation by allowing the simultaneous measurement of both trait categories. These approaches allow for repeated or continuous monitoring of both endocrine status and behavior, thus enabling more accurate phenotyping of individuals. Improved estimates of individual (co)variation in hormone concentrations could thus help to resolve the inherent challenges of understanding hyper-labile traits (Dingemanse et al., 2010).

Rapid declines in amphibian populations are associated with multiple sources of aquatic degradation and known stressors such as water acidification and reductions in dissolved oxygen (Kiesecker et al., 2001). Incorporating non-invasive reproductive and stress physiology methods in amphibian research can provide valuable tools for understanding the role of endocrine dysregulation in amphibian declines and setting conservation priorities, particularly for endangered and threatened species with special restrictions on use. Recent studies have validated urinary steroid methods for amphibians (reviewed in Kindermann et al., 2012; Narayan, 2013). Urine sampling has the advantage of being minimally invasive and yet can provide samples that are concentrated enough to measure multiple steroids from the same sample, but typically involves subjecting the animal to handling stress (cloacal sampling), thereby potentially interfering with subsequent behavior and hormone secretion, and is limited to larger adult amphibians. Water-borne sampling methods have the potential to be used with minimal or no animal handling and for any size amphibian, and recent studies by Gabor et al. (2013a, 2016) validated water-borne methods for estimating CORT metabolite concentrations in three salamander species (*Eurycea* sp.) and an anuran (*Alytes obstetricans*). Here we extend the validation of water-borne hormones by applying it to an anuran species—the túngara frog (*Physalaemus pustulosus*). We validated water-borne procedures for three steroids—corticosterone, CORT; 17β-estradiol, E$_2$; and progesterone, PROG—testing the idea that concentrations of hormones excreted in a water column reflect physiologically relevant and biologically informative concentrations in the animal through pharmacological challenges; dose-response assays; time courses to identify peak excretion; recovery determination; parallelism; correlation estimates for concentrations of hormones in water, plasma and whole body homogenates. We used high performance liquid chromatography-mass spectrometry (HPLC-MS) to identify and compare the various steroid species in tissues and water samples.

2. Methods

2.1. The system

Túngara frogs are small anurans (ca. 30 mm snout-to-vent length, SVL) found throughout Mesoamerica (Weigt et al., 2005) that can be maintained in captivity. During the breeding season males spend hours each night immersed in water while advertising vocally and females hydrate their clutches and oviposit while immersed in water (Ryan, 1985). Though not tested here, we assume that immersion in water is not a potent stressor.

Most research on this species has explored the behavioral ecology of mating using wild-caught adults, for which it has become a model system (Ryan, 2010; Ryan, 2011). A growing number of studies have also examined hormone-behavior relationships in both wild (Baugh and Ryan, 2017; Chakraborty and Burmeister, 2009; Chakraborty and Burmeister, 2010; Lynch and Wilczynski, 2005; Lynch and Wilczynski, 2006; Marler and Ryan, 1996) and captive populations (Baugh and Ryan, 2010; Baugh, Hoke and Ryan, 2012; Lynch et al. 2006; O'Connell et al. 2011), and they are increasingly studied in the context of global change biology (Brem and Lips, 2008; Forero-Medina et al., 2010; Gallmetzer and Schulze, 2015; Turriago et al., 2015; Rodriguez-Brenes et al., 2016).

2.2. Animals and experimental design

Adult túngara frogs were transferred from a captive breeding colony at the University of Texas at Austin to Swarthmore College where they were maintained and housed communally for 6 months prior to experimentation (10 frogs per terraria at 27 °C, ambient humidity, L:D 12:12). Frogs were prevented from breeding during the three months prior to the experiment. Frogs were randomly assigned to treatments and dosages, maintained individually in small critter cages (9 × 4 × 5 in.; LxWxH) and fed an *ad libitum* diet of fruit flies. Before experimentation, each frog was marked with a unique toe-clip combination. Frogs were weighed (to the nearest 0.01 g) and measured for SVL (to the nearest 0.01 mm) before experimentation and immediately prior to injections (mean ± SD: 0.72 ± 0.13 g; 22.10 ± 1.72 mm). We collected all samples in June and July 2016.

We conducted three validation phases. In *Phase 1* we performed a dose response and time course of water-borne hormones after administering intraperitoneal challenges of ACTH (for CORT in males and females) or hCG (for E$_2$ and PROG in females). Parallelism for water-borne samples (and optimal sample dilutions for enzyme immunoassay; EIA) and recovery efficiency following extraction of water samples was also calculated. In order to compare circulating and water-borne hormone concentrations, in *Phase 2* we repeated this process with selected dosages and time-courses proved effective in *Phase 1* and sacrificed the animals for the collection of blood and whole body homogenate samples. This permitted us to estimate parallelism and recovery efficiency for plasma and homogenates samples (and optimal sample dilutions for EIA) as well as estimate the correlation among the concentrations of hormones in water, plasma and homogenates. In *Phase 3*, we identified and quantified the concentration of steroid species present in pooled samples of water, plasma and homogenate using HPLC-MS (conducted at West Coast Metabolomics (UC-Davis)); replicate pools were also analyzed by EIA for comparison of these two quantification techniques.

For the collection of water samples, "frog water" was prepared by dissolving the following solutes in 30 L of reverse osmosis (RO) water: 1.2 g CaCl$_2$, 1.38 g MgSO$_4$, 1.08 g KHCO$_3$, 0.9 g NaHCO$_3$ and 0.038 g of a commercial trace-element mix. We added frog water to 100 mL beakers and manually introduced a single frog to each beaker. Sample collection was conducted between 0800 and 1200 to control for diel rhythms in hormone secretion. After completion of each water bath, frogs were manually removed and each sample was immediately filtered to remove large particles and then maintained at −80 °C for up to 5 months prior to extraction. Control water samples (i.e., no frog) were collected at each phase to evaluate possible contamination (all control samples registered at or below the detection limits of the EIA).

The vehicle (0.9% NaCl solution) was used to dissolve adrenocorticotropic hormone (ACTH from porcine pituitary; Sigma Cat. No. A6303) and human chorionic gonadotropin (hCG; Sigma Cat. No. C1063). ACTH is the pituitary secretogue in the hypothalamic-pituitary-interenal (HPI) axis that induces CORT secretion when it binds to receptors in the adrenal cortices, and hCG

stimulates ovulation. Hormones and vehicle were stored at 4 °C for up to 24 h prior to injection. Individual dosages were mass-specific (see *Phase 1* below) and all injections were intra-peritoneal (Baugh and Ryan, 2017; Baugh and Ryan, 2010).

2.2.1. Phase 1a: CORT in water: Timelines and dose-response following ACTH challenge

Frogs were injected with one of three dosages (N = 5 females and 5 males per dosage)–(1) vehicle; (2) low ACTH: 0.5 μg g^{-1}; (3) high ACTH: 5 μg g^{-1}–and held for 60 min before being transferred to a series of four 60-min water baths (40 mL frog water) to provide a 4-h timeline of CORT excretion (i.e. repeated measures sampling scheme; Fig. 1).

2.2.2. Phase 1b: E₂ and PROG in water: Timelines and dose-response following hCG challenge

Frogs were injected with one of three dosages (N = 10 females per dosage)–(1) vehicle; (2) low hCG: 50 IU g^{-1}; (3) high hCG: 500 IU g^{-1}–and held for 24 h to elicit sexually proceptive behavior in captive female túngara frogs that mirrors the behavior of wild-caught amplectant females (see Baugh and Ryan, 2010). Following this 24-h delay, females were individually transferred to a single water bath (40 mL frog water) for 60 min.

2.2.3. Phase 2a: CORT in water, plasma and whole body homogenates: Timelines following ACTH challenge

Based on the results of *Phase 1a*, we selected the low ACTH dosage (0.5 μg g^{-1}) for *Phase 2a*. Frogs were injected with vehicle (N = 4 males & 3 females) or ACTH (N = 13 males & 12 females) (see Fig. 2). Frogs were placed in a 60-min pre-injection water bath, followed by a holding period of 30 min. Next, each frog was injected, held for 15 min, and then placed in two sequential 60-min water baths. All water baths in *Phase 2a* experiments used 25 mL frog water (instead of 40 mL) in 100 mL beakers to hasten the SPE step. Within 3 min after removal from the final water bath we collected between 5 and 40 μL of whole blood from each frog via cardiac puncture using heparinized microhematrocrit capillary

tubes and immediately centrifuged samples in their microhematocrit tubes for 5 min at 5000 RPM. The percentage hematocrit was estimated for each sample using a hematocrit reader (Ample Scientific) and the plasma fraction was removed using specialty pipette tips (VWR). Samples were stored in microcentrifuge tubes at −80 °C until assaying.

Immediately following blood collection, we weighed and decapitated each frog, placed them inside 15 mL conical tubes and submerged in a slurry of dry ice and methanol. We recorded the latency between removing the frog from the final water bath and the completion of tissue freezing (handling duration). Frozen specimens were maintained at −80 °C until they were homogenized. To homogenize, we thawed specimens and added a mass-specific volume (2 μL mg^{-1}) of assay buffer based on the post-blood collection mass of each frog (1:2 dilution). We homogenized this mixture in 15-mL conical tubes on wet ice (Scilogex D160, 7-mm tissue probe, LLC, Rocky Hill, CT). Homogenates were then centrifuged at 3750 RPM in a refrigerated centrifuge for 20 min and supernatant was collected and maintained at −80 °C until further processing.

2.2.4. Phase 2b: E₂ and PROG in water, plasma and whole body homogenates: Timelines following hCG challenge

Based on the results of *Phase 1b*, we selected the high hCG dosage (500 IU g^{-1}) for *Phase 2b*. Frogs were injected with vehicle (N = 5 females) or hCG (N = 19 females). Frogs were placed in a 60 min pre-injection water bath, held for 60 min, and then injected and held again for 24 h. Each frog was then placed in a 60 min post-injection water bath (25 mL frog water in 100 mL beakers). Immediately following the post-injection water bath, blood and homogenate samples were collected and processed as described in *Phase 2a*.

2.2.5. Phase 3: High performance liquid chromatography-mass spectrometry

A duplicate subset of pooled samples from *Phase 2a* and *2b* were processed through the extraction and drying stage and then shipped on dry ice to the core services at West Coast Metabolomics
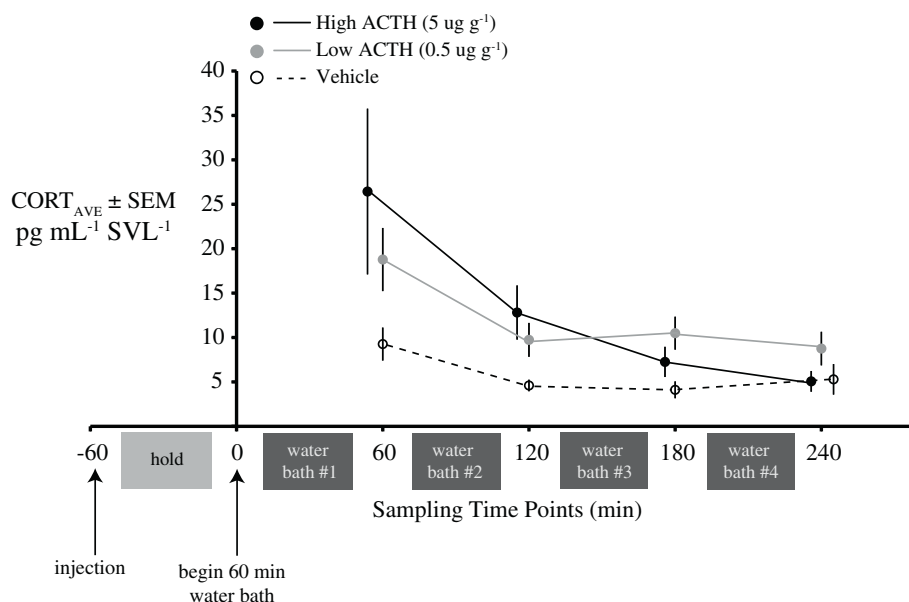
Fig. 1. Individual male (N = 5 per treatment group) and female (N = 5 per treatment group) combined water-borne CORT values (mean ± SEM) for vehicle, low dose ACTH (0.5 μg g^{-1}) and high dose ACTH (5 μg g^{-1}). ACTH or vehicle (0.9% saline) injection occurred at −60 min. Frogs were held in terraria for 60 min following injection and then introduced to a water bath at 0 min time-point. All water baths were 60 min in duration and were sequential (i.e. repeated measures). For graphical purposes, the x-axis intercepts are shifted for some points so that error bars do not overlay.
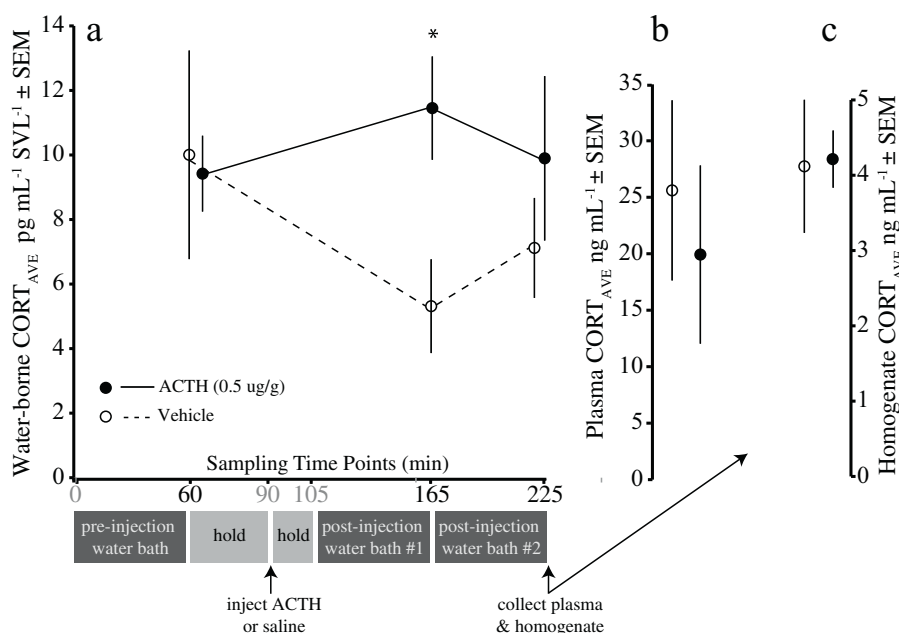
Fig. 2. CORT concentrations for ACTH (0.5 μg g^{-1}) and vehicle (0.9% saline) injected frogs for 3 water samples, a plasma sample and a whole body homogenate sample (mean ± SEM). For graphical purposes, the x-axis intercepts are shifted slightly so that error bars do not overlay. (a) Water-borne CORT concentrations for ACTH (N = 24) and vehicle (N = 7) injected frogs are corrected for each animal's SVL and are depicted at the end of each of the three 60-min water bath periods. (b) Plasma CORT concentrations for ACTH injected frogs (N = 16) and vehicle injected frogs (N = 4). (c) Whole body homogenate CORT concentrations for ACTH injected frogs (N = 25) and vehicle injected frogs (N = 7). *p = 0.05.

(University of California-Davis) for HPLC-MS analysis. The other subset was analyzed by EIA. We prepared six pooled samples each from the *Phase 2a* and *2b* experiments containing (1) pre-secretagogue injection water (ACTH for *Phase 2a*; hCG for *Phase 2b*), (2) post-secretagogue injection water, (3) vehicle injected plasma, (4) vehicle-injected homogenate, (5) secretagogue-injected plasma, and (6) secretagogue-injected homogenate. We also analyzed two plasma samples (10 μL) collected from an individual wild-caught amplectant female túngara frog before and after oviposition. We removed 3 mL aliquots from each experimental water sample following filtering and prior to SPE and corrected for this lost fraction of water in their final estimates. From each homogenate and plasma sample we removed 0–25 μL and 0–5 μL, respectively, as available, resulting in pool volumes of each sample type that were equivalent to the experimental samples processed by EIA.

Dried samples were resuspended in 100 μL of a 1:1 solution of HPLC grade methanol and acetonitrile. Samples were processed using HPLC-MS (Waters Acquity/SciEx QTrap 6500) using a targeted metabolite and steroid panel designed to quantify 30 steroid species. Four internal standards were used for calibration: 17-hydroxyprogesterone, estradiol, PROG and testosterone. Steroid limits of quantification (LOQ) ranged between 0.5 nM and 500 nM, including 2.5 for CORT, 5.0 nM for estradiol and 3.0 nM for PROG (see Supplementary materials S1).

2.3. Extraction and reconstitution

2.3.1. Water samples

After thawing filtered samples, we removed a 3-mL subsample from each of the 40-mL water samples collected in *Phase 1* experiments and pooled them within each dosage group and time-point to perform parallelism, optimize dilutions, determine recovery and examine the dose-response and time course of excretion for pooled samples (in addition to individual samples). Pooled and individual water samples were extracted using columns for solid-phase extraction (SPE; Sep-Pak C$_{18}$, 500 mg sorbent; Waters Corp., Milford, MA). SPE has been shown to result in reliable analyte recovery and remove interfering compounds (Lee and Goeger, 1998; Newman et al., 2008) and has been used successfully for amphibian water-borne hormones (Gabor et al., 2013a, 2013b, 2015). Columns were first activated with 4 mL of methanol (ACS grade) and equilibrated with 4 mL RO water. Samples were then slowly drawn through the columns under 15 bar of vacuum pressure using a 24-port manifold system (United Chemical Technologies, LLC, Bristol PA). An additional 4 mL of RO water was then processed through each column. We slowly eluted each column with 4 mL of methanol (HPLC grade) into borosilicate vials and then dried samples under nitrogen gas in a 37 °C water bath using an evap-o-rack manifold (Cole-Parmer, Bunker CT). We cleaned all parts from the vacuum manifold that were in contact with samples by passing 25 mL methanol through each port and allowing them to dry prior to the next use.

Dried samples were resuspended in a mixture of 5% absolute ethanol and 95% assay buffer provided in the EIA kits (to a total volume of 250 μL, designated as the 1:1 dilution). A small volume of absolute ethanol has been shown to enhance steroid recovery (Newman et al., 2008). Samples were then vortexed for 30 s, covered with aluminum foil and allowed to reconstitute overnight at 4 °C. Pooled samples were processed by EIA at this 1:1 dilution and were further diluted (see *Parallelism and recovery*) to determine optimal dilution and parallelism prior to the assaying of individual frog samples.

2.3.2. Plasma and homogenate samples

Pools of plasma and homogenates were collected for parallelism, to determine optimal sample dilution and for recovery determination. We used a double diethyl-ether liquid extraction procedure for plasma and homogenate samples because this extraction method is effective with small sample volumes and results in high recoveries for plasma (Baugh et al., 2012); moreover, homogenate samples proved too viscous for processing by SPE as the silica

matrix quickly becomes impregnated with tissue. For liquid extraction, plasma and homogenate samples were vortexed and subsamples (plasma: 5–10 μL; homogenates: 200–600 μL) were added to borosilicate vials. Then 200 μL of RO water was added to each vial in order to increase the aqueous volume for ease of decanting. In a fume hood, 2 mL of diethyl-ether was added to each vial, vortexed and placed in a dry ice and methanol slurry until the aqueous layer was frozen. The organic layer was then decanted to a clean vial and the frozen layer was allowed to thaw; this process was then repeated. The ether extracts were then dried under nitrogen gas in a 37 °C water bath using the evap-o-rack manifold, resuspended in 150 μL assay buffer and allowed to equilibrate overnight at 4 °C. Subsequent dilutions were performed for parallelism and optimal sample dilution determination.

2.4. Parallelism and recovery

All sample pools used for parallelism and recovery determination were processed (extraction method) identically to experimental samples with the exception that pools used for recovery determination were stripped of endogenous steroids and spiked with known concentrations of commercial steroids prior to extraction.

2.4.1. Water
CORT: A pooled water sample from the low ACTH treatment was assayed at 1:1 (i.e. 250 μL assay buffer), serially diluted at 1:2, 1:4, and 1:8 and then assayed. A separate set of pooled water samples from the low ACTH treatment group were stripped of endogenous steroids using 7 mg mL^{-1} of dextran-coated activated charcoal. These samples (N = 9) were then vortexed and incubated at 37 °C for 4 h and centrifuged 3 times following Delehanty et al. (2015). Supernatant was collected and spiked at one of three concentrations using purified CORT (supplied by kit; N = 3 replicates per concentration): low (312.5 pg mL^{-1}), medium (1250 pg mL^{-1}) and high (5000 pg mL^{-1}).

E$_2$: A pooled water sample from the high hCG treatment was assayed at 1:2 (i.e. 500 μL assay buffer), serially diluted at 1:4, 1:8, and 1:16 and then assayed. A separate set of pooled water samples from the high hCG treatment group were stripped (see above) and spiked at one of three concentrations using purified 17-β estradiol (supplied by kit; N = 6 replicates per concentration): low (156.25 pg mL^{-1}), medium (625 pg mL^{-1}) and high (2500 pg mL^{-1}).

PROG: Processing of a pooled water sample from the high hCG treatment was identical to that described for E$_2$ above but was spiked using purified PROG rather than estradiol.

2.4.2. Plasma and homogenates
CORT: A pool of plasma from the low ACTH treatment was serially diluted in assay buffer at 5 concentrations (1:15–1:240). A separate set of pooled plasma samples (N = 3) from the low ACTH treatment group were stripped of endogenous steroids. For all plasma and homogenate stripping we used 70 mg mL^{-1} of dextran-coated activated charcoal (see above). Samples were spiked at 10,000 pg mL^{-1} using purified CORT.

A pool of homogenate from the low ACTH treatment was serially diluted in assay buffer at 7 concentrations (1:3–1:192). A separate set of pooled homogenate samples (N = 3) from the low ACTH treatment group were stripped of endogenous (see above) and spiked at 3000 pg mL^{-1} using purified CORT.

E$_2$: A pool of plasma from the high hCG treatment was serially diluted in assay buffer at 4 concentrations (1:25–1:200). A separate set of pooled plasma samples (N = 2) from the high hCG treatment group were stripped of endogenous steroids (see above) and spiked at 10 pg mL^{-1} using purified estradiol.

A pool of homogenate from the high hCG treatment was serially diluted in assay buffer at 4 concentrations (1:3.75–1:30). A separate set of pooled homogenate samples (N = 2) from the high hCG treatment were stripped of endogenous steroids (see above) and spiked at 9.6 pg mL^{-1} using purified estradiol.

PROG: There was insufficient plasma from the hCG experiment to quantify PROG and thus we only assayed homogenates. A pool of homogenate from the high hCG treatment was serially diluted in assay buffer at 5 concentrations (1:0.5–1:8). A separate set of pooled homogenate samples (N = 2) from the high hCG treatment were stripped of endogenous steroids (see above) and spiked at 9.6 pg mL^{-1} using purified PROG (supplied by kit). Because PROG is less concentrated than E$_2$ in homogenates, it is critical that dilution for E$_2$ (1:6) be carried out after PROG is assayed.

2.5. Enzyme immunoassays

We estimated steroid concentrations using commercial EIA kits (DetectX® kits, Arbor Assays, Ann Arbor, MI) for CORT (Cat. No. K014, Donkey anti-Sheep IgG), 17-β estradiol (plasma and homogenates: Cat. No. KB30 (this kit is optimized for the typically low concentrations of circulating E$_2$), Donkey anti-Sheep IgG; water: Cat. No. K030, Goat anti-Rabbit IgG), and PROG (Cat. No. K025, Goat anti-Mouse IgG).

Reconstituted samples and kit reagents were allowed to reach room temperature prior to use and samples were vortexed prior to plating. We randomly assigned samples to wells and assayed them in duplicate along with blanks, standards, stripped samples, and stripped/spiked samples. We accepted the average of duplicate wells. Samples were assayed following manufacturer instructions (for the concentrations of standards see Supplementary materials S2–S9). We read plates at 450 nm on a Versa$_{max}$ microplate reader with SoftMax Pro software using a four-parameter curve fitting equation (Molecular Devices, Sunnyvale CA). Intra- and inter-assay coefficients of variation (CV) were estimated by including three stripped and spiked samples per plate and thus incorporated cumulative technical error during extraction and assaying. Experimental samples exceeding a 15% CV (between duplicate wells) were re-assayed until CV values met this criterion. The assays have detection limits and sensitivities, respectively, of 16.9 pg mL^{-1} and 18.6 pg mL^{-1} (CORT); 26.5 pg mL^{-1} and 39.6 pg mL^{-1} (E$_2$ in water); 2.05 pg mL^{-1} and 2.21 pg mL^{-1} (E$_2$ in plasma and homogenates); and 52.9 pg mL^{-1} and 47.9 pg mL^{-1} (PROG). The cross-reactivity of the antiserum for the CORT kit is 100% for CORT, 12.3% for des-oxycorticosterone, 0.76% for tetrahydrocorticosterone, 0.62% for aldosterone and 0.24% for PROG. The cross-reactivity of the antiserum for the plasma/homogenate E$_2$ kit is 100% for E$_2$, 3.2% for estrone sulfate, and 2.5% for estrone. The cross-reactivity of the antiserum for the water E$_2$ kit is 100% for E$_2$ and 0.73% for estrone. The cross-reactivity of the antiserum for the PROG kit is 100% for PROG, 188% for 3α-hydroxy-PROG, 172% for 3β-hydroxy-progesterone, 147% for 11α-hydroxy-progesterone, 7.0% for 5α-dihydroprogesterone, 5.9% for pregnenolone, and 2.7% for 11β-hydroxy-progesterone.

2.6. Statistics

Individual water-borne hormone concentrations were corrected for each frog's body size by dividing by SVL (Gabor et al., 2013a). We used conventional parametric statistical methods as described in Results. Hormone data were log$_{10}$-transformed prior to analysis to improve residuals. Parametric assumptions (normality, equality of variance and covariance, and sphericity) were met unless indicated otherwise. We used SPSS (version 21, IBM) for all statistical analyses.

3. Results

3.1. CORT from ACTH experiments

3.1.1. Phase 1: Water

Results from pooled samples in *Phase 1* indicated that a 1:1 dilution (250 μL reconstitution volume) was optimal in terms of the linear portion of the standard curve, and therefore all subsequent samples were reconstituted to this volume. Serial dilutions were parallel to the standard curve (Δ slope = 0.07, SE = 0.80, t_8=0.09, p = 0.93) and recovery efficiencies were high (84.8–99.3%) with low CVs (Supplementary materials S9). Stripped (and unspiked) samples registered near the detection limit of the assay (mean: 28.9 pg mL^{-1})—we subtracted this background concentration from the stripped/spiked samples when calculating percent recovery.

Pooled samples suggested that peak water-borne CORT concentrations were present at 60-min post-injection time-point (post-injection water bath #1) and that low and high ACTH dosages yielded similar CORT levels, which were elevated compared to vehicle (Supplementary materials S10). This pattern was confirmed using a repeated measures ANOVA (within-subject effect: time-point; between-subject effects: dosage and sex) for the individual samples (Fig. 1): there was a main effect of time-point ($F_{1.6,35.2}$ = 19.448, p < 0.0001) due to decreasing CORT following the 60-min peak, and a main effect of treatment group ($F_{2,22}$ = 6.360, p = 0.007). Post hoc comparisons indicated that the vehicle treatment group differed from the low ACTH (p = 0.007) and the high ACTH treatment groups (p = 0.044), but the low and high ACTH groups did not differ from each other (p = 0.703). Males and females did not differ in CORT profiles: there was no main effect of sex ($F_{1,22}$ = 0.03, p = 0.88) nor any interactions between sex, dose and time-point (p > 0.3 for all 2- and 3-way interactions; Supplementary materials S10–S15).

3.1.2. Phase 2: water, plasma and homogenates

Serial dilutions of plasma and homogenates were parallel to the standard curve (plasma: Δ slope = 0.07, SE = 0.77, t_9 = 0.09, p = 0.93; homogenates: Δ slope = 0.03, SE = 0.21, t_{11} = 0.17, p = 0.87) and recovery efficiencies were uniformly high (plasma: mean ± SD: 88.9% ± 2.1%; Supplementary materials S2; homogenates: mean ± SD: 98.8% ± 5.9%; Supplementary materials S5). Pooled samples showed that the optimal sample dilution was 1:15 for plasma and 1:6 for homogenates, which we used in assays for subsequent individual samples. The intra- and inter-assay CVs were 7.6% and 9.2% for water and 7.3% and 8.8% for plasma and homogenate plates, respectively.

Given the dose-response results from *Phase 1*, we used the low ACTH dose for *Phase 2* experiments, as has been used successfully in other amphibians (Narayan et al., 2011). We limited the time course for water-borne CORT to a pre-injection time-point and 2 post-injection time-points (60 and 120 min). As with *Phase 1*, there were no sex differences in any of the CORT measures, including plasma and homogenate concentrations (all p > 0.2), and thus males (N = 17) and females (N = 15) were pooled for statistical analyses. Compared to vehicle treated frogs, ACTH-injected frogs exhibited an increase in water-borne CORT in water bath #1 (75 min post-injection) and returned to baseline in water bath #2 (135 min after injection; Fig. 2a). A repeated measures ANOVA with post hoc comparisons for the water-borne CORT demonstrated a significant interaction between treatment and time-point ($F_{2,28}$ = 3.66, p = 0.039) driven by a significant decrease in CORT in the vehicle group in the post-injection water bath #1 (p = 0.036) and a non-significant increase in the ACTH treatment (p = 0.11) at that time-point, resulting in a significant interaction

between the treatment groups at this first post-injection time-point (p = 0.05). There were no significant differences between treatment groups in either the pre-injection water bath or the post-injection water bath #2 (all p > 0.5). There was no difference between vehicle and ACTH groups in the plasma CORT samples (t_{18} = 1.0, p = 0.319), and hematocrit estimates for the plasma were similar between the treatment groups (vehicle: 11.3 ± 4.2%; ACTH: 9.7 ± 1.1%). There was no correlation between handling duration (mean ± SEM, range: 343 ± 13.2, 245–480 s) and plasma CORT concentrations (r = 0.34, p = 0.14, N = 20). Likewise, there was no difference between treatment groups in the whole body homogenate CORT samples (t_{30} = 0.11, p = 0.91) and no correlation between handling duration (mean ± SEM, range: 343 ± 13.2, 245–480 s) and homogenate CORT concentrations (r = 0.16, p = 0.40, N = 32). The lack of a treatment effect for plasma and homogenates might be explained by the fact that treatment groups had returned to parity by the time tissues were harvested (see post-injection water bath #2 in Fig. 2a); however, this cannot explain the pattern observed in PROG (see Phase 2 PROG).

Pre-injection water-borne CORT concentrations were positively correlated with the first post-injection water-borne time-point and likewise, concentrations at the two post-injection time-points were positively correlated (Supplementary materials S16). We found positive correlations among media types (Fig. 3): (1) water-borne CORT from post-injection water bath #2 were positively correlated with plasma CORT (r = 0.46, p = 0.04, N = 20) and homogenate CORT (r = 0.52, p = 0.002, N = 32); (2) water-borne CORT from post-injection water bath #1 were positively correlated with homogenate CORT (r = 0.44, p = 0.013, N = 32); and (3) plasma concentrations were positively correlated with homogenate concentrations (r = 0.81, p < 10^{-5}, N = 20). Hematocrit (%) and plasma CORT concentrations were not correlated (r = 0.38, p = 0.13, N = 17), and plasma and post-injection water bath #1 CORT concentrations were not correlated (r = 0.08, p = 0.73, N = 20).

## 3.2. Estradiol and PROG from hCG experiments

3.2.1. Phase 1: water-borne E$_2$

Results from pooled samples in *Phase 1* indicated that a 1:2 dilution (500 μL reconstitution volume) was optimal in terms of the linear portion of the standard curve, and therefore all subsequent samples were reconstituted to this volume (Supplementary materials S7). Serial dilutions were parallel to the standard curve (Δ slope = 0.07, SE = 1.17, t_5 = 0.06, p = 0.95) and recovery efficiencies varied between moderate and high (61.4–110.9%) with low CVs (Supplementary materials S7). Stripped (and unspiked) samples registered below the detection limit of the assay.

Females injected with hCG had higher E$_2$ than vehicle injected females (Fig. 4a). A repeated measures ANOVA showed a main effect of treatment group ($F_{2,25}$=9.555, p = 0.001) and post hoc comparison showed that this treatment effect was driven by significant differences between vehicle and the low hCG group (p = 0.002) as well as vehicle and the high hCG group (p = 0.003). There was no difference between low and high hCG (p = 0.996). Two samples had high concentrations of E2 but were within the linear portion of the standard curve. Exclusion of these two potential statistical outliers did not affect the outcome of the analyses.

Water-borne E$_2$ was positively correlated with water-borne PROG in both the vehicle (Spearman's rho = 0.83, p = 0.003) and high hCG treatment groups (Spearman's rho = 0.76, p = 0.028, respectively), as well as when all three treatment groups were combined (Spearman's rho = 0.55, p = 0.002). This correlation was not present in the low hCG treatment (Spearman's rho = 0.19, p = 0.60; Supplementary materials S17–S20).
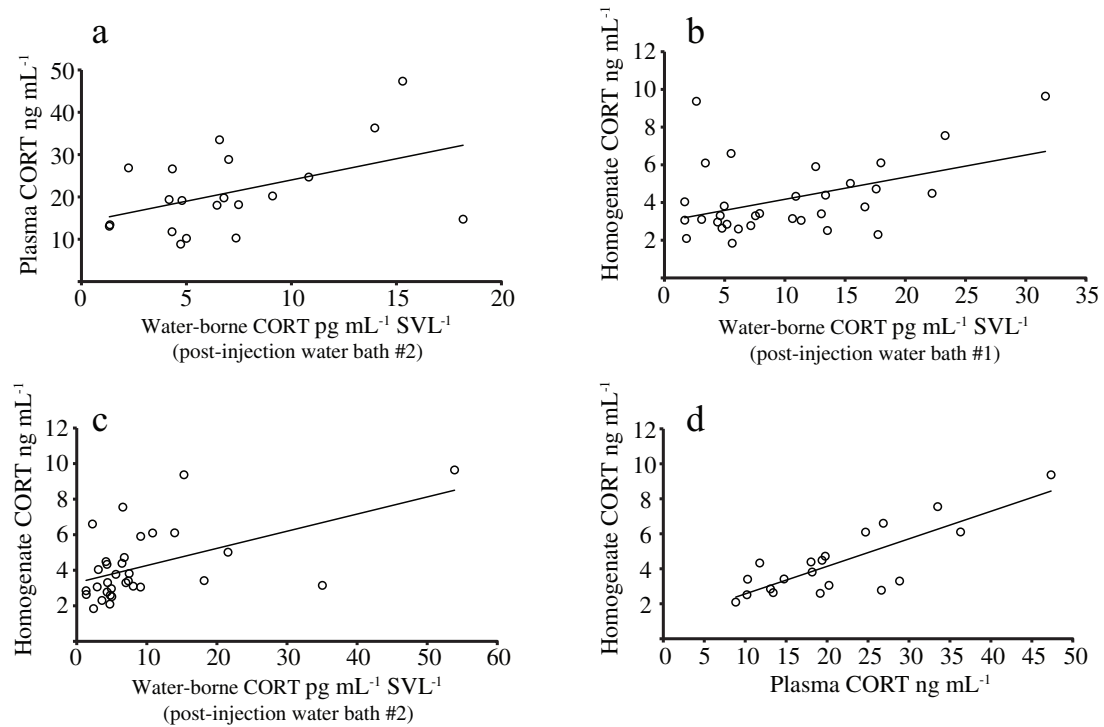
Fig. 3. Correlations (Pearson's) between CORT concentrations among tissues. (a) plasma concentrations were positively correlated with water-borne concentrations (post-injection water bath #2). (b–c) whole body homogenate concentrations were positively correlated with water-borne concentrations in post-injection water bath #1 and #2. (d) plasma and whole body homogenate concentrations were positively correlated.
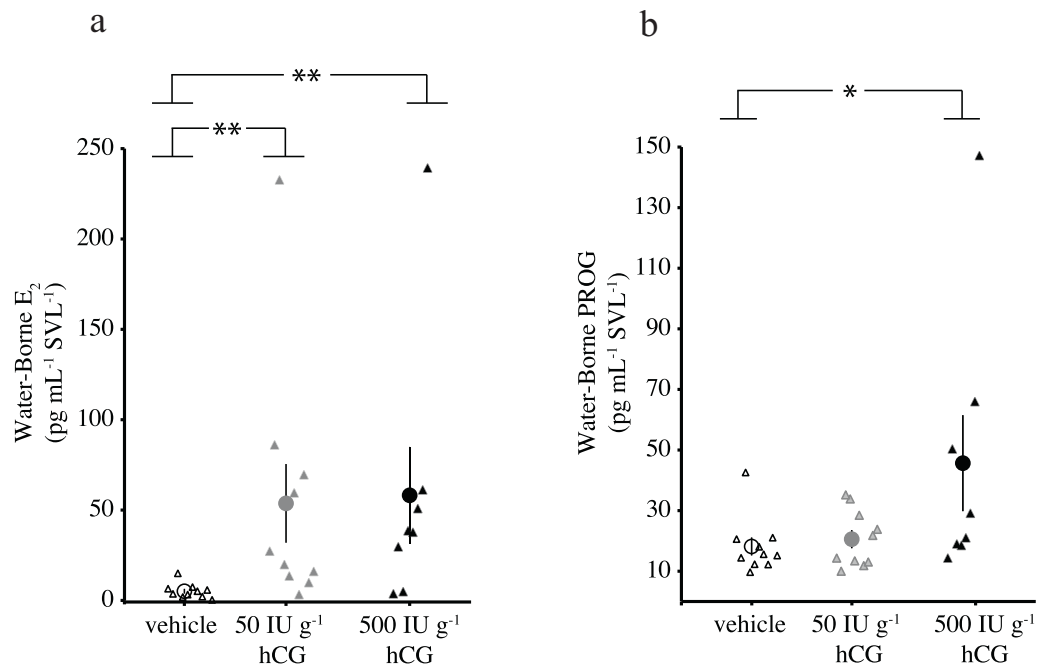
Fig. 4. Individual samples (triangles) of (a) water-borne 17-β estradiol or (b) water-borne PROG at one of three doses of hCG. Circles represent the mean (±SD).

3.2.2. Phase 2: E_2 in water, plasma and homogenates

Serial dilutions of plasma and homogenates were parallel to the standard curve (plasma: Δ slope = 0.18, SE = 2.77, t_6 = 0.06, p = 0.95; homogenates: Δ slope = 0.03, SE = 2.29, t_6 = 0.01, p = 0.99) and recovery efficiencies were uniform and relatively low (plasma: mean ± SD: 46.8% ± 6.2%; Supplementary materials S4; homogenates: mean ± SD: 125.5% ± 5.7%; Supplementary materials S3).

Pooled samples showed that the optimal sample dilution was 1:50 for plasma and 1:6 for homogenates, which we used in assays for subsequent individual samples. The intra- and inter-assay CVs, respectively, were 19.1% and 5.4% for water and 2.4% and 11.2% for plasma and homogenate plates.

Given the dose-response results from *Phase 1*, we used the high hCG dose for *Phase 2* experiments, which has been used previously

in eliciting reproductive readiness in this species (Baugh and Ryan, 2010). Results from the pre-injection water bath showed that for water-borne E_2, vehicle and hCG treatment groups had similar E_2 concentrations before injection. Further, hCG treated frogs exhibited a non-significant elevation in water-borne E_2 following injection whereas vehicle treatment groups did not change (Fig. 5a). However, a repeated measures ANOVA demonstrated a non-significant interaction between treatment and time-point for water-borne E_2 ($F_{1,19} = 0.58$, $p = 0.45$; Fig. 5a). Plasma E_2 concentrations were also higher in the hCG compared to vehicle treatments but this difference was also not significant ($t_{16.4} = 1.75$, $p = 0.09$; equal variances not assumed; Fig. 5b). The average hematocrit of the plasma was similar between the hCG ($7.4 \pm 1.2\%$; $N = 16$) and vehicle-injected frogs ($7.1 \pm 3.4\%$; $N = 5$). In contrast, E_2 concentrations in homogenates were significantly higher in hCG compared to vehicle treated frogs ($t_{19.4} = 2.13$, $p = 0.04$; equal variances not assumed; Fig. 5c).

Concentrations of E_2 in the plasma were positively correlated with water-borne E_2 concentrations in the pre-injection water bath ($r = 0.51$, $p = 0.03$, $N = 18$; Fig. 6a). There was a positive but non-significant correlation between plasma E_2 and water-borne E_2 from the post-injection water bath ($r = 0.43$, $p = 0.06$, $N = 19$; Fig. 6b). E_2 concentrations in homogenates were positively correlated with water-borne concentrations in post-injection water bath ($r = 0.48$, $p = 0.02$, $N = 22$; Fig. 6c). Further, E_2 concentrations in plasma and homogenates were positively correlated ($r = 0.767$, $p < 10^{-4}$, $N = 19$; Fig. 6d). Concentrations of water-borne E_2 at pre- and post-injection time-points were not correlated ($r = 0.18$, $p = 0.44$, $N = 21$). Lastly, hematocrit (%) and plasma E_2 concentrations were not correlated ($r = 0.29$, $p = 0.27$, $N = 19$).

3.2.3. Phase 1: water-borne PROG

Results from pooled samples in *Phase 1* indicated that a 1:2 dilution (500 μL reconstitution volume) was optimal in terms of the linear portion of the standard curve, and therefore all subsequent samples were reconstituted to this volume (Supplementary materials S8). Serial dilutions were parallel to the standard curve (Δ slope = 0.07, SE = 0.76, $t_7 = 0.09$, $p = 0.93$) and recovery efficiencies varied between low and moderate (39.7–50.2%) with low CVs (Supplementary materials S8). Stripped (and unspiked) samples registered below the detection limit of the assay.

Females injected with hCG had higher PROG than vehicle injected females (Fig. 4b).

An ANOVA showed that there was a main effect of treatment group ($F_{2,25} = 3.741$, $p = 0.038$), and post hoc tests showed that this treatment effect was driven by significant differences between vehicle and the high HCG group ($p = 0.040$); the other two comparisons were not significant: vehicle versus low hCG ($p = 0.891$); low versus high hCG ($p = 0.098$). One sample had high PROG but was within the linear portion of the standard curve. Exclusion of this potential statistical outliers did not affect the outcome of the analyses.

3.2.4. Phase 2: PROG in water and homogenates

Serial dilutions of homogenates were parallel to the standard curve (Δ slope = 0.03, SE = 0.34, $t_8 = 0.01$, $p = 0.99$) and recovery efficiencies were high (mean $\pm$ SD: 94.2% $\pm$ 13.2%; Supplementary materials S6). From pooled samples we found that the optimal sample dilution was 1:0.5 for homogenates, which we used in assays for subsequent individual samples. The intra- and inter-assay CVs, respectively, were 17.7% and 4.7% for water and 12.9% and 12.2% for homogenate plates.

We found that both vehicle and hCG treatment groups experienced a similar and small elevation in water-borne PROG following injection (Fig. 7a). The repeated measures ANOVA revealed a non-significant interaction between treatment and time-point ($F_{1,21} = 0.003$, $p = 0.96$). In contrast, homogenate PROG concentrations for hCG injected frogs were significantly higher than in vehicle injected frogs ($t_{22} = 2.55$, $p = 0.018$; equal variances not assumed).

Concentrations of PROG in the homogenates were not correlated with post-injection ($r = 0.09$, $p = 0.69$, $N = 23$; Fig. 8a) or pre-injection water-borne PROG concentrations ($r = 0.063$, $p = 0.77$, $N = 24$; Fig. 8b). In contrast, there was a positive correlation between concentrations of water-borne PROG at the two time-points ($r = 0.64$, $p = 0.002$, $N = 23$; Fig. 8c). Lastly, we found an unexpected negative correlation between water-borne PROG and E_2 in homogenates, present in both the pre- and post-injection time-points (Supplementary materials S21). Because there was no correlation between these two products in homogenates ($r = -0.12$, $p = 0.57$, $N = 23$), this suggests some inter-dependency between these two hormones in the context of clearance.
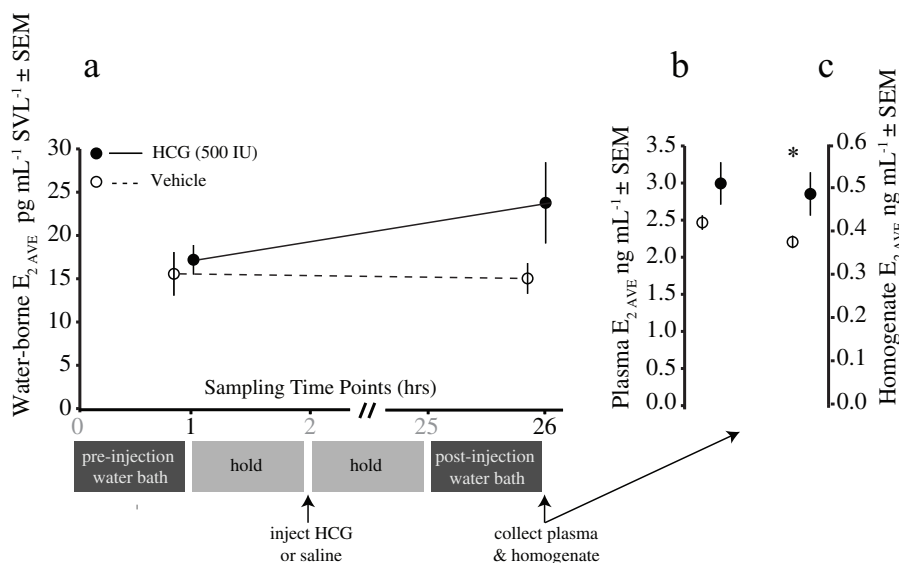
Fig. 5. 17-β estradiol (E_2) concentrations for hCG (500 IU g^{-1}) and vehicle (0.9% saline) injected female frogs for two water samples, a plasma sample and a whole body homogenate sample (mean $\pm$ SEM). For graphical purposes, the x-axis intercepts are shifted slightly so that error bars do not overlay. (a) Water-borne E_2 concentrations for HCG (N = 16) and vehicle (N = 5) injected frogs are corrected for each animal's SVL and are depicted at the end of each of the two 60-min water bath periods.
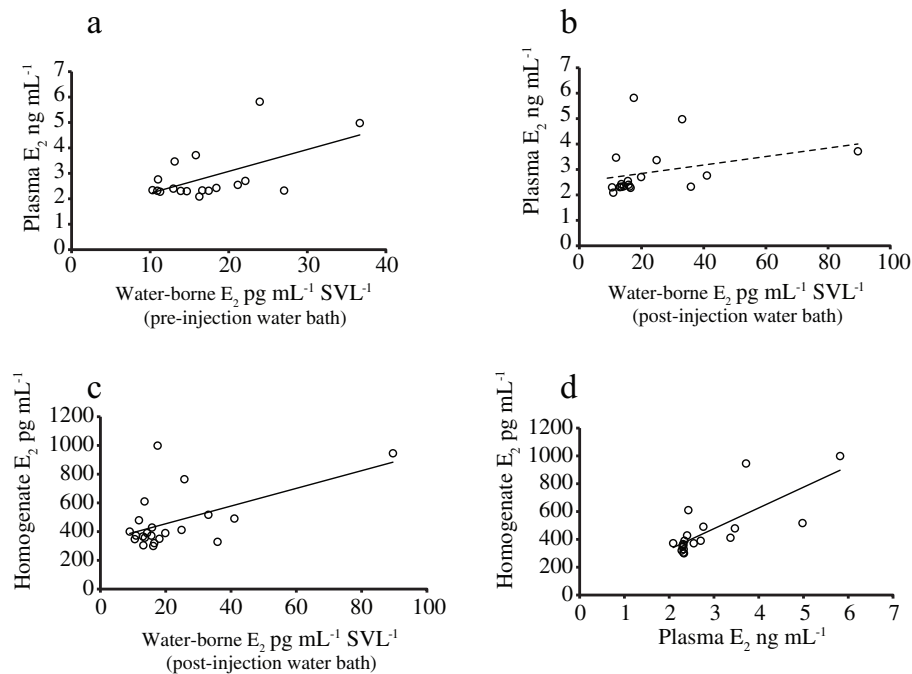
Fig. 6. PROG concentrations for hCG (500 IU g^{-1}) and vehicle (0.9% saline) injected female frogs for two water samples and a whole body homogenate sample (mean ± SEM). For graphical purposes, the x-axis intercepts are shifted slightly so that error bars do not overlay. (a) Water-borne PROG concentrations for hCG and vehicle injected frogs are corrected for each animal's SVL and are depicted at the end of the pre-injection (N_{veh} = 5, N_{hCG} = 19) and post-injection (N_{veh} = 5, N_{hCG} = 18) water bath periods. (b) Whole body homogenate PROG concentrations for hCG injected frogs (N = 19) and vehicle injected frogs (N = 5).
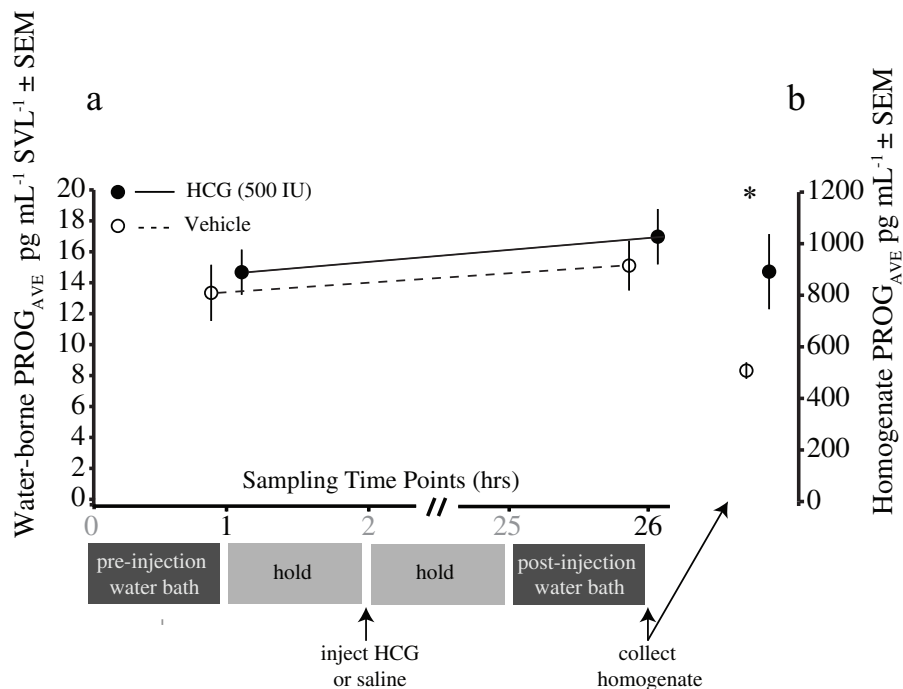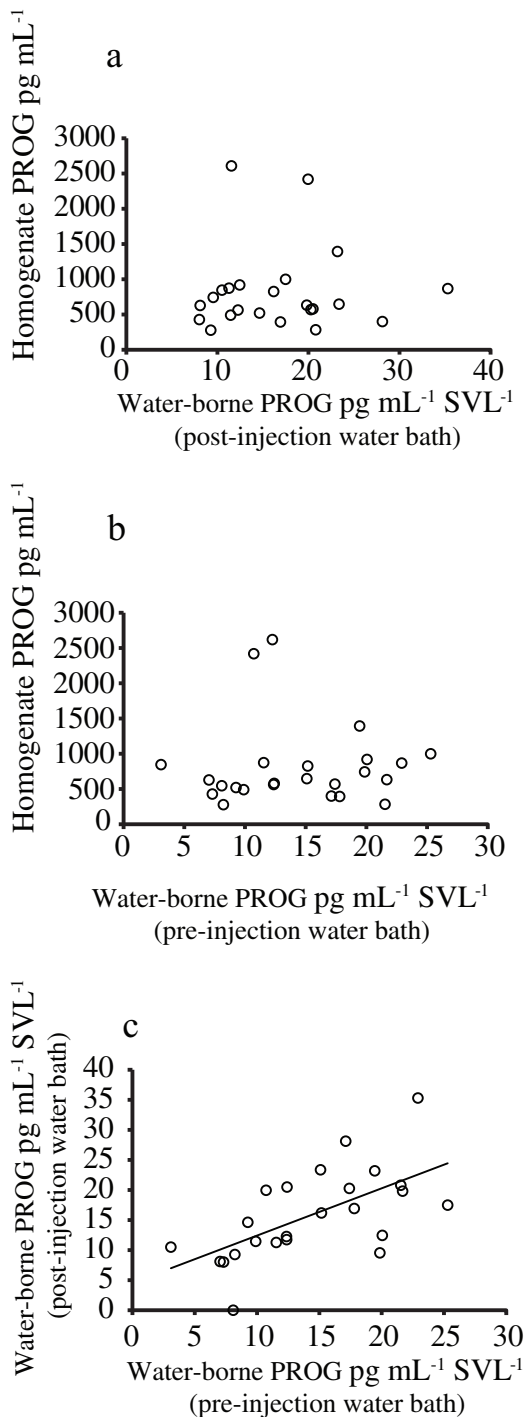

Fig. 7. Correlations (Pearson's) between 17-β estradiol (E$_2$) concentrations among tissues. All E$_2$ data were log$_{10}$-transformed prior to analysis to improve residuals (untransformed data are depicted here). Plasma concentrations were positively correlated with water-borne concentrations in the pre-injection water bath (a) and there was a positive trend (dashed line) in the post-injection water bath (b). (c) whole body homogenate concentrations were positively correlated with water-borne concentrations in post-injection water bath. (d) plasma and whole body homogenate concentrations were positively correlated.

3.3. HPLC-Mass spectrometry

Water samples contained the largest diversity of steroids, followed by homogenates and finally plasma (see Supplementary materials S1 for table of complete HPLC-MS results).

3.3.1. ACTH experiments

WATER: CORT concentrations were lower in the pre-injection compared to the post-injection pools and generally similar to the values estimated in the duplicate pooled samples processed by EIA (HPLC-MS: pre: 87.8 pg mL^{-1}; post: 289.8 pg mL^{-1}; EIA: pre:

Fig. 8. Correlations (Pearson's) between PROG concentrations among tissues. All PROG data were $\log_{10}$-transformed prior to analysis to improve residuals (untransformed data are depicted here). (a–b) whole body homogenate PROG concentrations were not correlated with either the pre-injection or post-injection water-borne concentrations. (c) There was a positive correlation between pre- and post-injection water-borne concentrations.

224 pg mL^{-1}; post: 261 pg mL^{-1}). This was accompanied by a decrease in cortisol and several other steroids (Supplementary materials S1). Compared to plasma and homogenates, water-borne cortisol was enriched and was typically found in higher concentrations than CORT.

PLASMA: Plasma CORT concentrations were similar in the saline and ACTH injected treatments, mirroring the results from the individual samples (see Fig. 2). Likewise the HPLC-MS concentrations

were generally in agreement with the duplicate pooled samples processed by EIA (HPLC-MS: saline: 20.2 ng mL^{-1}; ACTH: 11.9 ng mL^{-1}; EIA: saline: 15.4 ng mL^{-1}; ACTH: 9.9 ng mL^{-1}). Plasma cortisol levels were approximately 10% the concentration of CORT. The only other abundant steroid found in plasma was etiocholanolone (Supplementary materials S1).

HOMOGENATES: CORT concentrations in homogenates were approximately 25% the concentrations observed in plasma, and were lower in the saline compared to the ACTH treatment, mirroring the results from the individual samples (see Fig. 2). Likewise the HPLC-MS concentrations were in close agreement with the duplicate pooled samples processed by EIA (HPLC-MS: saline: 4.3 ng mL^{-1}; ACTH: 5.5 ng mL^{-1}; EIA: saline: 4.26 ng mL^{-1}; ACTH: 5.6 ng mL^{-1}). Also abundant in homogenates were cortisol, etiocholanolone, androstanediol, and estradiol (Supplementary materials S1).

3.3.2. hCG experiments

Water: Estradiol concentrations were lower in the pre-injection compared to the post-injection pool, mirroring results from the individual samples (Fig. 5), and lower (but in register) compared to values estimated in duplicate pooled samples processed by EIA (HPLC-MS: pre: 88.1 pg mL^{-1}; post: 163.7 pg mL^{-1}; EIA: pre: 409 pg mL^{-1}; post: 562 pg mL^{-1}). PROG concentrations were similar in the pre- and post-injection pools, as found in the individual samples (see Fig. 7), and lower (but in register) compared to values estimated in duplicate pooled samples processed by EIA (HPLC-MS: pre: 98.3 pg mL^{-1}; post: 64.0 pg mL^{-1}; EIA: pre: 292 pg mL^{-1}; post: 287 pg mL^{-1}). These pre- to post-hCG injection effects were accompanied by changes in other steroids, notably a large increase in estriol and a decrease in androstenediol and androstanediol (Supplementary materials S1).

Plasma: In contrast to the EIA results, plasma estradiol and PROG were undetectable by HPCL-MS in our captive frog samples (the wild caught female had low but detectable levels of estradiol in plasma). Other steroids were low with the exception of CORT, which was higher in the hCG treatment (saline: 2.3 ng mL^{-1}; hCG: 35.4 ng mL^{-1}), and dihydroprogesterone, which was similar in the saline and hCG treatments (Supplementary materials S1).

Homongenates: Also in contrast to the EIA results, estradiol was undetectable by HPCL-MS in homogenates. PROG was undetectable in the saline treatment but present in the hCG treatment (278.8 pg mL^{-1}), as we found in the individual samples (see Fig. 7). A variety of other steroids were abundant in homogenates and might differ between saline and hCG treatments (Supplementary materials S1).

Wild-caught breeding female: Plasma steroid concentrations were similar between pre- and post-oviposition time-points for the female collected in amplexus during the breeding season in Panama. Similar to the captive frogs, plasma CORT was much more abundant than cortisol and PROG was undetectable. In contrast to the captive frogs, plasma estradiol concentrations were detectable and decreased after oviposition (pre 0.5 ng mL^{-1}; post: 0.3 ng mL^{-1}) and beta-pregnanolone was abundant (post-oviposition: 7.7 ng mL^{-1}) (Supplementary materials S1).

4. Discussion

We found that water-borne assays present a non-invasive, accurate and biologically informative means for sampling HPI and HPG axis steroids in individual adult túngara frogs. Partial immersion in a water bath for one hour coupled with solid-phase extraction yielded steroid concentrations quantifiable using commercial EIA kits. Compared to blood sampling, which provides an instantaneous snapshot of endocrine status, water-borne timelines

present an opportunity to integrate the endocrine state of an individual over a longer window of time, preferable for many questions (e.g. developmental or chronic endocrine regulation, baseline, repeatability). The higher diversity of steroids present in water compared to tissues may reflect this longer timeline of integration and thus the opportunity to detect less abundant steroid species, in addition to the conversion to various metabolites during clearance. In a similar validation study using a larger anuran (grey treefrogs; *Hyla versicolor*), we found that a 30-min water bath was sufficient for estimating all three of these steroids (Baugh, unpubl. data). It will be important for future studies to determine the minimum duration for a water bath and thereby ascertain the limits to the temporal resolution of this approach, which will likely vary as a function of the size and developmental stage of the organism (Gabor et al., 2013a, 2016).

Our use of pharmacological challenges yielded the predicted dose-response relationships, with increasing water-borne CORT, E_2 and PROG following injection with the upstream secretagogues. We also found that water-borne CORT and E_2 were positively correlated with plasma and whole-body homogenate concentrations, indicating that this non-invasive method reflects physiologically relevant peripheral concentrations and thus could serve as an alternative for these more conventional techniques. Water-borne steroids offer important advantages for conservation biologists working with threatened or small amphibians or without the expertise to sample blood (e.g. cardiac punctures), which can be an important limiting factor for many small amphibian species. Quantifying and monitoring steroid hormones in amphibians has become increasingly important as some of the major threats to this group appear to impact an animal's endocrine status, e.g. through elevated CORT in chytrid-infected frogs and dysregulation of the HPG axis in response to estrogenic endocrine disrupting chemicals (reviewed in Narayan, 2013; Gabor et al., 2013b; Gabor et al., 2015). This technique could also prove practical and informative for behavioral endocrinologists who are interested in repeated or chronic endocrine monitoring (e.g. estimating 'baseline' CORT), or who seek experimental designs that involve simultaneous or sequential measurements of behavior and hormones without experimenter impacts (Davis and Gabor, 2015). Our results indicate that water-borne samples of all three hormones provide useful information at the individual level—in *Phase 2* we found positive correlations between multiple water sampling time-points in our repeated measures design (Fig. 8c; S16–S20).

Our results are similar to validations of this technique with fresh water fishes, in which free hormones are passed directly from gill tissue to the water column (reviewed in Scott and Ellis, 2007; Scott et al., 2008; Sebire et al., 2007; Fisher et al. 2014). In anurans, water-borne steroid levels likely reflect the joint contributions of excreted free native hormone (e.g. 17-β estradiol) from the skin and mucus membranes and metabolic products excreted through urine (e.g. estrone). In the present study, solid-phase extraction of certain water samples resulted in a visible layer of yellow discoloration at the column's air-sorbent interface that was captured in the elutant. This may reflect urine in the sample, thereby potentially elevating measured steroid concentrations in those samples and thus introducing unexplained variance (Narayan et al. 2013). This might also explain the greater diversity of steroids found in water compared to tissue, particularly the presence of estrone, a common excretory estrogen, and androstenedione, which is produced by the adrenals in response to binding ACTH and can be converted to testosterone and estrone.

4.1. CORT

We found that water-borne concentrations CORT were correlated across sampling time points and reflected concentrations in plasma and whole body homogenates. CORT concentrations in plasma and homogenates were strongly correlated. In conjunction with the dose-response studies, these correlations indicate that this water-borne method is capturing biologically meaningful CORT estimates across a predictable timeline of elevation followed by return to baseline. The high and uniform recovery efficiencies and strong parallelism we found for water-borne CORT suggests that solid-phase extraction and EIA methods effectively reduce interfering substances and provide excellent specificity without loss of analyte (Newman et al., 2008). We found that a relatively high concentration (a 1:1 dilution, i.e. reconstitution to 250 μL) is optimal for water-borne CORT, suggesting a limit to the number of analytes that can be estimated from a single sample without the use of multiplexing approaches. Lastly, the HPLC-MS results showed that water samples contain a mixture of various glucocorticoids, including an enrichment of cortisol and, to a lesser extent CORT, as well cortisol sulfate and cortisone. In contrast, plasma contained principally CORT, as documented in other amphibians (Norris and Carr, 2013). The enrichment of cortisol in water was not expected; future studies of amphibian glucocorticoids should consider measuring this steroid along with CORT and conjugated forms.

In contrast to blood sampling, water sampling has the disadvantage of requiring confinement of subjects in small vessels, which itself might induce a stress response (Gabor et al., 2013a). Although this possibility was not tested in the current study, we did observe a decrement in CORT in the vehicle treatment in *Phase 1* during the second water bath, possibly indicating recovery from the stress of injection and confinement (Fig. 1). In *Phase 2*, however, we also measured pre-injection water-borne CORT and found that vehicle injection resulted in a decrease in CORT compared to the pre-injection time-point (Fig. 2). Together these findings might indicate that handling and confinement induce an initial CORT response and that recovery (via negative feedback) occurs during the first hour after the initial stressor, but that ACTH injections overcome this endogenous negative feedback. In other words, injection *per se* might not itself be a potent endocrine stressor in this species. In contrast, Gabor et al. (2016) showed that San Marcos salamanders (*Eurycea nana*) respond with a CORT increase following injection, and Baugh et al. (2017a,b) reported that sexual behavior in male (but not female) túngara frogs is inhibited by injection *per se*.

The relevance of measuring glucocorticoids in anurans extends beyond the study of stress, metabolism and coping behavior. For example, recent work in green treefrogs (*Hyla cineria*) has demonstrated that in addition to gonadal steroids, circulating levels of CORT are important in understanding adult male behavior during contests (Leary, 2014; Davis and Gabor, 2015) and female mate choice behavior (Davis and Leary, 2015). Further, we know that ecologically relevant threats and stressors experienced early in life can result in dramatic impacts on developmental decisions and life history trade-offs in vertebrates (reviewed in Crespi et al., 2013), including in anurans (Touchon et al., 2013; Warkentin, 2011). Hence, there is potentially high utility for non-invasive methods for repeatedly measuring CORT profiles, especially because it might be the dynamics of CORT secretion—and not single snapshots—that prove informative for understanding the hormonal and behavioral (co)variation (Baugh et al., 2013; Baugh et al., 2014; Baugh et al. 2017a,b).

4.2. E_2

In contrast to water-borne CORT, we found that water-borne E_2 concentrations at the pre-injection time-point were not correlated with the post-injection time-point. This difference might be observed if hCG injections resulted in a larger increase in water-borne E_2 than ACTH injections did for CORT, or if the interval (24 h) was too long. There were, however, positive correlations

between water-borne E_2 and PROG, suggesting that these two steroids covary positively, which is not surprising given that E_2 and PROG are known to be elevated in female túngara frogs immediately prior to oviposition (Lynch and Wilczynski, 2005) and that hCG results in elevations in E_2 and PROG (present study). We also found positive correlations between concentrations of E_2 in water and plasma as well as water and homogenates at both the pre- and post-injection time-points, which, given the elevation in water-borne E_2 following hCG injection, suggests that plasma E_2 is relatively stable within-individuals across this 24-h period despite the hCG injections. As with CORT, the positive correlations observed for water-plasma and water-homogenate indicate that excreted E_2 integrated over a one-hour time window reflects physiologically relevant concentrations in the female.

In conjunction with the dose-response studies, which showed the predicted increase in E_2 following hCG injection in all sample types, these correlations indicate that this water-borne method is capturing biologically meaningful E_2 estimates. We also found high and uniform recovery efficiencies and strong parallelism for water-borne E_2, implying that solid-phase extraction and EIA methods effectively reduce interfering substances, providing specificity without loss of analyte. We found that a relatively high sample concentration (a 1:2 or 1:4 dilution of the reconstituted sample) is optimal for water-borne E_2 in hCG injected females, which allows for quantification of multiple analytes from a single sample. Lastly, the HPLC-MS results showed that estradiol was the principal estrogen in water and was sometimes accompanied by estrone.

4.3. PROG

As predicted, water-borne concentrations of PROG did increase following hCG injection—suggesting that there is utility in estimating this steroid in water—and were correlated with the post-injection time-point, as with water-borne CORT. Given that hCG injections resulted in only a moderate increase in water-borne PROG, this within-individual consistency in water-borne PROG is not surprising. However, there was a relatively large increase in homogenate PROG following hCG injection, and in contrast to both CORT and E_2 we did not detect correlations between water-borne and homogenate PROG concentrations (plasma PROG was not estimated for individual samples). Possible explanations for these results are (1) other hormones cross-react with the PROG antibody in the EIA at much higher rates than E_2 and CORT (see Methods); (2) PROG concentrations in the animal fluctuate at a shorter or longer time scales than were resolved with our time courses; (3) circulating concentrations of PROG are not reflected in homogenates (in a separate study of gray treefrogs, we found that plasma PROG, not estimated in the current study due to limited plasma volumes, was indeed positively correlated with water-borne PROG (Baugh unpubl. data)); or (4) PROG is converted enzymatically to other products at higher rates compared to CORT and E_2 (PROG is an important precursor hormone). For example, a study by Lutz et al. (2001) in *Xenopus laevis* showed that PROG is rapidly converted to androstenedione in ovarian tissue and that these tissues are more sensitive to androgens than PROG (via androgen receptors). This finding emphasizes the need to better understand the role of androgens in promoting reproductive readiness in female frogs. In wild-caught female túngara frogs, plasma PROG is known to increase at reproductive readiness and as plasma androgen concentrations decrease (Lynch and Wilczynski, 2005). In studies of two species of North American treefrogs, PROG injections coupled with prostaglandins were sufficient to induce reproductive behavior in females, and plasma PROG levels were positively correlated with the intensity of female reproductive behavior (Gordon and Gerhardt, 2009; Ward et al., 2015). Thus, PROG is likely an important endocrine signal involved in reproductive readiness in female frogs. Irrespective

of the mechanistic basis for the more complicated nature of water-borne PROG, the lack of a correlation between water-borne and whole body homogenate PROG, as well as the relatively low recovery of PROG, suggest limitations to accurately estimating PROG in water. Lastly, the HPLC-MS results showed that PROG was abundant in water samples, despite undetectable levels in plasma where dihydroprogesterone was the primary metabolite.

5. Conclusions

The protocol validated here in adult túngara frogs demonstrates that water-borne CORT and E_2 increase in response to pharmacological challenges, correlate with concentrations in the animals and are confirmed by HPLC-MS. Water-borne PROG concentrations increased moderately following stimulation with hCG and were detected by HPLC-MS, suggesting that PROG is excreted as a native hormone and exhibits some dose-responsiveness; however, we did not find a correlation between water and whole body PROG concentrations, suggesting that PROG may be complicated by factors involved conversion and clearance. We found evidence of repeatability in all three water-borne steroids, suggesting that this method has promise for behavioral endocrinologists interested in estimating stable endocrine correlates of behavioral differences in amphibians, which are known to carry over from the tadpole stage (see Wilson and Krause, 2012). Because hormone studies in tadpoles are often limited to whole body homogenates, the links between water, plasma and whole body homogenates in adults shown here suggests that water-borne methods should be considered as a method for earlier life stages in amphibians.

Beyond showing the biological relevance of water-borne sampling, the utility of comparisons between circulating and water-borne steroid concentrations will depend on the focus of study. There is nothing sacrosanct about circulating levels; in terms of function, we would ideally estimate the concentrations of the steroids bound by receptors in specific target tissues and do so over a relevant time window of integration. Circulating levels and other peripheral estimates (e.g. homogenates) provide an instant snapshot proxy for this, and thus suffer from intrinsic (e.g. secretion periodicity) and extrinsic (e.g. unknown stressors encountered prior to blood sampling) factors influencing the sample. Excreted concentrations measured in water, as demonstrated here, provide a longer integration time, thereby reducing variance due to intrinsic pulsatility, but suffer from the complications of clearance (e.g. variation in rates and conversion species). The water-borne method we present here may offer a preferable or sole option in the many situations where (1) the stress caused by handling and bleeding needs to be minimized, (2) an integrated measure is desirable because it better reflects chronic stress (e.g. Sheriff et al., 2011; Dickens and Romero, 2013), or (3) repeated measures are required to address a particular question or estimate true baseline concentrations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.01.025.

References

Baugh, A.T., Ryan, M.J., 2010. The development of sexual behaviour in túngara frogs. J. Comp. Psychol. 124, 66–80.

Baugh, A.T., Hoke, K.L., Ryan, M.J., 2012. Development of communication behaviour: receiver ontogeny in túngara frogs and a prospectus for a behavioural evolutionary development. Sci. World J. 2012, 1–10.

Baugh, A.T., Hau, M., Naguib, M., van Oers, K., 2013. Initial reactivity and magnitude of the acute stress response linked to exploratory behavior in great tits (Parus major). Gen. Comp. Endocrinol. 189, 96–104.

Baugh, A.T., Ryan, M.J., 2017. Vasotocin induces sexually dimorphic effects of acoustically-guided behavior in a tropical frog (Physalaemus pustulosus). J. Comp. Physiol. A. 203, 265–273.

Baugh, A.T., van Oers, K., Dingemanse, N., Hau, M., 2014. Baseline and stress-induced glucocorticoid concentrations are not repeatable but covary within individual great tits (Parus major). Gen. Comp. Endocrinol. 208, 154–163.

Baugh, A.T., Davidson, S.C., Hau, M., van Oers, K., 2017a. Temporal dynamics of the HPA axis linked to exploratory behavior in a wild European songbird (Parus major). Gen. Comp. Endocrinol. 250, 104–112.

Baugh, A.T., Senft, R.A., Firke, M., Lauder, A., Schroeder, J., Meddle, S.L., van Oers, K., Hau, M., 2017b. Risk-averse personalities have a systemically potentiated neuroendocrine stress axis: a multilevel experiment in Parus major. Horm. Behav. 93, 99–108.

Blickley, J.L., Word, K.R., Krakauer, A.H., Phillips, J.L., Sells, S.N., Taff, C.C., Wingfield, J.C., Patricelli, G.L., 2012. Experimental chronic noise is related to elevated fecal corticosteroid metabolites in lekking male greater sage grouse (Centrocercus urophasianus). PLoS One 7 (11), e50462. https://doi.org/10.1371/journal.pone.0050462.

Brem, F.M.R., Lips, K.R., 2008. Batrachochytrium dendrobatidis infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. Diseases Aquat. Organ. 81, 189–202.

Breuner, C.W., Hahn, T.P., 2003. Integrating stress physiology, environmental change, and behavior in free-living sparrows. Horm. Behav. 43, 115–123.

Chakraborty, M., Burmeister, S.S., 2009. Estradiol induces sexual behavior in female túngara frogs. Horm. Behav. 55, 106–112.

Chakraborty, M., Burmeister, S.S., 2010. Sexually dimorphic androgen and estrogen receptor mRNA expression in the brain of túngara frogs. Horm. Behav. 58, 619–627.

Cockrem, J.F., Silverin, B., 2002. Sight of a predator can stimulate a corticosterone response in the great tit (Parus major). Gen. Comp. Endocrinol. 125, 248–255.

Crespi, E.J., Williams, T.D., Jessop, T.S., Delehanty, B., 2013. Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals? Func. Ecol. 27, 93–106.

Davis, D.R., Gabor, C.R., 2015. Behavioral and physiological antipredator responses of the San Marcos salamander, Eurycea nana. Physiol. Behav. 139, 145–149.

Davis, A.G., Leary, C.J., 2015. Elevated stress hormone diminishes the strength of female preferences for acoustic signals in the green treefrog. Horm. Behav. 69, 119–122.

Delehanty, B., Hossain, S., Jen, C.C., Crawshaw, G.J., Boonstra, R., 2015. Measurement of free glucocorticoids: quantifying corticosteroid-binding globulin binding affinity and its variation within and among mammalian species. Conserv. Physiol. 3, 1–13. https://doi.org/10.1093/conphys/cov020.

Dickens, M.J., Romero, L.M., 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. Gen. Comp. Endocrinol. 191, 177–189.

Dingemanse, N.J., Edelaar, P., Kempenaers, B., 2010. Why is there variation in baseline glucocorticoid levels. Trends Ecol. Evol. 25, 261–262.

Fisher, E.K., Harris, R.M., Hofmann, H.A., Hoke, K.L., 2014. Predator exposure alters stress physiology in guppies across timescales. Horm. Behav. 65, 165–172.

Forero-Medina, G., Joppa, L., Pimm, S.L., 2010. Constraints to species' elevational range shifts as climate change. Conserv. Biol. 25, 163–171.

Gabor, C.R., Bosch, J., Fries, J.N., Davis, D.R., 2013a. A non-invasive water-borne hormone assay for amphibians. Amphib-Reptil 34, 151–162.

Gabor, C.R., Fisher, M.C., Bosch, J., 2013b. A non-invasive stress assay shows that tadpole populations infected with Batrachochytrium dendrobatidis have elevated corticosterone levels. PLoS One 8, e56054.

Gabor, C.R., Fisher, M.C., Bosch, J., 2015. Elevated corticosterone levels and changes in amphibian behavior are associated with Batrachochytrium dendrobatidis (Bd) infection and Bd lineage. PLoS One 10, e0122685.

Gabor, C.R., Zabierek, K.C., Kim, D.S., Alberici da Barbiano, L., Mondelli, M.J., Bendik, N.F., Davis, D.R., 2016. A non-invasive water-borne assay of stress hormones in aquatic salamanders. Copeia 2016, 172–181.

Gallmetzer, N., Schulze, C.H., 2015. Impact of oil palm agriculture on understory amphibians and reptiles: a Mesoamerican perspective. Glob. Ecol. Conserv. 4, 95–109.

Glennemeier, K.A., Denver, R.J., 2002. Small changes in whole-body corticosterone content affect larval Rana pipiens fitness components. Gen. Comp. Endocrinol. 127, 16–25.

Gordon, N.M., Gerhardt, H.C., 2009. Hormonal modulation of phonotaxis and advertisement call-preferences in the gray treefrog (Hyla versicolor). Horm. Behav. 55, 121–127.

Hayes, T.B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A.A., Vonk, A., 2002. Hermaphroditic, demasculanized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc. Nat. Acad. Sci. U.S.A. 99, 5476–5480.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M., 2006. Pesticide mixtures, endocrine disruption,

and amphibian declines: are we underestimating the impact? Environ. Health Perspec. 114, 40–50.

Kiesecker, J.M., Blaustein, A.R., Belden, L.K., 2001. Complex causes of amphibian population declines. Nature 410, 681–684.

Kindermann, C., Narayan, E.J., Hero, J.M., 2012. Urinary corticosterone metabolites and chytridiomycosis disease prevalence in a free-living population of male Stony Creek frogs (Litoria wilcoxii). Comp. Biochem. Physiol. Part A Molec. Integ. Physiol. 162, 171–176.

Koolhaas, J.M., Bartolomucci, A., Buwalda, B., de Boer, S.F., Flugge, G., Korte, S.M., Meerlo, P., Murison, R., Olivier, B., Palanza, P., Richter-Levin, G., Sgoifo, A., Steimer, T., Stiedl, O., van Dijk, G., Wohr, M., Fuchs, E., 2011. Stress revisited: a critical evaluation of the stress concept. Neurosci. Biobehav. Rev. 35, 1291–1301.

Leary, C.J., 2014. Close-range vocal signals elicit a stress response in male green treefrogs: resolution of an androgen-based conflict. Anim. Behav. 96, 39–49.

Lee, C., Goeger, D.E., 1998. Interference of 6b-Hydroxycortisol in the quantification of urinary free cortisol by immunoassay and its elimination by solid-phase extraction. Clin. Biochem. 31, 229–233.

León-Olea, M., Martyniuk, C.J., Orlando, E.F., Ottinger, M.A., Rosenfeld, C.S., Wolstenholme, J.T., Trudeau, V.L., 2014. Current concepts in neuroendocrine disruption. Gen. Comp. Endocrinol. 203, 158–173.

Lutz, L.B., Cole, L.M., Gupta, M.K., Kwist, K.W., Auchus, R.J., Hammes, S.R., 2001. Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation. Proc. Nat. Acad. Sci. U.S.A. 98, 13728–13733.

Lynch, K.S., Crews, D., Ryan, M.J., Wilczynski, W., 2006. Hormonal state influences aspects of female mate choice in the túngara frog (Physalaemus pustulosus). Horm. Behav. 49, 450–457.

Lynch, K.S., Wilczynski, W., 2005. Gonadal steroids vary with reproductive stage in a tropically breeding female anuran. Gen. Comp. Endocrinol. 143, 51–56.

Lynch, K.S., Wilczynski, W., 2006. Social regulation of plasma estradiol concentration in a female anuran. Horm. Behav. 50, 101–106.

Marler, C.A., Ryan, M.J., 1996. Energentic constraints and steroid hormone correlates of male calling behaviour in the túngara frog. J. Zool. Lond. 240, 397–409.

McEwen, B., Wingfield, J.C., 2003. The concept of allostasis in biology and biomedicine. Horm. Behav. 43, 2–15.

McMahon, T.A., Halstead, N.T., Johnson, S., Raffel, T.R., Romansic, J.M., Crumrine, P.W., Boughton, R.K., Martin, L.B., Rohr, J.R., 2011. The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. Environ. Health Perspect. 119, 1098–1103.

McMahon, T.A., Boughton, R.K., Martin, L.B., Rohr, J.R., 2017. Exposure to the herbicide atrazine nonlinearly affects tadpole corticosterone levels. J. Herpetol. 51, 270–273.

Narayan, E.J., 2013. Non-invasive reproductive and stress endocrinology in amphibian conservation physiology. Conserv. Physiol. 1, 2013. https://doi.org/10.1093/conphys/cot011.

Narayan, E.J., Cockrem, J., Hero, J.-M., 2011. Urinary corticosterone metabolite responses to capture and captivity in the cane toad (Rhinella marina). Gen. Comp. Endocrinol. 173, 371–377.

Narayan, E.J., Cockrem, J., Hero, J.-M., 2013. Changes in serum and urinary corticosterone and testosterone during short-term capture and handling in the cane toad (Rhinella marina). Gen. Comp. Endocrinol. 191, 225–230.

Newman, A.E.M., Chin, E.H., Schmidt, K.L., Bond, L., Wynne-Edwards, K.E., Soma, K.K., 2008. Analysis of steroids in songbird plasma and brain by coupling solid-phase extraction to radioimmunoassay. Gen. Comp. Endocrinol. 155, 503–510.

Norris, D.O., Carr, J.A., 2013. Vertebrate Endocrinology. Elsevier Inc..

O'Connell, L.A., Ding, J.H., Ryan, M.J., Hofmann, H.A., 2011. Neural distribution of the nuclear progesterone receptor in the túngara frog, Physalaemus pustulosus. J. Chem. Neuroanatomy 41, 137–147.

Rodríguez-Brenes, S., Rodriguez, D., Ibáñez, R., Ryan, M.J., 2016. Spread of Amphibian Chytrid Fungus across Lowland Populations of Túngara Frogs in Panamá. PLoS ONE 11, e0155745.

Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under three minutes good enough? Comp. Biochem. Physiol. A 140, 73–79.

Ryan, M.J., 1985. The Túngara Frog: A Study in Sexual Selection and Communication. University of Chicago Press, Chicago.

Ryan, M.J., 2010. Túngara frog: a model for sexual selection and communication. In: Encyclopedia of Animal Behavior, pp. 453–461.

Ryan, M.J., 2011. Sexual selection: a tutorial from the túngara frog. In: Losos, J.B. (Ed.), In Light of Evolution, Essays from the Laboratory and Field. Roberts and Company, Greenwood Village CO, pp. 185–203.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. Endoc. Rev. 21, 55–89.

Scott, A.P., Ellis, T., 2007. Measurement of fish steroids in water—a review. Gen. Comp. Endocrinol. 153, 392–400.

Scott, A.P., Hirschenhauser, K., Bender, N., Oliveira, R., Earley, R.L., Sebire, M., Ellis, T., Pavlidis, M., Hubbard, P.C., Huertas, M., 2008. Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. Behaviour 145, 1307–1328.

Sebire, M., Katsiadaki, I., Scott, A.P., 2007. Non-invasive measurement of 11-ketotestosterone, cortisol and androstenedione in male three-spined stickleback (Gasterosteus aculeatus). Gen. Comp. Endocr. 153, 30–38.

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–887.

Tennessen, J.B., Parks, S.E., Langkilde, T., 2014. Traffic noise causes physiological stress and impairs breeding migration behaviour in frogs. Conserv. Physiol. 2 (1). https://doi.org/10.1093/conphys/cou032.

Thaker, M., Lima, S.L., Hews, D.K., 2009. Acute corticosterone elevation enhances antipredator behaviors in male tree lizard morphs. Horm. Behav. 56, 51–57.

Touchon, J.C., McCoy, M.W., Vonesh, J.R., Warkentin, K.M., 2013. Effects of plastic hatching timing carry over through metamorphosis in red-eyed treefrogs. Ecology 94, 850–860.

Turriago, J.L., Parra, C.A., Bernal, M.H., 2015. Upper thermal tolerance in anuran embryos and tadpoles at constant and variable peak temperatures. Can. J. Zool. 93, 267–272.

Van Rooij, P., Martel, A., Haesebrouck, F., Pasmans, F., 2015. Amphibian chytridiomycosis: a review with focus on fungus-host interactions. Vet Res. 46. https://doi.org/10.1186/s13567-015-0266-0.

Ward, J.L., Love, E.K., Baugh, A.T., Gordon, N.M., Bee, M.A., 2015. Progesterone and prostaglandin F2α induce species-typical female preferences for male sexual displays in Cope's gray treefrog (Hyla chrysoscelis). Physiol. Behav. 152, 280–287.

Warkentin, K.M., 2011. Plasticity of hatching in amphibians: Evolution, trade-offs, cues and mechanisms. Integr. Comp. Biol. 51, 111–127.

Weigt, L.A., Crawford, A.J., Rand, A.S., Ryan, M.J., 2005. Biogeography of the túngara frog, Physalaemus pustulosus: a molecular perspective. Mol. Ecol. 14, 3857–3876.

Wilson, A.D.M., Krause, J., 2012. Personality and metamorphosis: is behavioral variation consistent across ontogenetic niche shifts? Behav. Ecol. 23, 1316–1323.

1   **List of Supplementary Materials**

2

3   **S1:** Table of all HPLC-MS results.

4   **S2:** Parallelism and recovery for plasma corticosterone.

5   **S3:** Parallelism and recovery for whole body homogenate 17-β estradiol.

6   **S4:** Parallelism and recovery for plasma 17-β estradiol.

7   **S5:** Parallelism and recovery for whole body homogenate corticosterone.

8   **S6:** Parallelism and recovery for whole body homogenate progesterone.

9   **S7:** Parallelism and recovery for water-borne 17-β estradiol.

10   **S8:** Parallelism and recovery for water-borne progesterone.

11   **S9:** Parallelism and recovery for water-borne corticosterone.

12   **S10:** Pooled water-borne corticosterone concentrations at different time-points post-injection in

13   the vehicle, low and high ACTH treatment groups (sexes combined).

14   **S11:** Average male and female water-borne corticosterone concentrations at different time-points

15   post-injection in the low dose of ACTH treatment group (0.5 μg g^{-1}).

16   **S12:** Average male and female water-borne corticosterone concentrations at different time-points

17   post-injection in the vehicle treatment group (saline).

18   **S13:** Average male and female water-borne corticosterone concentrations at different time-points

19   post-injection in the high dose of ACTH treatment group (5 μg g^{-1}).

20   **S14:** Average female water-borne corticosterone concentrations at different time-points post-

21   injection in the vehicle, low and high ACTH treatment groups.

22   **S15:** Average male water-borne corticosterone concentrations at different time-points post-

23   injection in the vehicle, low and high ACTH treatment groups.

24   **S16:** Correlations between water-borne corticosterone at the various water bath time-points.

25   **S17:** Correlation between water-borne 17-β estradiol and water-borne progesterone in 50 IU

26   HCG injected females.

27   **S18:** Correlation between water-borne 17-β estradiol and water-borne progesterone in 500 IU

28   HCG injected females.

29   **S19:** Correlation between water-borne 17-β estradiol and water-borne progesterone in vehicle

30   injected females.

31   **S20:** Correlation between water-borne 17-β estradiol and water-borne progesterone in females

32   (all three injections treatments pooled).

33   **S21:** Correlations between water-borne progesterone (at the various water bath time-points) and

34   17-β estradiol in whole body homogenates.

HPLC-MS Results for Physalaemus pustulosus (Tungara Frog) samples: Water, Plasma & Whole Body Homogenates
This table accompanies the article by titled: Validation of water-borne steroid hormones in a tropical frog (Physalaemus pustulosus)

name	formula	acc mass	ESI mode	MRM	ret time	count	DP	CE	EP	CXP	LOD (nM)	LOQ (nM)
cortisol	C21H30O5	363.217	(+) ESI	363.1/121.1	1.93	14	50	35	10	15	1	5
corticosterone	C21H30O4	347.222	(+) ESI	347.2/121.1	3.59	14	50	35	10	15	0.5	2.5
etiocholanolone	C19H30O2	291.232	(+) ESI	273.1/255.2	11.58	14	45	20	10	18	1	10
testosterone	C19H28O2	289.216	(+) ESI	289.2/109	6.37	14	20	28	10	12	0.1	0.5
estradiol	C18H24O2	273.185	(+) ESI	255/159	5.7	14	35	22	10	18	0.5	5
dihydroprogesterone	C21H32O2	317.248	(+) ESI	317.1/299.1	15.8	14	40	15	10	10	25	100
progesterone	C21H30O2	315.232	(+) ESI	315.1/109.1	13.8	14	40	30	10	15	0.25	3
beta-pregnanolone	C21H34O2	319.263	(+) ESI	301.2/283.1	11.97	14	30	18	10	18	2	20
androstenedione	C19H26O2	287.201	(+) ESI	287.2/109.1	7.71	14	50	35	10	15	0.5	2
androstanediol	C19H32O2	293.248	(+) ESI	275.1/257.2	6.83	14	20	20	10	16	1	5
trans-androsterone	C19H30O2	291.232	(+) ESI	273.1/255.2	9.09	14	50	18	10	25	5	25
cortexone	C21H30O3	331.227	(+) ESI	331.1/109.1	7.26	14	40	39	10	15	0.3	3
estriol	C18H24O3	289.180	(+) ESI	271.1/253.1	1.33	14	30	15	10	11	2	10
dehydroepiandrosterone sulfat	C19H28O5S	369.497	(+) ESI	271.1/253.2	3.35	14	40	20	10	25	10	40
20-hydroxyprogesterone	C21H32O2	317.248	(+) ESI	317.1/299.1	11.07	14	50	24	10	15	50	250
androstenediol	C19H30O2	291.232	(+) ESI	273.2/255	5.57	14	50	18	10	12	5	25
estrone	C18H22O2	271.169	(+) ESI	271.1/253.2	7.62	14	30	15	10	11	2	10
17α-Hydroxyprogesterone	C21H30O3	331.227	(+) ESI	331.2/109.1	8.49	14	40	30	10	13	0.5	2.5
cortisone	C21H28O5	361.201	(+) ESI	361.1/163.1	1.97	14	50	35	10	11	0.2	1
cortisol sulfate	C21H30O8S	443.173	(+) ESI	363.1/121.1	1.5	14	50	35	10	15	5	20
dehydroepiandrosterone	C19H28O2	289.216	(+) ESI	271.1/253.1	7.88	14	30	15	10	11	5	25
17-OH pregnenolone	C21H32O3	333.242	(+) ESI	297.2/279.1	7.41	14	45	18	10	25	200	500
dihydrotestosterone	C19H30O2	291.232	(+) ESI	291.1/255.1	9.54	14	50	18	10	15	0.5	2.5
2-methoxyestradiol	C19H26O3	303.195	(+) ESI	285/189	7.18	14	25	25	10	15	0.5	2.5
pregnenolone	C21H32O2	317.248	(+) ESI	317.2/299.1	13.93	14	40	15	10	15	100	250
allo-pregnanolone	C21H34O2	319.263	(+) ESI	301.1/283.2	15.7	14	30	17	10	20	3	15
cortexolone	C21H30O4	347.222	(+) ESI	347.2/109.1	3.94	14	50	43	10	15	0.5	5
aldosterone	C21H28O5	361.201	(+) ESI	361.2/343.1	1.48	14	10	24	10	25	0.5	5
cis-androsterone	C19H30O2	291.232	(+) ESI	273.2/255.1	12.55	14	20	18	10	15	1	10
testosterone glucuronide	C25H36O8	465.248	(+) ESI	289.1/109	2.22	14	40	30	10	10	10	25

S1: Table of HPLC-MS results (Page 1 of 8)

name	W1_ACTH_Tung_Pool: pre-0.5 ACTH injection water pool water Physalaemus pustulosus (Tungara Frog) pg/mL	W2_ACTH_Tung_Pool: post-0.5 ACTH injection water pool water Physalaemus pustulosus (Tungara Frog) pg/mL
cortisol	998.4862049	193.630262
corticosterone	87.84985388	289.8478732
etiocholanolone	0	830.3937306
testosterone	284.5503484	185.4290488
estradiol	93.29737298	125.2095452
dihydroprogesterone	0	0
progesterone	377.6139504	66.41934085
beta-pregnanolone	0	0
androstenedione	35.70466664	35.46890066
androstanediol	0	569.9646145
trans-androsterone	738.3168662	536.7880283
cortexone	0	0
estriol	0	3100.325394
dehydroepiandrosterone sulfate	0	0
20-hydroxyprogesterone	0	0
androstenediol	0	0
estrone	324.8650194	0
17α-Hydroxyprogesterone	0	0
cortisone	0	29.04183786
cortisol sulfate	8551.35085	4480.436818
dehydroepiandrosterone	0	801.8726341
17-OH pregnenolone	0	0
dihydrotestosterone	231.584218	0
2-methoxyestradiol	0	0
pregnenolone	1300.608207	0
allo-pregnanolone	0	0
cortexolone	0	0
aldosterone	0	0
cis-androsterone	0	0
testosterone glucuronide	0	0

S1: Table of HPLC-MS results (Page 2 of 8)

name	W1_HCG_Tung_Pool: pre-500 HCG injection water pool water Physalaemus pustulosus (Tungara Frog) pg/mL	W2_HCG_Tung_Pool: post-500 HCG injection water pool water Physalaemus pustulosus (Tungara Frog) pg/mL
cortisol	333.678176	248.9701808
corticosterone	0	60.08731752
etiocholanolone	404.8706541	468.013149
testosterone	160.0940893	100.1956606
estradiol	88.10889511	163.694018
dihydroprogesterone	0	0
progesterone	98.33056132	64.0229765
beta-pregnanolone	0	301.2459144
androstenedione	15.0298299	16.69427991
androstanediol	611.1318832	0
trans-androsterone	556.8516478	615.9096769
cortexone	0	0
estriol	0	2018.152311
dehydroepiandrosterone sulfate	0	0
20-hydroxyprogesterone	0	0
androstenediol	544.7650699	0
estrone	0	0
17α-Hydroxyprogesterone	0	0
cortisone	16.55727181	0
cortisol sulfate	0	0
dehydroepiandrosterone	370.3931394	0
17-OH pregnenolone	0	0
dihydrotestosterone	0	0
2-methoxyestradiol	0	0
pregnenolone	0	0
allo-pregnanolone	0	0
cortexolone	0	0
aldosterone	0	0
cis-androsterone	0	0
testosterone glucuronide	0	0

S1: Table of HPLC-MS results (Page 3 of 8)

Sample ID / Sample Type / Species / Units (corrected for dilution and tissue volume u

name	Tung_0A_ACTH_plasma_pool (5 ul plasma) plasma Physalaemus pustulosus (Tungara Frog) ng/mL	Tung_0A_ACTH_homogenate_pool (200 ul homogenate) homogenate Physalaemus pustulosus (Tungara Frog) ng/mL
Sample ID		
Sample Type		
Species		
Units (corrected for dilution and tissue volume		
cortisol	2.348364714	3.462066139
corticosterone	20.17052131	4.324345584
etiocholanolone	26.06399976	183.1952526
testosterone	0.322443778	0
estradiol	0	1.005770377
dihydroprogesterone	0	0
progesterone	0	0.234597213
beta-pregnanolone	0	0
androstenedione	0	0
androstanediol	0	9.931794112
trans-androsterone	0	0
cortexone	0	0
estriol	0	9.30226319
dehydroepiandrosterone sulfate	0	1279.670448
20-hydroxyprogesterone	0	1.91356148
androstenediol	0	0
estrone	0	38.46023654
17α-Hydroxyprogesterone	0	0.140970381
cortisone	0	0
cortisol sulfate	0	0
dehydroepiandrosterone	0	0
17-OH pregnenolone	0	0
dihydrotestosterone	0	0
2-methoxyestradiol	0	3.152612711
pregnenolone	0	0
allo-pregnanolone	0	0
cortexolone	0	0
aldosterone	0	0
cis-androsterone	0	0
testosterone glucuronide	0	0

S1: Table of HPLC-MS results (Page 4 of 8)

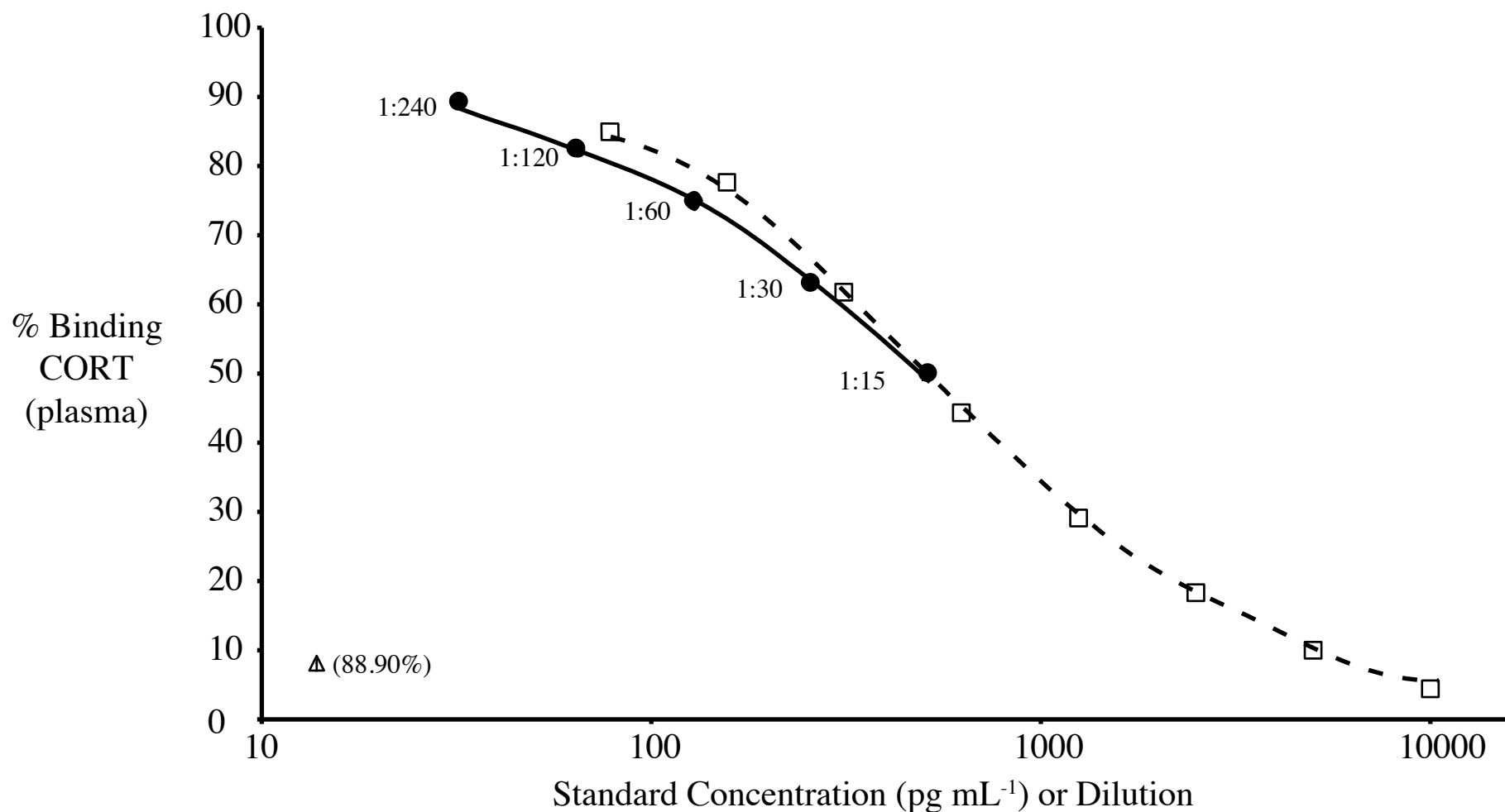| name	Tung_0.5A_ACTH_plasma_pool (5 ul plasma)	Tung_0.5A_ACTH_homogenate_pool (200 ul homogenate)
Sample ID	Tung_0.5A_ACTH_plasma_pool (5 ul plasma)	Tung_0.5A_ACTH_homogenate_pool (200 ul homogenate)
Sample Type	plasma	homogenate
Species	Physalaemus pustulosus (Tungara Frog)	Physalaemus pustulosus (Tungara Frog)
Units (corrected for dilution and tissue volume	ng/mL	ng/mL
cortisol	2.409941576	5.304617595
corticosterone	11.93148773	5.461870949
etiocholanolone	29.54930019	112.7481957
testosterone	0	0.016244945
estradiol	0	1.140749696
dihydroprogesterone	0	0
progesterone	0	0
beta-pregnanolone	0	0
androstenedione	0	0
androstanediol	0	3.758484863
trans-androsterone	0	1.292216223
cortexone	0	0
estriol	0	14.09774031
dehydroepiandrosterone sulfate	0	1111.853843
20-hydroxyprogesterone	0	3.292887206
androstenediol	0	0
estrone	0	3.552908019
17α-Hydroxyprogesterone	0	0.213393394
cortisone	0	0
cortisol sulfate	0	0
dehydroepiandrosterone	0	0
17-OH pregnenolone	0	0
dihydrotestosterone	0	0
2-methoxyestradiol	0	0
pregnenolone	0	0
allo-pregnanolone	0	0
cortexolone	0	0
aldosterone	0	0
cis-androsterone	0	0
testosterone glucuronide	0	0

S1: Table of HPLC-MS results (Page 5 of 8)

name	Tung_0H_HCG_plasma_pool (10 ul plasma)	Tung_0H_HCG_homogenate_pool (200 ul homogenate)
Sample Type	plasma	homogenate
Species	Physalaemus pustulosus (Tungara Frog)	Physalaemus pustulosus (Tungara Frog)
Units (corrected for dilution and tissue volume use	ng/mL	pg/mL
cortisol	1.173597162	346.0249996
corticosterone	2.298223968	7477.955699
etiocholanolone	13.86090436	28243.41385
testosterone	0.787267893	312.2275987
estradiol	0	0
dihydroprogesterone	196.9507106	51564.41691
progesterone	0	0
beta-pregnanolone	0	14484.29647
androstenedione	0	0
androstanediol	0	0
trans-androsterone	0	0
cortexone	0	203.8262643
estriol	0	0
dehydroepiandrosterone sulfate	0	0
20-hydroxyprogesterone	0	0
androstenediol	0	3831.389485
estrone	0	0
17α-Hydroxyprogesterone	0	0
cortisone	0	0
cortisol sulfate	0	0
dehydroepiandrosterone	0	0
17-OH pregnenolone	0	22384.56582
dihydrotestosterone	0	0
2-methoxyestradiol	0	0
pregnenolone	0	0
allo-pregnanolone	0	0
cortexolone	0	0
aldosterone	0	0
cis-androsterone	0	0
testosterone glucuronide	0	0

S1: Table of HPLC-MS results (Page 6 of 8)

name	Tung_500H_HCG_plasma_pool (10 ul plasma) plasma Physalaemus pustulosus (Tungara Frog) ng/mL	Tung_500H_HCG_homogenate_pool (200 ul homogenate) homogenate Physalaemus pustulosus (Tungara Frog) pg/mL
cortisol	1.233652036	354.2508738
corticosterone	35.41680769	9425.347887
etiocholanolone	13.48782472	42209.1299
testosterone	0.103122252	745.3625917
estradiol	0	0
dihydroprogesterone	227.7553465	45672.95398
progesterone	0	278.7604092
beta-pregnanolone	0	16385.26928
androstenedione	0	0
androstanediol	0	8486.547672
trans-androsterone	0	0
cortexone	2.254183901	429.5125951
estriol	0	0
dehydroepiandrosterone sulfate	0	11123.12585
20-hydroxyprogesterone	0	1215.230706
androstenediol	0	0
estrone	0	0
17α-Hydroxyprogesterone	0	0
cortisone	0	0
cortisol sulfate	0	0
dehydroepiandrosterone	0	0
17-OH pregnenolone	0	0
dihydrotestosterone	0	0
2-methoxyestradiol	0	0
pregnenolone	0	0
allo-pregnanolone	0	0
cortexolone	0	0
aldosterone	0	0
cis-androsterone	0	0
testosterone glucuronide	0	0

S1: Table of HPLC-MS results (Page 7 of 8)

Sample ID
Sample Type
Species
Units (corrected for dilution and tissue volume i

name	Tung_wild_female2_pre-oviposition_amplectant_plasma (10 ul plasma)	Tung_wild_female1_post-oviposition_amplectant_plasma (10 ul plasma)
Sample Type	plasma (NO EIA DATA)	plasma (NO EIA DATA)
Species	Physalaemus pustulosus (Tungara Frog)	Physalaemus pustulosus (Tungara Frog)
Units (corrected for dilution and tissue volume used)	ng/mL	ng/mL
cortisol	1.291846033	1.34476408
corticosterone	30.60212371	28.18104556
etiocholanolone	17.29323317	18.15315631
testosterone	0.223542351	0
estradiol	0.530628291	0.339312734
dihydroprogesterone	213.5335752	201.9239139
progesterone	0	0
beta-pregnanolone	0	7.69201043
androstenedione	0	0
androstanediol	0	0
trans-androsterone	0	0
cortexone	0	1.058388436
estriol	0	0
dehydroepiandrosterone sulfate	0	0
20-hydroxyprogesterone	0	0
androstenediol	0	0
estrone	0	0
17α-Hydroxyprogesterone	0	0
cortisone	0	0
cortisol sulfate	0	0
dehydroepiandrosterone	0	0
17-OH pregnenolone	0	0
dihydrotestosterone	0	0
2-methoxyestradiol	0	0
pregnenolone	0	0
allo-pregnanolone	0	0
cortexolone	0	0
aldosterone	0	0
cis-androsterone	0	0
testosterone glucuronide	0	0

S1: Table of HPLC-MS results (Page 8 of 8)

S2: Parallelism for plasma corticosterone using the Arbor Assays Corticosterone EIA DetectX (Cat. No. k014). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of five dilutions indicated on the graph. Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed subsequent plasma CORT samples at a 1:15 dilution. The open triangle represents mean (± standard deviation) percent binding for high concentration (10000 pg mL^{-1}; CV = 2.3%) spike recovery samples (3 replicates assayed in duplicate) and percent recovery is shown in parentheses (x-axis intercept is arbitrary). A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.071, SE = 0.768, t_9 = 0.093, p = 0.928).
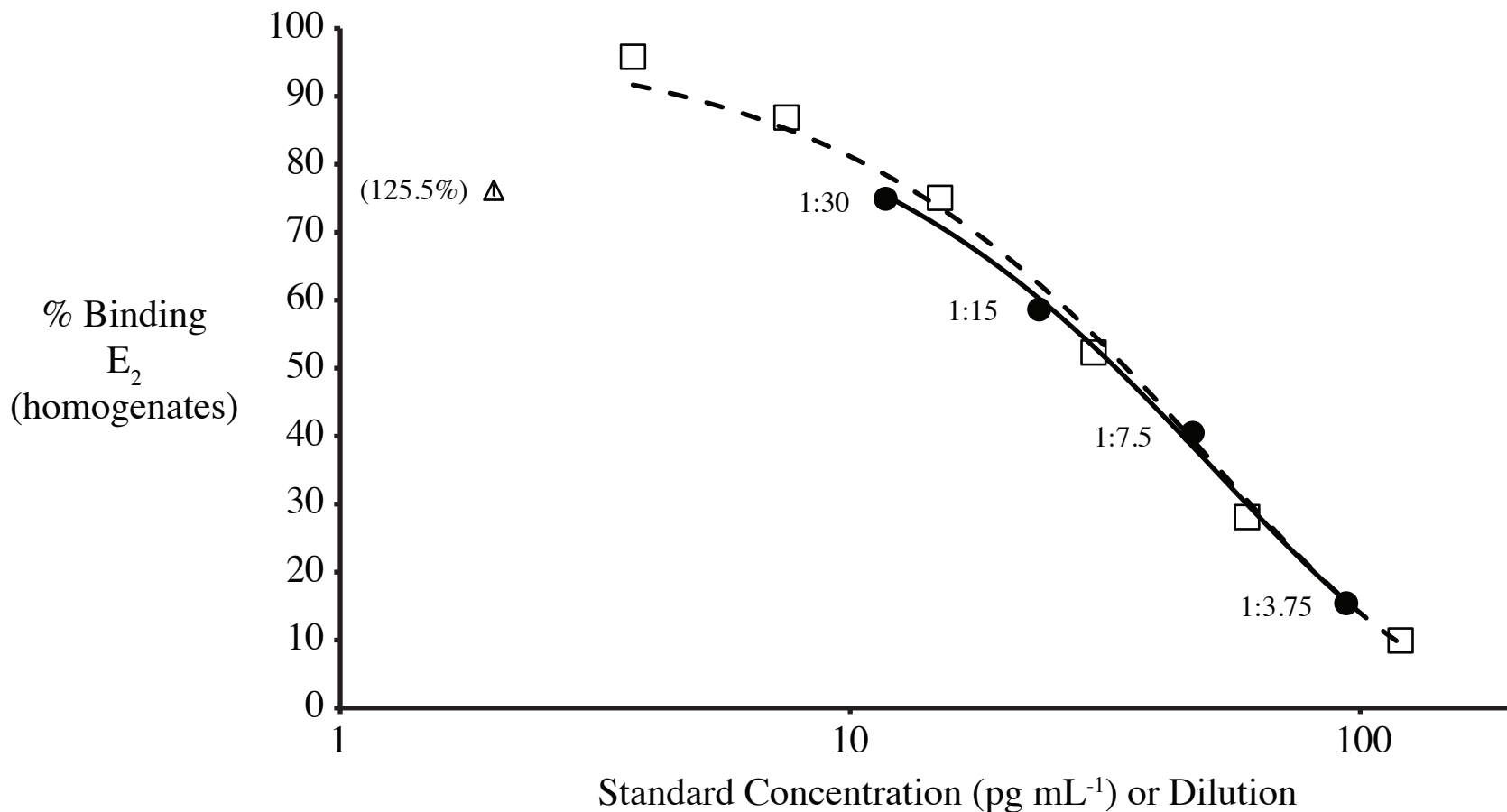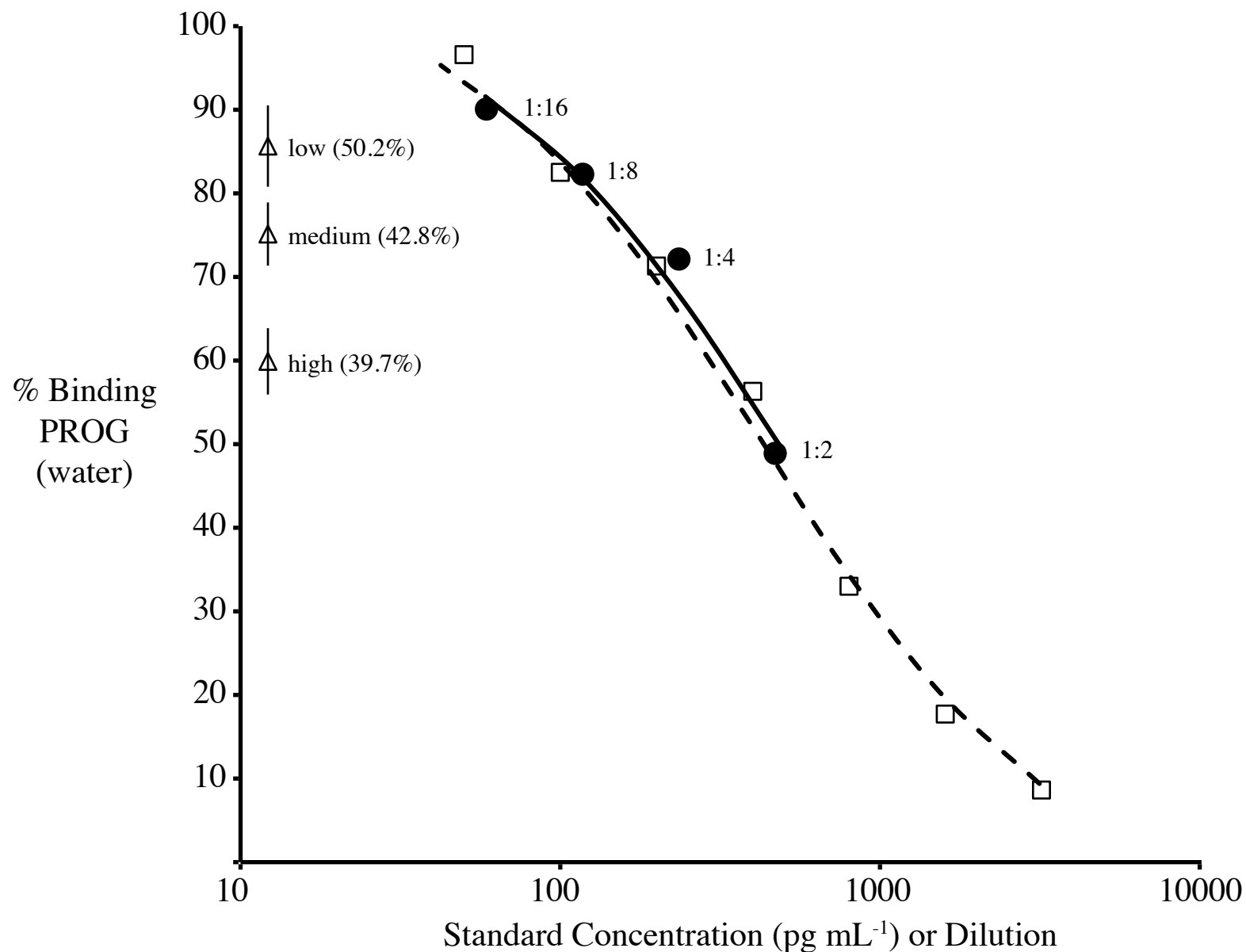
S3: Parallelism for whole animal homogenate 17-β estradiol using the Arbor Assays 17-β Serum Estradiol EIA DetectX (Cat. No. KB30). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of four dilutions indicated on graph. Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed subsequent homogenate samples at a 1:6 dilution. Open triangles represent mean (± standard deviation) percent binding for two spike recovery samples (9.6 pg mL^{-1}; CV = 4.5%; replicates of each run in duplicate wells) and percent recovery is shown in parentheses. A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.029, SE = 2.29, t_6 = 0.012, p = 0.990).
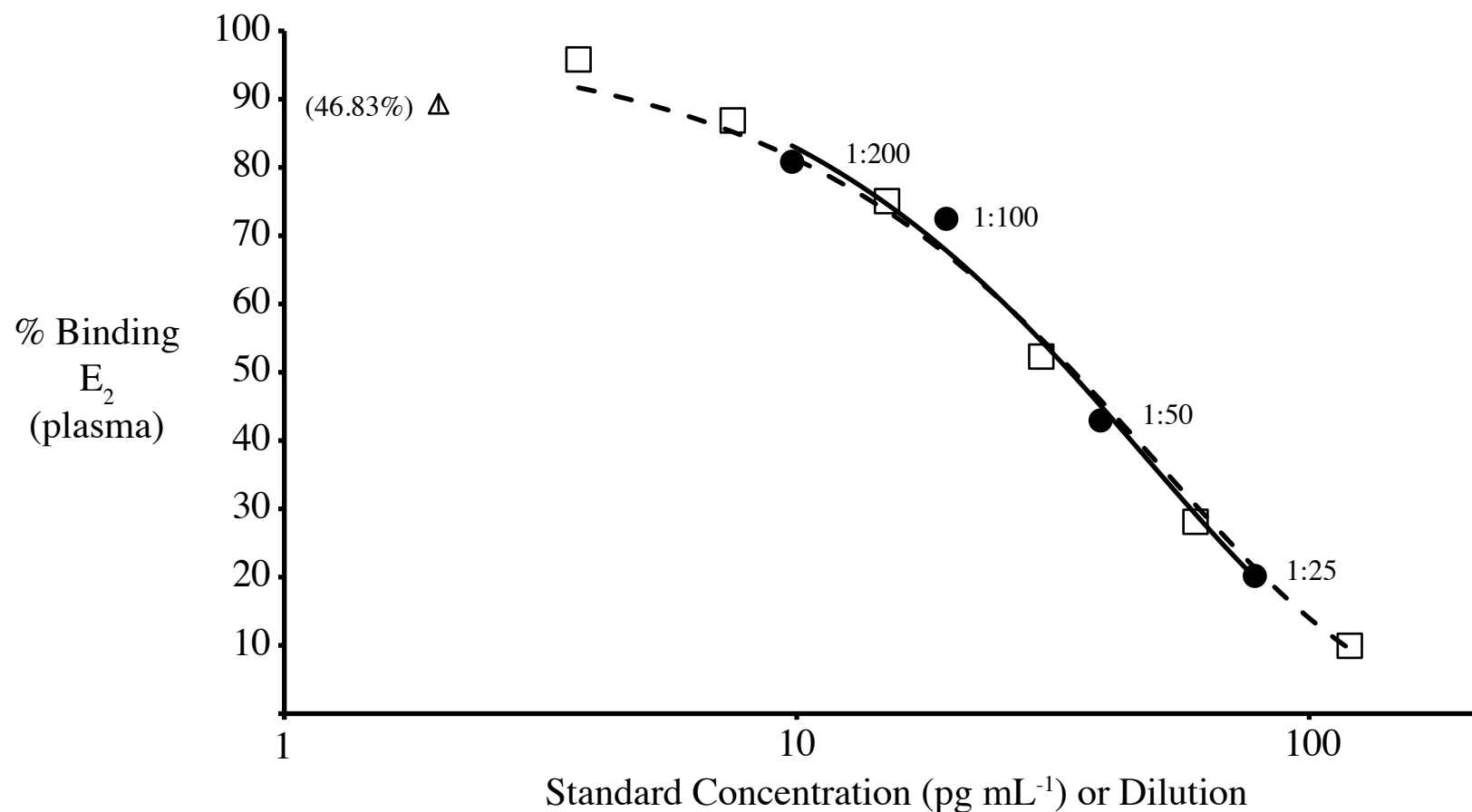
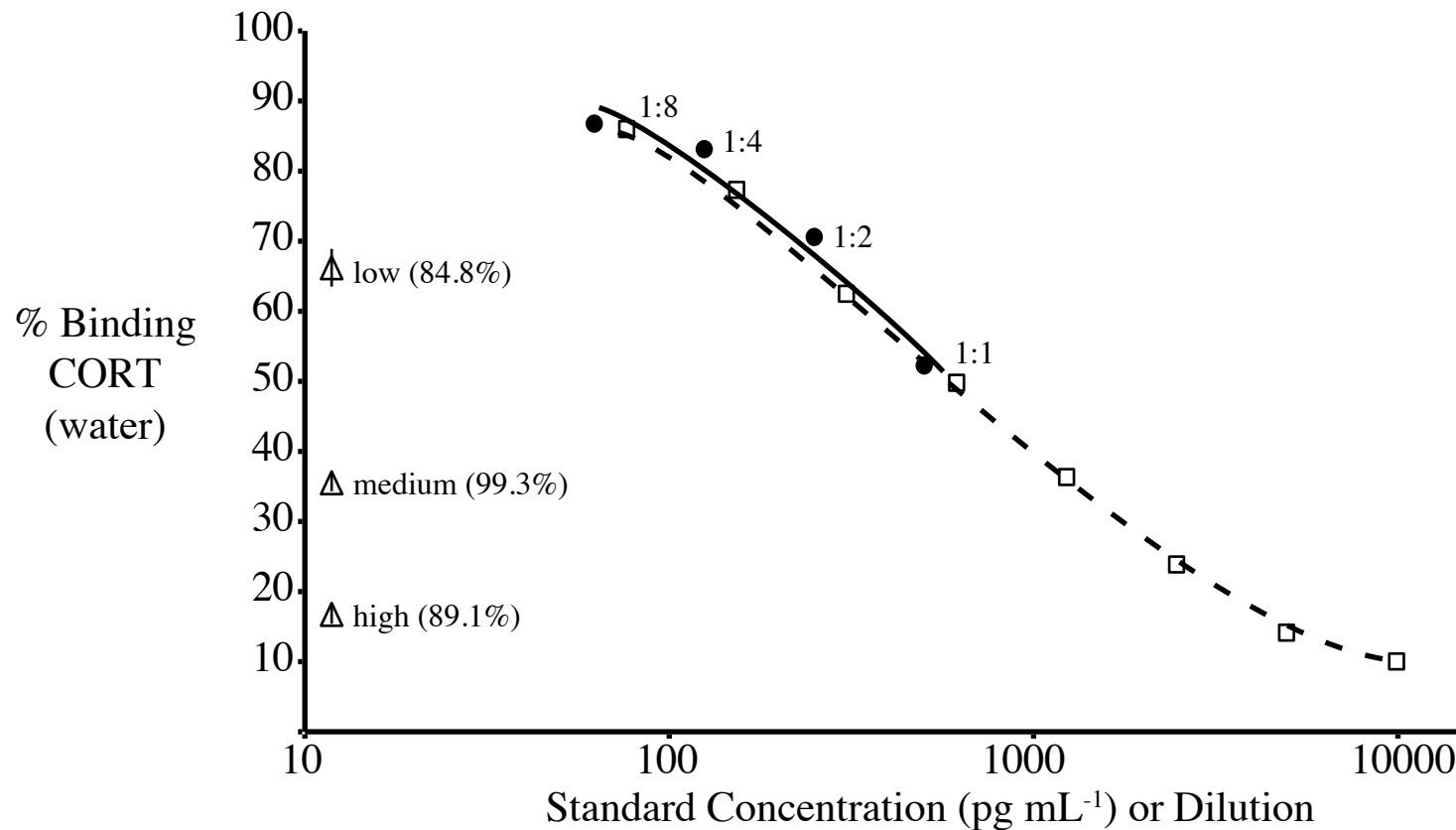**S4**: Parallelism for plasma 17-β estradiol using the Arbor Assays 17-β Serum Estradiol EIA DetectX (Cat. No. KB30). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of four dilutions indicated on graph. Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed subsequent plasma samples at a 1:50 dilution. Open triangles represent mean (± standard deviation) percent binding for two spike recovery samples (10 pg mL^{-1}; CV = 13.10%; replicates of each run in duplicate wells) and percent recovery is shown in parentheses. A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.178, SE = 2.77, t_6 = 0.064, p = 0.951).
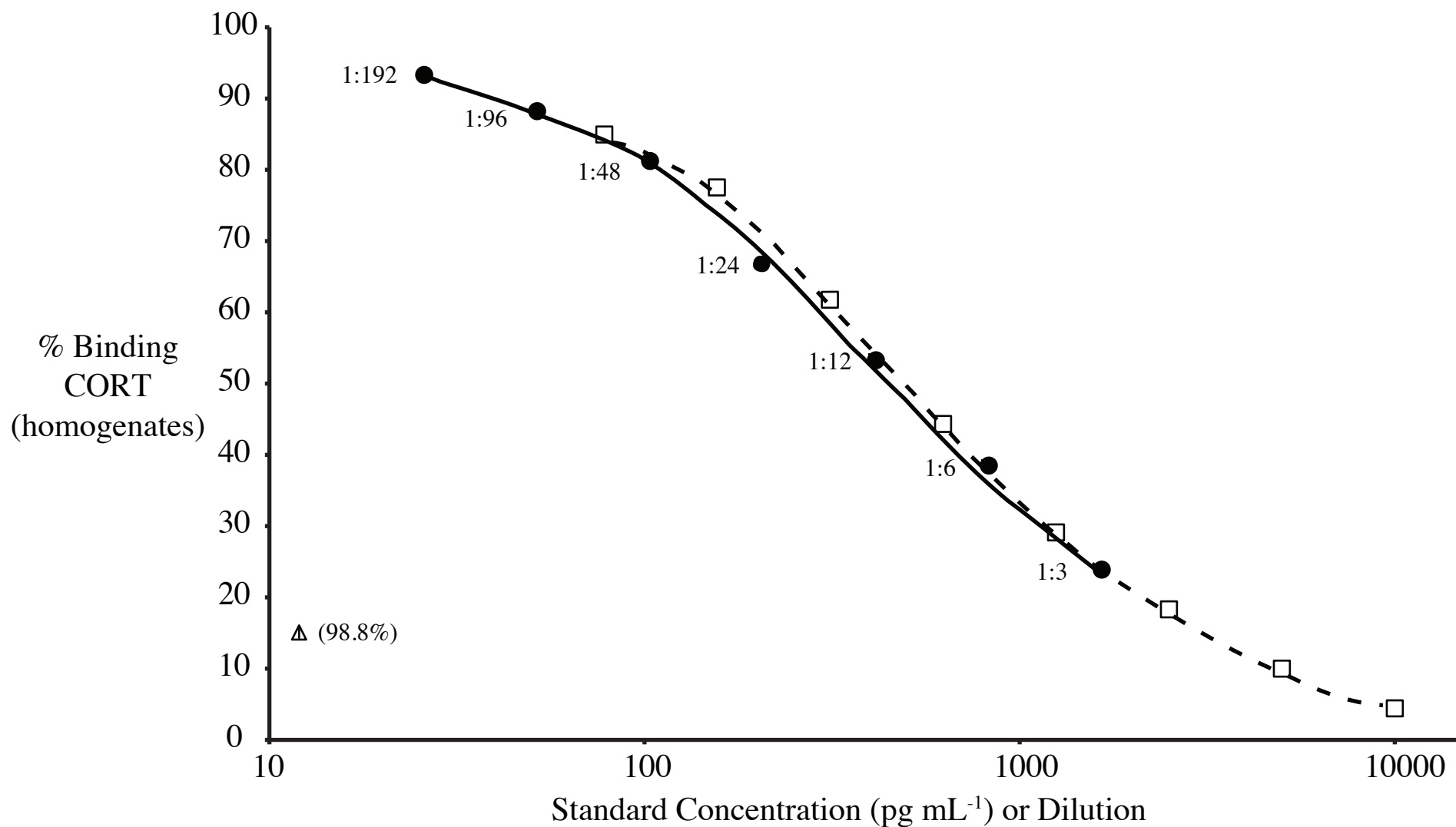
S5: Parallelism for whole body homogenates with the Arbor Assays Corticosterone EIA DetectX (Cat. No. k014). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of seven dilutions indicated on the graph. Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed all subsequent water borne CORT samples at a 1:6 dilution. The open triangle represents mean (± standard deviation) percent binding for high concentration (3000 pg mL^{-1}; CV = 6.5%) spike recovery samples (3 replicates assayed in duplicate) and percent recovery is shown in parentheses (x-axis intercept is arbitrary). A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.03, SE = 0.205, t_{11} = 0.165, p = 0.872).
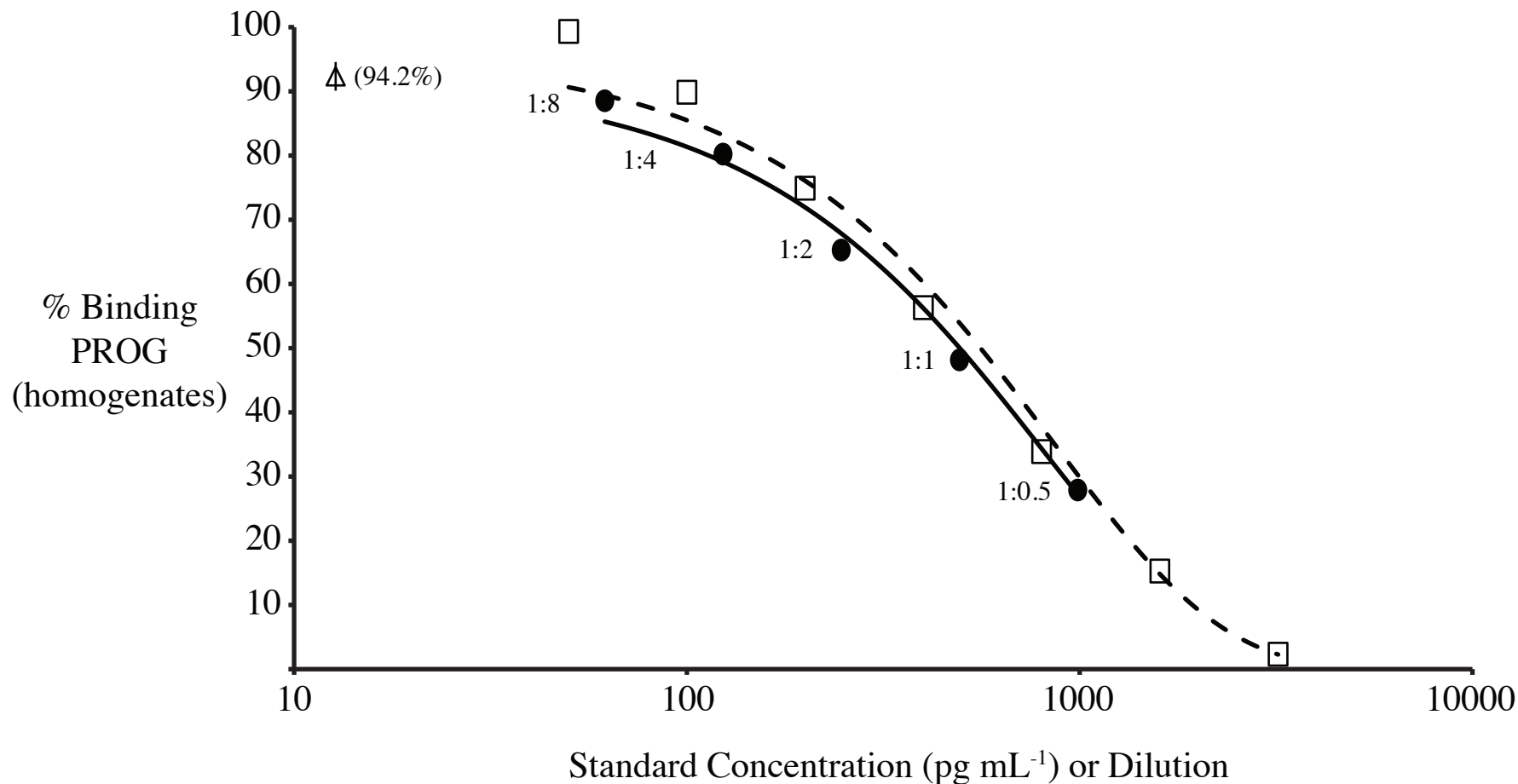
S6: Parallelism for whole animal homogenate progesterone using the Arbor Assays Progesterone EIA DetectX (Cat. No. K025). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of five dilutions indicated on graph. Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed subsequent homogenate samples at a 1:0.5 dilution. Open triangles represent mean (± standard deviation) percent binding for two spike recovery samples (9.6 pg mL^{-1}; CV = 14.1%; replicates of each run in duplicate wells) and percent recovery is shown in parentheses. A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.034, SE = 0.337, t$_8$ = 0.013, p = 0.990).
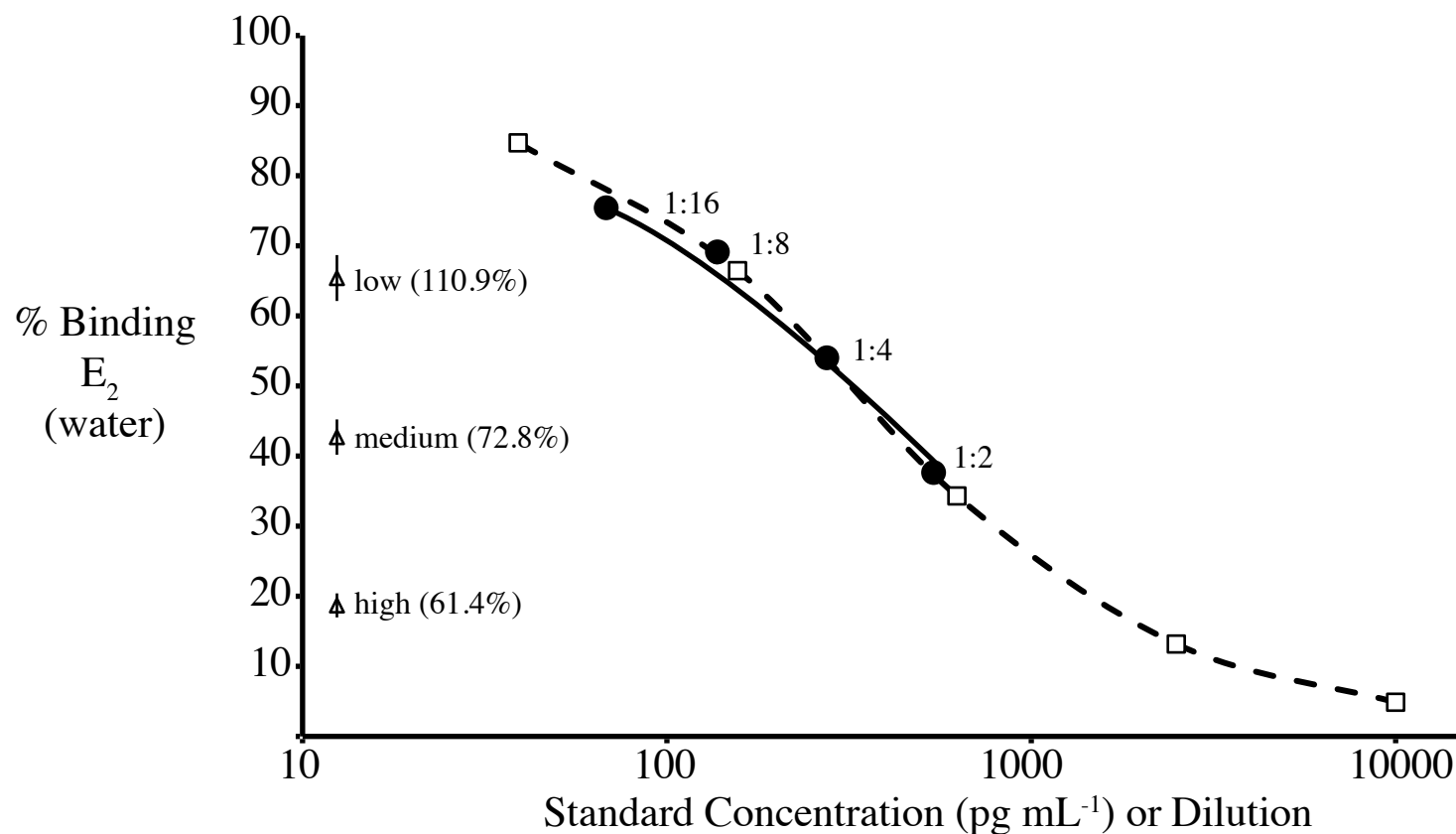
S7: Parallelism for water-borne 17-β estradiol (E2) using Arbor Assays 17-β Estradiol EIA DetectX (Cat. No. K030). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of four dilutions indicated on graph (treatment group: 500 IU/g dose of hCG). Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed all subsequent water borne E2 samples at a 1:2 dilution. Open triangles represent mean (± standard deviation) percent binding for low (CV = 17.1%), medium (CV=10.6%) and high concentration (CV=11.2%) spike recovery samples (n=6 replicates each assayed in duplicate) and percent recovery is shown in parentheses. A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.072, SE = 1.17, t_5 = 0.061, p = 0.953).

**S8**: Parallelism for water-borne progesterone (PROG) samples using the Arbor Assays Progesterone EIA DetectX (Cat. No. K025). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of four dilutions indicated on graph (treatment group: 500 IU/g dose of hCG). Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed all subsequent water borne progesterone samples at a 1:2 dilution. Open triangles represent mean (± standard deviation) percent binding for low (CV = 28.8%), medium (CV = 17.2%) and high (CV = 15.0%) concentration spike recovery samples (n=6 replicates each assayed in duplicate) and percent recovery is shown in parentheses. A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.07, SE = 0.761, t_7 = 0.096, p = 0.926).

S9: Parallelism for water-borne corticosterone using the Arbor Assays Corticosterone EIA DetectX (Cat. No. k014). Dashed line and open squares indicate the standard curve for a single plate and the solid line and solid circles depict a pooled sample at one of four dilutions indicated on graph (Low ACTH treatment group: 0.5ug/g ACTH) at the 60 min time point. Curve fitting performed using a four parameter equation (Softmax Pro). Based on this parallelism, we processed all subsequent water borne CORT samples at a 1:1 dilution (i.e., 250 ul assay buffer reconstitution). Open triangles represent mean (± standard deviation) percent binding for low (312.5 pg mL^{-1}; CV = 18.1%), medium (1250 pg mL^{-1}; CV = 2.3%) and high (5000 pg mL^{-1}; CV = 7.5%) concentration spike recovery samples (3 replicates of each run in duplicate wells) and percent recovery is shown in parentheses. A comparison of slopes demonstrated that the standard curve and dilution curve were parallel (Δ slope = 0.07, SE = 0.801, t$_8$ = 0.091, p = 0.929).
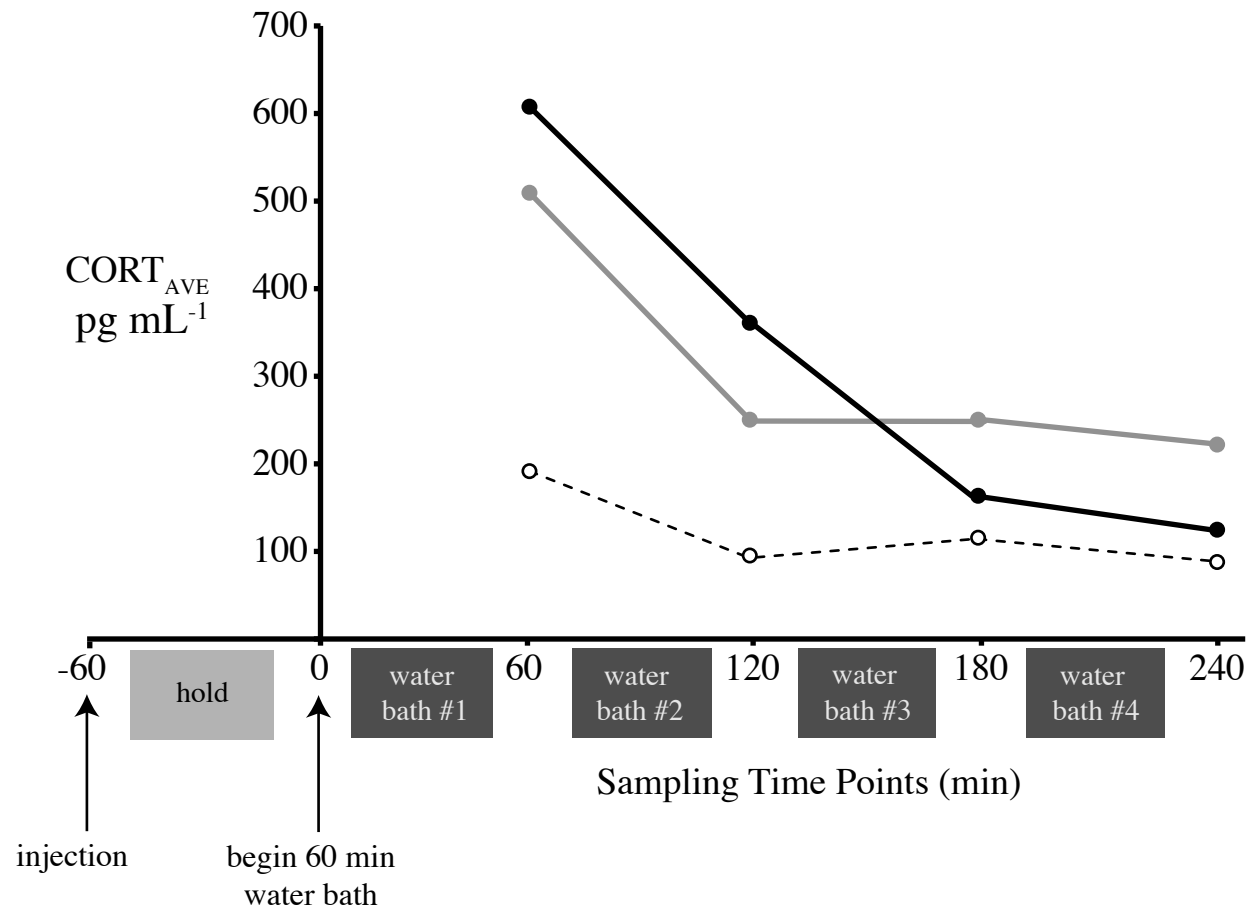
S10: Pooled (5 male and 5 female frogs pooled per point) water borne CORT values (mean) for vehicle, Low dose ACTH (0.5 ug/g) and High dose ACTH (5 ug/g). ACTH or vehicle (0.9% saline) injection occured at -60 min. Frogs were held in terraria for 60 min following injection and then introduced to a water bath (40 mL frog water) at 0 min timepoint. All water baths were 60 min duration, repeated measures. For graphical purposes, the x-axis intercepts are shifted for some points so that error bars do not overlay. Symbols: Open circles (dashed line) is vehicle; Closed grey circles and lines are Low ACTH dose; Closed black circles and lines are High ACTH dose.
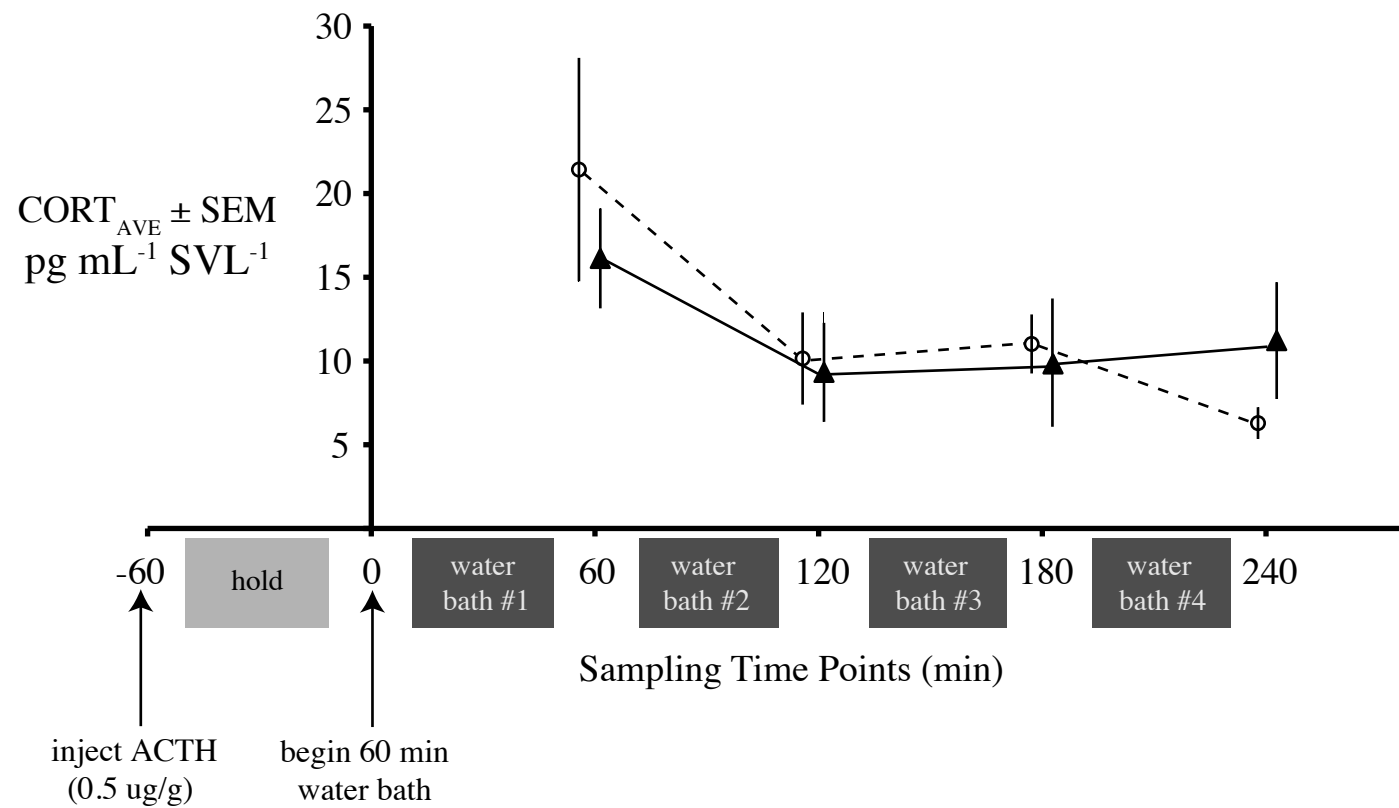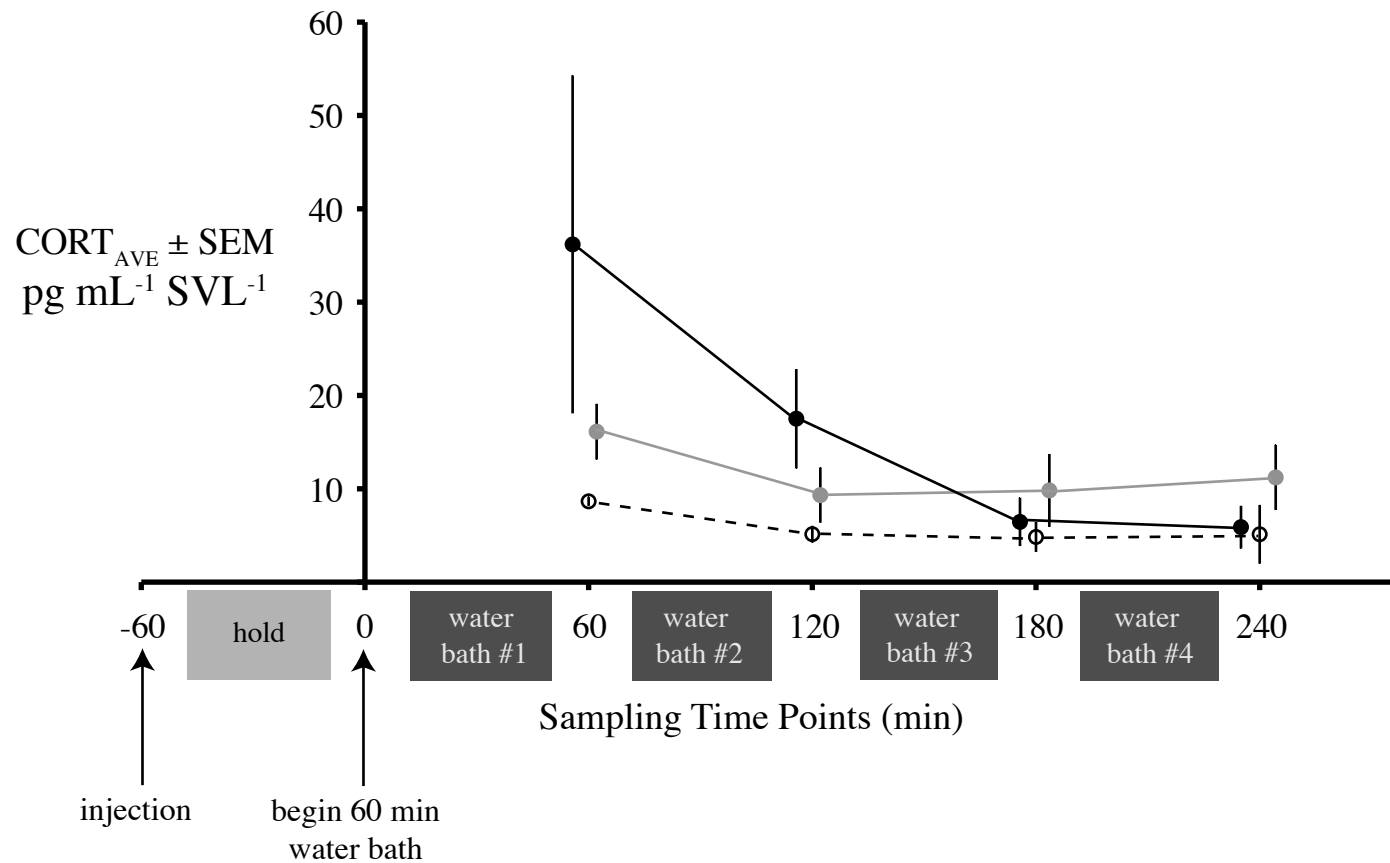
S11: Individual female (open circles and dashed line) and male (closed triangles and solid lines) (N=5 per treatment group) water borne CORT values (mean ± SEM) for Lo ACTH treatment (0.05 ug/g ACTH in 0.9% saline). Lo ACTH injection occured at -60 min. Frogs were held in terraria for 60 min following injection and then introduced to a water bath (40 mL frog water) at 0 min timepoint. All water baths were 60 min duration, repeated measures. For graphical purposes, the x-axis intercepts are shifted for some points so that error bars do not overlay. Symbols: Open circles (dashed line) is vehicle; Closed grey circles and lines are Low ACTH dose; Closed black circles and lines are High ACTH dose
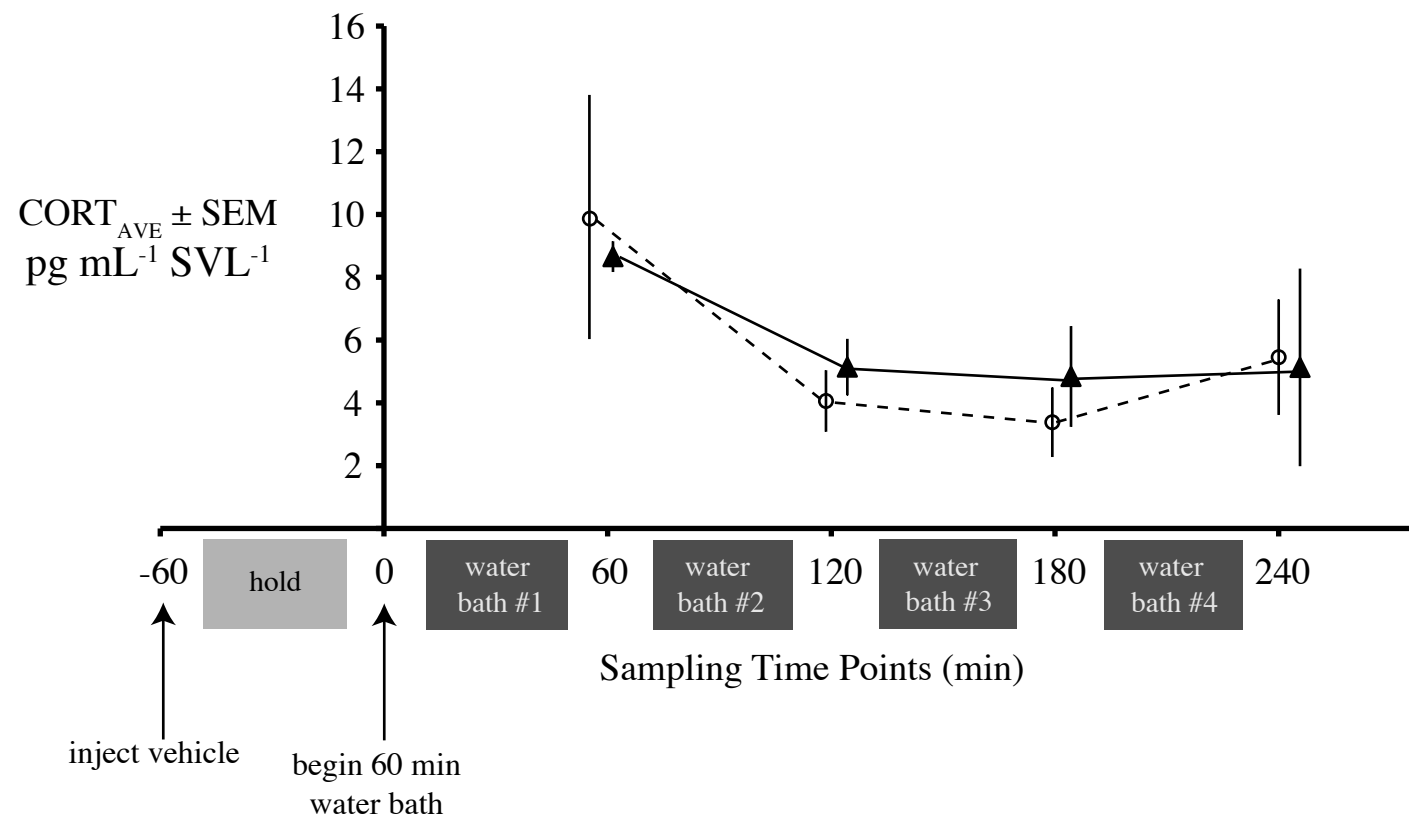
S12: Individual female (open circles and dashed line) and male (closed triangles and solid lines) (N=5 per treatment group) water borne CORT values (mean ± SEM) for vehicle (0.9% saline). Vehicle injection occured at -60 min. Frogs were held in terraria for 60 min following injection and then introduced to a water bath (40 mL frog water) at 0 min timepoint. All water baths were 60 min duration, repeated measures. For graphical purposes, the x-axis intercepts are shifted for some points so that error bars do not overlay. Symbols: Open circles (dashed line) is vehicle; Closed grey circles and lines are Low ACTH dose; Closed black circles and lines are High ACTH dose.
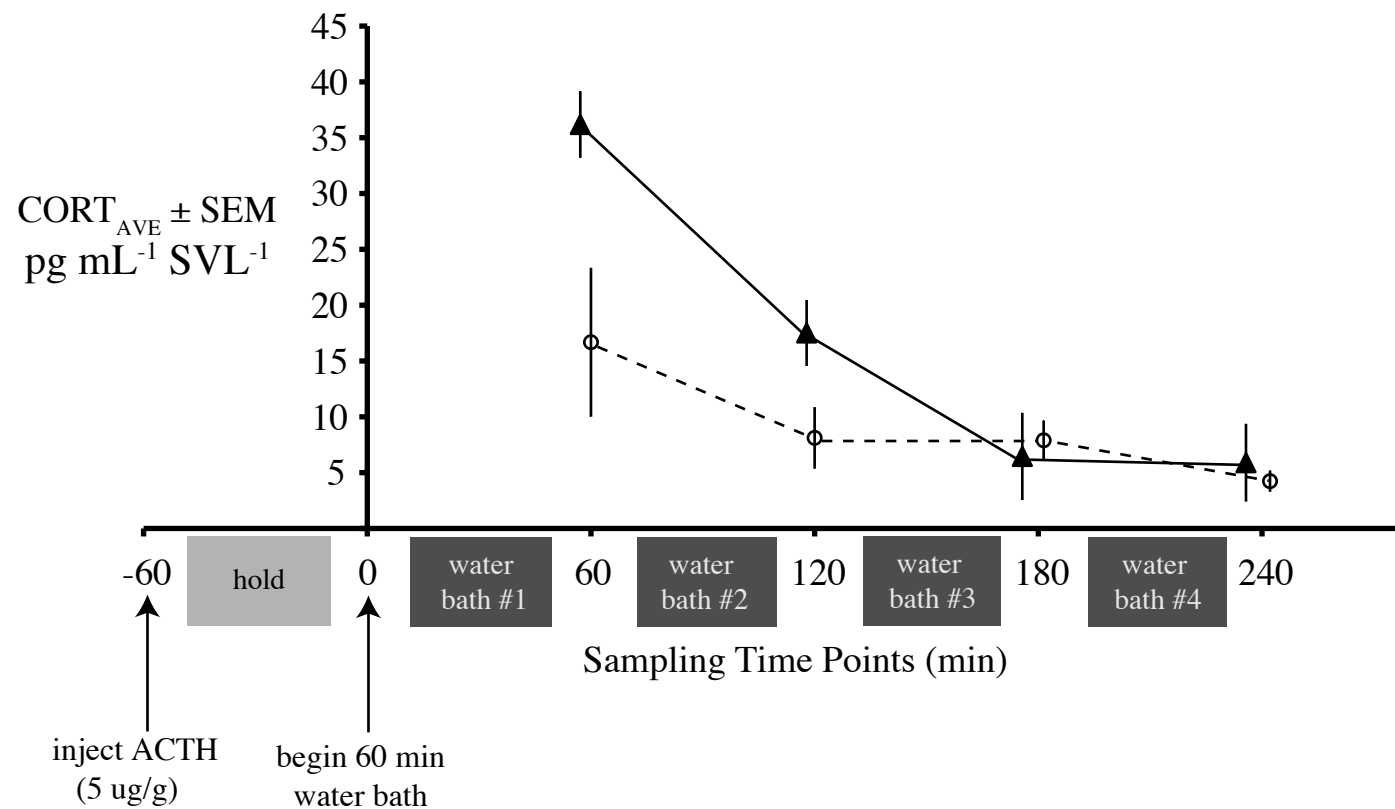
S13: Individual female (open circles and dashed line) and male (closed triangles and solid lines) (N=5 per treatment group) water borne CORT values (mean ± SEM) for Hi ACTH treatment (5 ug/g ACTH in 0.9% saline). Hi ACTH injection occured at -60 min. Frogs were held in terraria for 60 min following injection and then introduced to a water bath (40 mL frog water) at 0 min timepoint. All water baths were 60 min duration, repeated measures. For graphical purposes, the x-axis intercepts are shifted for some points so that error bars do not overlay. Symbols: Open circles (dashed line) is vehicle; Closed grey circles and lines are Low ACTH dose; Closed black circles and lines are High ACTH dose.
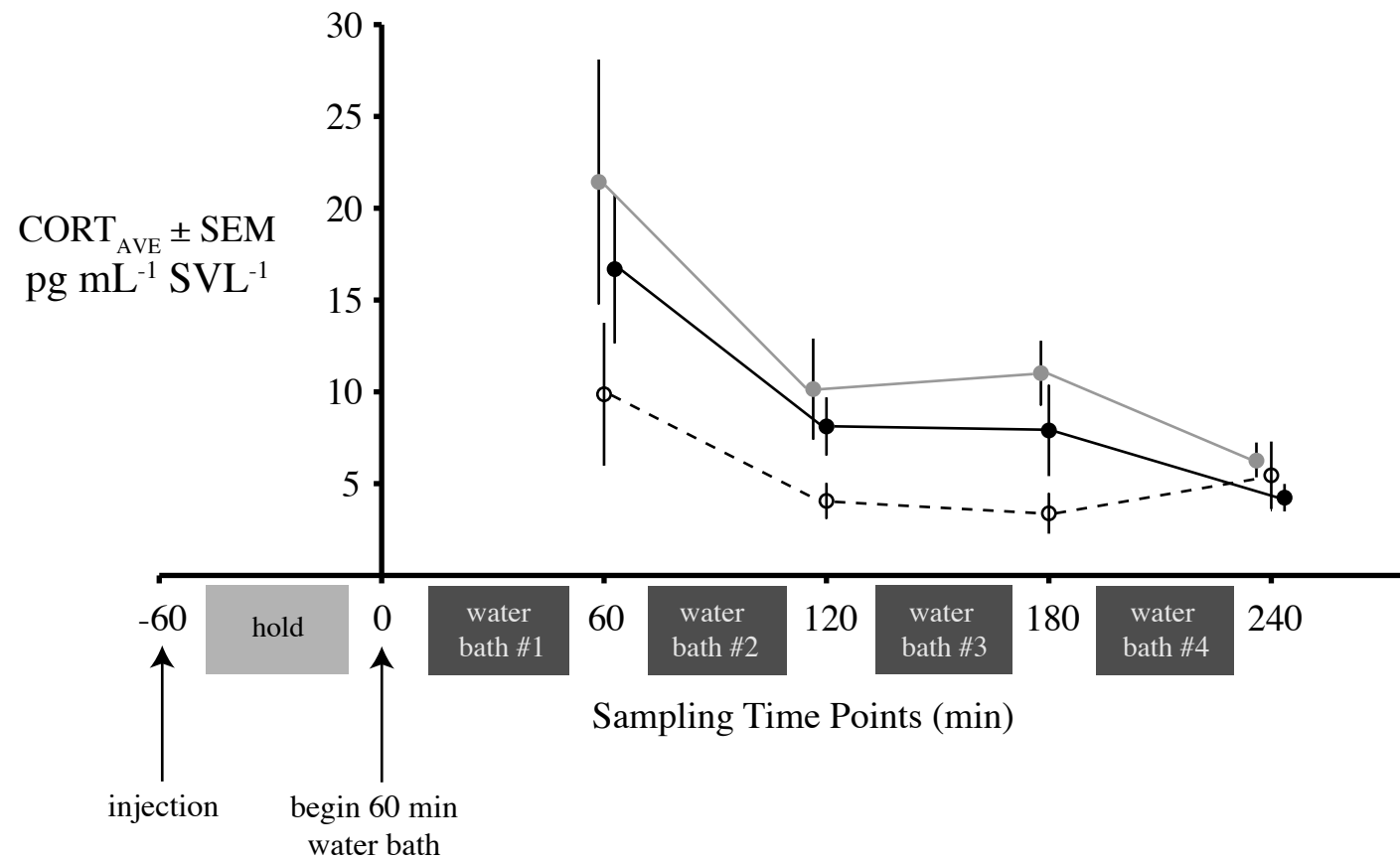
S14: Individual female (N=5 per treatment group) water borne CORT values (mean ± SEM) for vehicle, Low dose ACTH (0.5 ug/g) and High dose ACTH (5 ug/g). ACTH or vehicle (0.9% saline) injection occured at -60 min. Frogs were held in terraria for 60 min following injection and then introduced to a water bath (40 mL frog water) at 0 min timepoint. All water baths were 60 min duration, repeated measures. For graphical purposes, the x-axis intercepts are shifted for some points so that error bars do not overlay. Symbols: Open circles (dashed line) is vehicle; Closed grey circles and lines are Low ACTH dose; Closed black circles and lines are High ACTH dose.
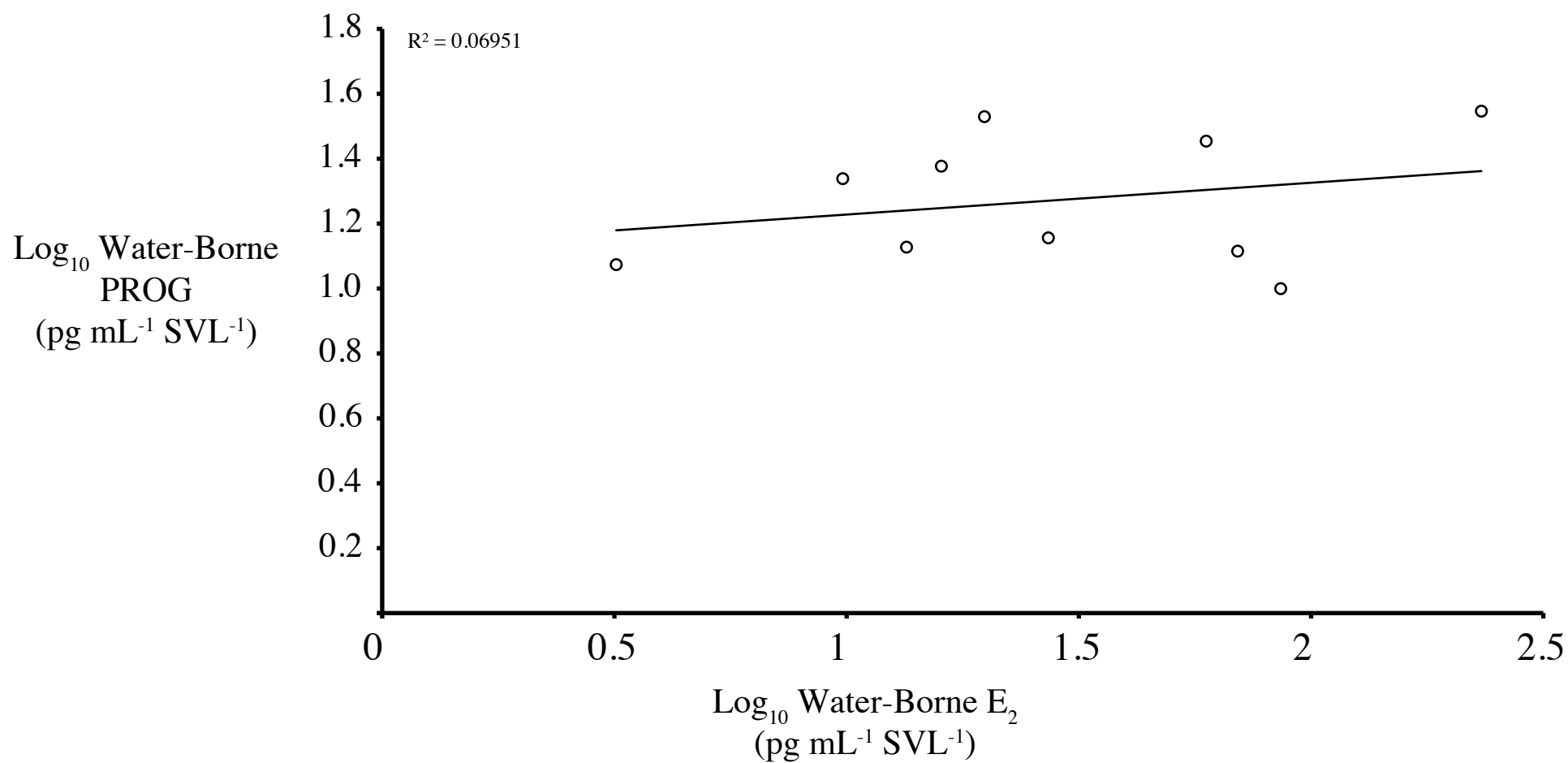
S15: Individual male (N=5 per treatment group) water borne CORT values (mean ± SEM) for vehicle, Low dose ACTH (0.5 ug/g) and High dose ACTH (5 ug/g). ACTH or vehicle (0.9% saline) injection occured at -60 min. Frogs were held in terraria for 60 min following injection and then introduced to a water bath (40 mL frog water) at 0 min timepoint. All water baths were 60 min duration, repeated measures. For graphical purposes, the x-axis intercepts are shifted for some points so that error bars do not overlay. Symbols: Open circles (dashed line) is vehicle; Closed grey circles and lines are Low ACTH dose; Closed black circles and lines are High ACTH dose.
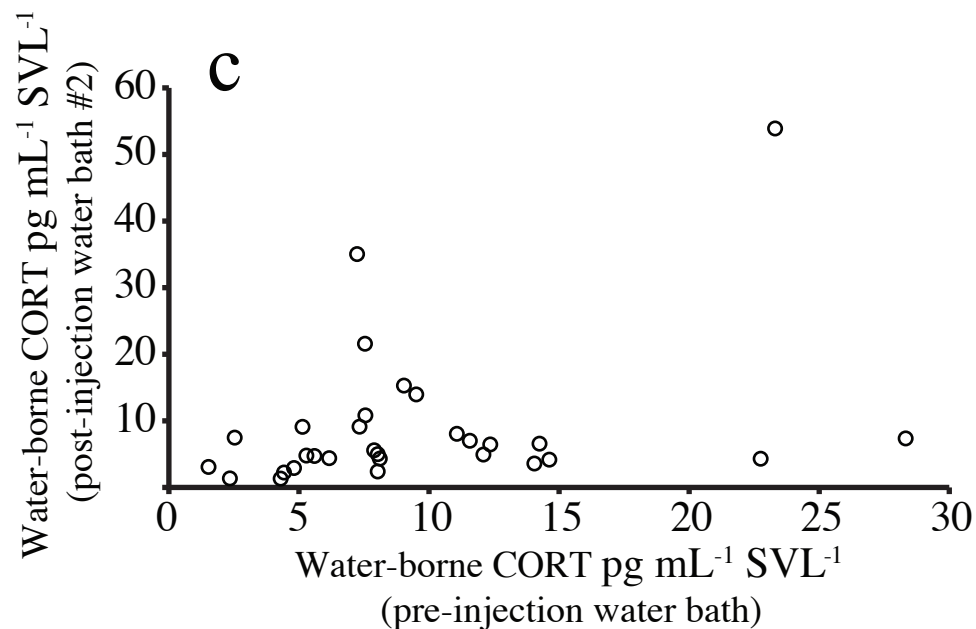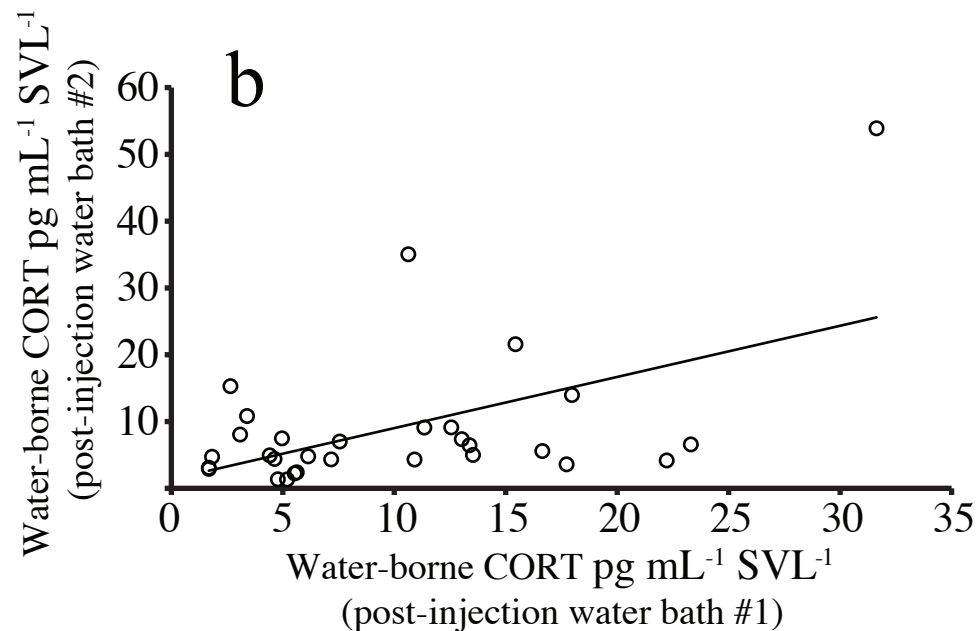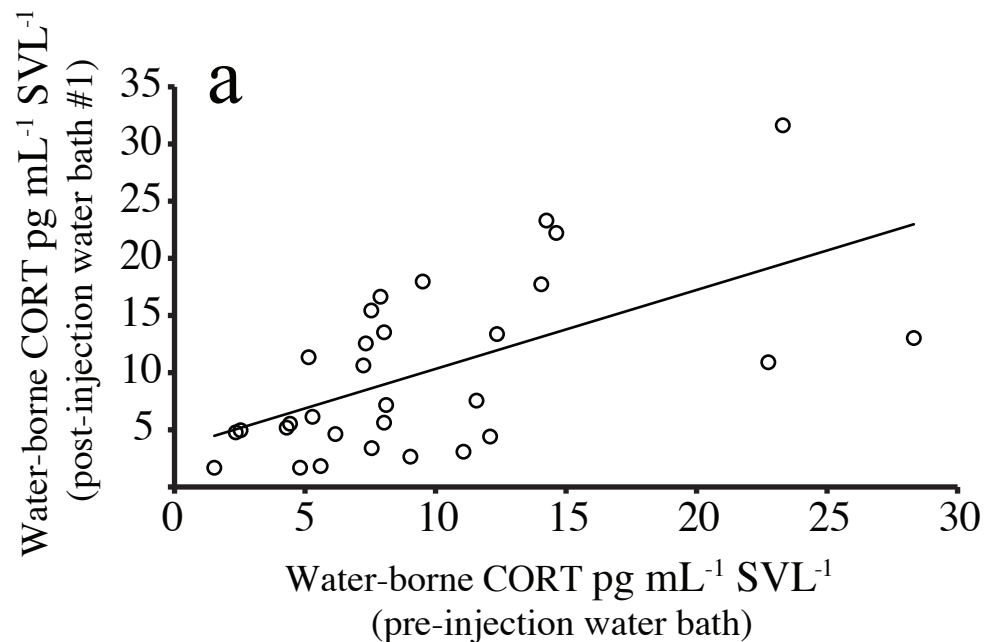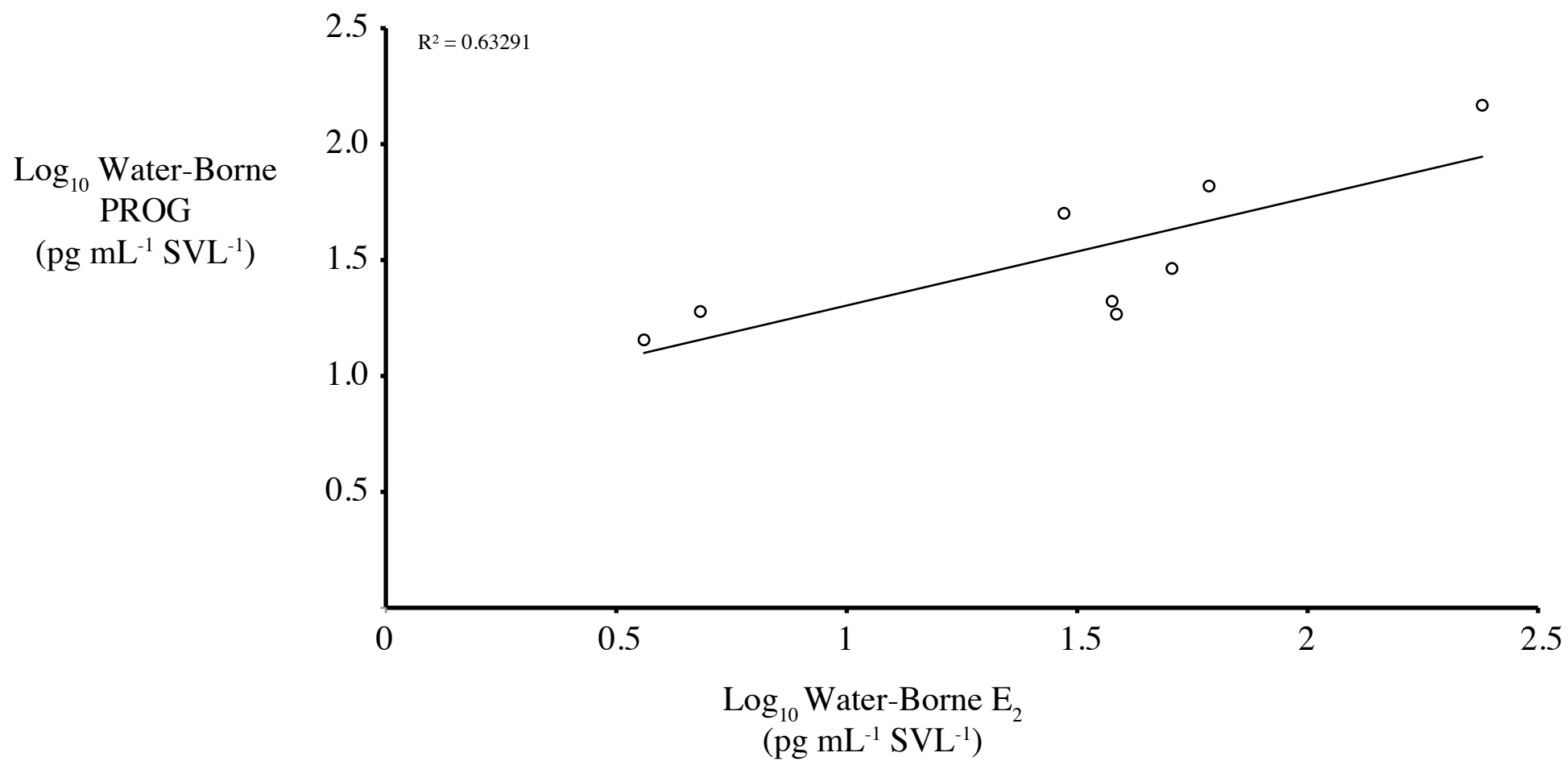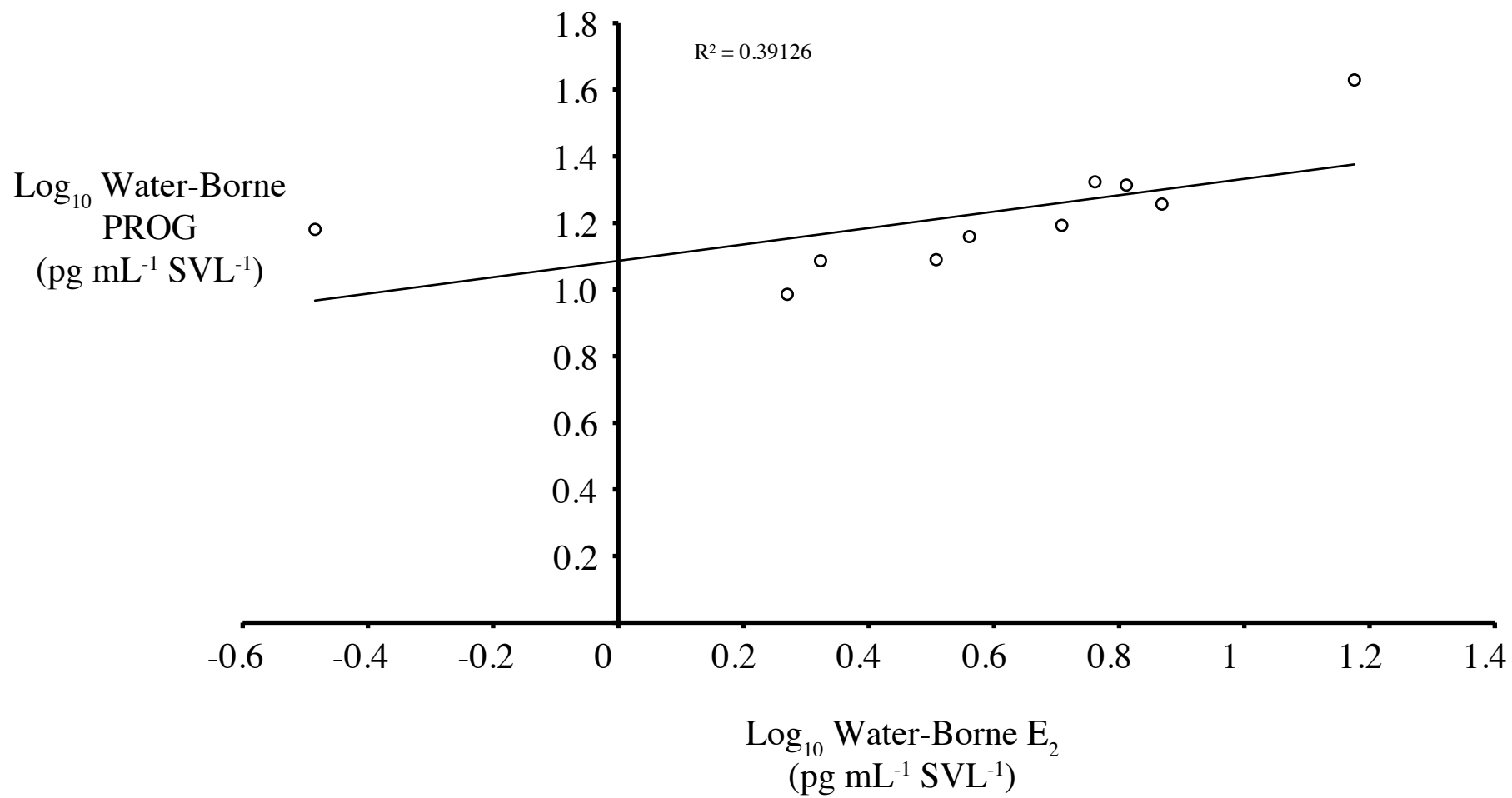
S16: Correalitons (Pearson's) between CORT concentrations among water samples. (a) the pre-injection time-point was positively correlated with the first post-injection time-point (r=0.58, p=0.001, n=31). (b) the first post-injection time-point was positively correlated with the second post-injection time-point (r=0.49, p=0.004, n=32). (c) the pre-injection time-point was not significantly correlated with the second post-injection time-point (r=0.31, p=0.08, n=31).
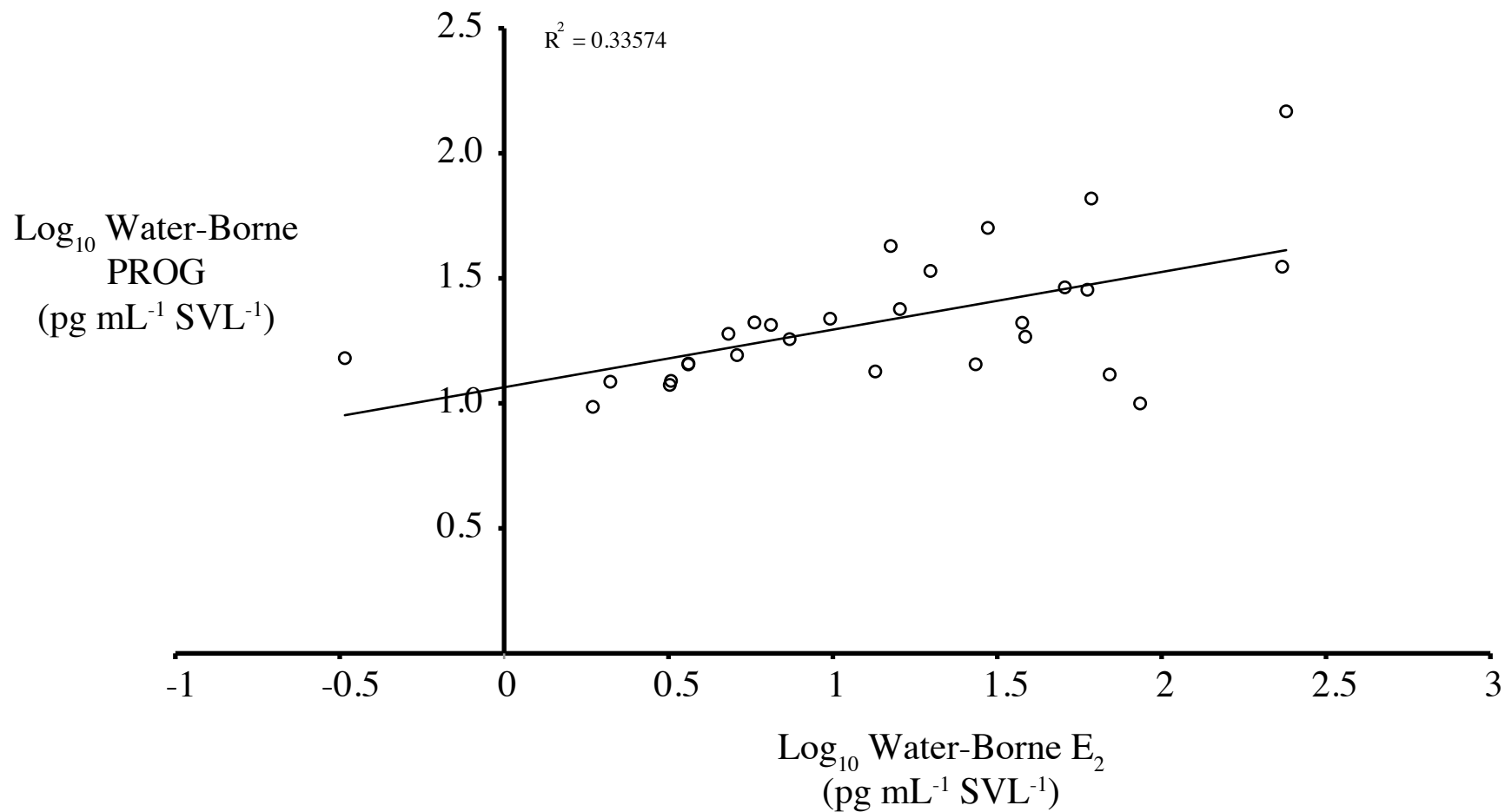
$R^2 = 0.06951$

Log$_{10}$ Water-Borne PROG (pg mL^{-1} SVL^{-1})

Log$_{10}$ Water-Borne E$_2$
(pg mL^{-1} SVL^{-1})

S17: 50IU HCG (Pearson's r=0.264, p=0.462, n=10; Spearmans rho=0.188, p=0.603, n=10);

$R^2 = 0.63291$

Log$_{10}$ Water-Borne PROG (pg mL^{-1} SVL^{-1})

Log$_{10}$ Water-Borne E$_2$ (pg mL^{-1} SVL^{-1})

S18: 500 IU HCG: persons r=0.796, p=0.018, n=8; spearmons rho=0.762, p=0.028, n=8).

Figure axes: Y-axis: Log$_{10}$ Water-Borne PROG (pg mL^{-1} SVL^{-1}); X-axis: Log$_{10}$ Water-Borne E$_2$ (pg mL^{-1} SVL^{-1})

$R^2 = 0.39126$

S19: Vehicle (Pearsons: r=0.626, p=0.053, n=10; Spearmans: rho=0.830, p=0.003);

S20: Pearson's correlation showed a significant and strong correlation between these two female reprod steroids: (residuals are reasonable gaussian, but a bit leptokurtic): r=0.579, p=0.001, n=28, R2=0.336; this correlation holds up with a Spearmans Rank Correlation non parametric: rho=0.549; p=0.002, n=28. This graph pools all three injection treatment groups (vehicle, 50IU g^{-1} HCG and 500IU g^{-1} HCG) together.
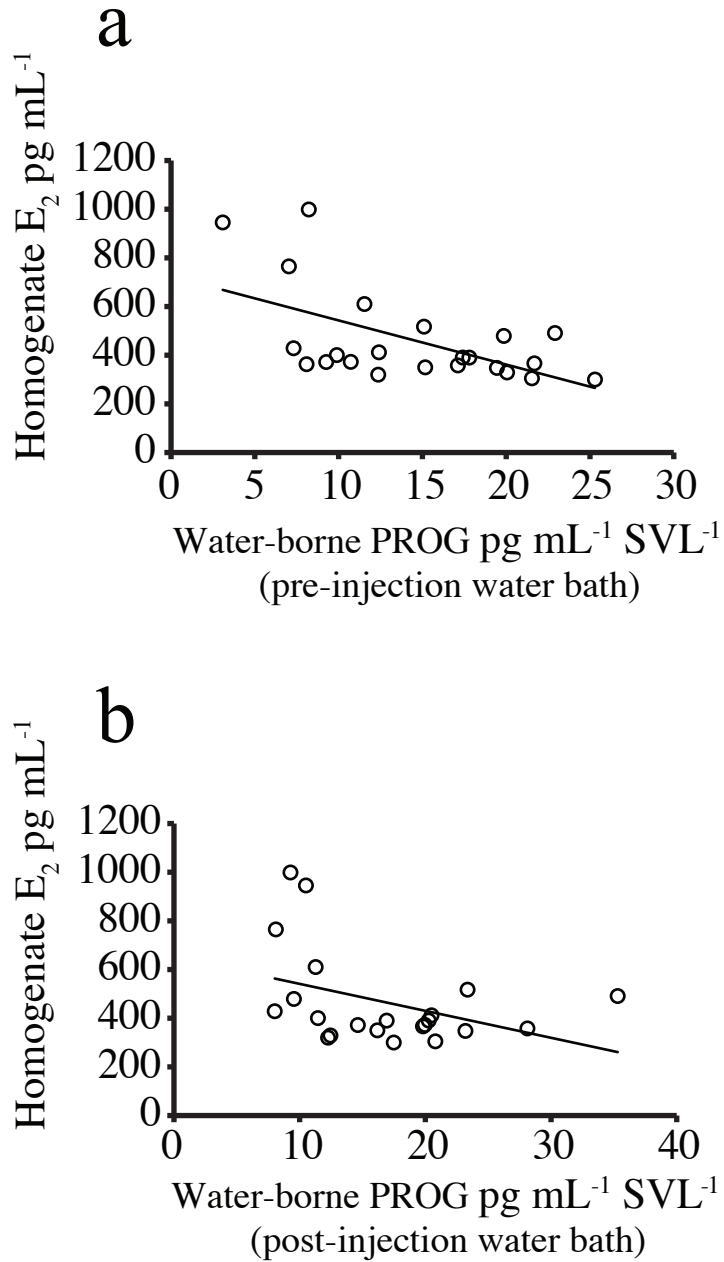
S21: Correlations (Pearson's) between concentrations of water-borne progesterone (PROG) and homogenate 17-β estradiol (E2). All data were $\log_{10}$ transformed prior to analysis to improve residuals (untransformed data are depicted here). (a) pre-injection water-borne PROG concentrations were negatively correlated with whole animal homogenate E2 concentrations ($r = -0.65$, $p=0.001$, $n=23$). (b) post-injection water-borne PROG concentrations were negatively correlated with whole animal homogenate E2 concentrations ($r = -0.47$, $p=0.026$, $n=22$).