1 SHORT COMMUNICATION: Fresh water acclimation elicits a decrease in plasma
2 corticosteroids in the euryhaline Atlantic stingray, *Dasyatis sabina*

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ABSTRACT

It is thought that the elasmobranch corticosteroid hormone 1α -hydroxycorticosterone (1α -B) functions as both a glucocorticoid (GC) and mineralocorticoid (MC). Classical antinatriuretic MC activities would run counter to the osmoregulatory strategy of euryhaline elasmobranchs acclimating to fresh water (FW). Therefore we hypothesize that FW acclimation will be accompanied by a decrease in plasma corticosteroids in these animals. However, events that activate the “fight-or-flight” response could mask changes associated with acclimation to lower salinities. To better define the MC role of corticosteroids in elasmobranchs, we designed a transfer system that allows the acclimation of Atlantic stingrays (*Dasyatis sabina*) from seawater (SW) to FW over 12 hours while minimizing other extraneous stressors. Blood and interrenal glands were sampled from one group of stingrays 24 hours after FW transfer, while another group was sampled two weeks after FW transfer. Two other groups served as mock-transfer controls in that they were treated and sampled in the same way, but remained in SW for the entire period. Plasma corticosteroids, osmolality, chloride, and urea were significantly lower in FW-acclimated stingrays (compared to mock-transfer stingrays) 24 hours after FW transfer. This pattern remained after two weeks in FW, with the exception that plasma corticosteroids returned to pre-acclimation levels. There were no significant differences between experimental groups in interrenal levels of mRNAs encoding key steroidogenic proteins (steroidogenic acute regulatory protein and cholesterol side chain cleavage enzyme). Temporally decreased corticosteroid levels during FW acclimation are consistent with the unique strategy of euryhaline elasmobranchs, whereby lower plasma osmolality is maintained in FW vs. SW environments to reduce hydromineral gradients.

Keywords: corticosteroids; 1α -hydroxycorticosterone; osmoregulation; elasmobranch; euryhaline

1. INTRODUCTION

Aquatic organisms living in coastal environments may experience frequent and significant changes in the salinity of the water in which they are living. In fishes, there are two primary physiological strategies to deal with changes in environmental osmolality. Euryhaline teleosts expend energy to maintain fairly constant osmolality by drinking water and secreting excess salt while in seawater (SW), and producing dilute urine while retaining and scavenging salt when salinity decreases (Smith, 1930; Keys, 1931; Krogh, 1937; Evans, 1980). On the other hand, the elasmobranch fishes retain high levels of nitrogenous compounds such as urea and trimethylamine oxide, increasing their osmolality equal to or just greater than that of SW, so that while in SW they do not need to drink, as water is gained osmotically (Smith, 1931b; Thorson, 1962). In fresh water (FW), however, elasmobranchs lessen the osmotic gradient by actively reducing plasma levels of solutes e.g. ions and nitrogenous compounds (Smith, 1931a; Piermarini and Evans, 1998; Pillans and Franklin, 2004; Hammerschlag, 2006).

Adrenocortical tissues in terrestrial vertebrates produce specific steroids to serve as glucocorticoid (GC; cortisol or corticosterone depending on the species) and mineralocorticoid (MC; aldosterone) steroid hormones. However, in fishes, interrenal tissue produces a dominant steroid thought to have both GC and MC properties. In elasmobranchs this steroid is 1α -hydroxycorticosterone (1α -B), a corticosteroid unique to this vertebrate subclass (Idler and Truscott, 1966). The putative GC and MC actions of 1α -B are supported by *in vitro* studies, which demonstrate that interrenal production of 1α -B is stimulated by both the stress hormone adrenocorticotrophic hormone (ACTH; Hazon and Henderson, 1985; Klesch and Sage, 1975; Nunez and Trant, 1999; O'Toole et al., 1990) and the osmoregulatory hormone angiotensin II (Armour et al., 1993a; Evans et al., 2010). Direct antinatriuretic MC actions of 1α -B have also

71 been demonstrated using heterologous bioassays, including *in vitro* stimulation of sodium
72 transport across isolated toad bladders and *in vivo* sodium retention in adrenalectomized mice
73 following 1α -B administration (Grimm et al., 1969; Idler et al., 1967).

74 Additional *in vivo* support for a MC role of 1α -B in elasmobranchs includes reduced
75 plasma osmolality, sodium and urea in interrenalectomized Atlantic stingrays (de Vlaming et al.,
76 1975) as well as elevated plasma 1α -B in dogfish (*Scyliorhinus canicula*) fed a low protein diet
77 (Armour et al., 1993b). In the latter study *S. canicula* were unable to maintain plasma urea due to
78 low protein availability but maintained osmolality via increased NaCl, leading to the hypothesis
79 that the elevated 1α -B may be responsible for increased ion retention. In the single study that has
80 examined changes in circulating 1α -B during salinity acclimation, 1α -B was higher in
81 stenohaline *S. canicula* 14 days after gradual (10% every 10 days) acclimation to 50% SW
82 (Hazon and Henderson, 1984). Chronically elevated 1α -B may therefore facilitate ion retention
83 in reduced salinities; however, the MC actions of 1α -B would be counterproductive during the
84 initial phase of acclimation characterized by decreasing concentrations of plasma ions.

85 We hypothesize that, in the absence of other stressors, plasma corticosteroids including
86 1α -B will decline during FW acclimation as part of the elasmobranch strategy to reduce plasma
87 osmolytes during acclimation to lower salinities. However, as there is evidence that 1α -B may
88 function as both GC and MC, studies attempting to isolate the MC actions of 1α -B may be
89 hampered by the confounding effects of stress on experimental animals. In such studies, care
90 must be taken to eliminate handling stress and other stressors that might stimulate the release of
91 ACTH, leading to changes in plasma corticosteroid concentrations that are not related to
92 osmoregulatory challenges. We therefore designed a transfer system that allowed the acclimation
93 of SW-adapted Atlantic stingrays (*Dasyatis sabina*) to FW with minimal stress. We used this

system to examine how acclimation from SW to FW alters blood corticosteroid concentrations and other blood chemistries. To see if such acclimation impinges on the capacity of the Atlantic stingray to produce corticosteroids, we also examined the expression of mRNAs encoding the rate-limiting steroidogenic proteins StAR (steroidogenic acute regulatory protein) and cytochrome P450_{scc} (cholesterol side-chain cleavage enzyme) in interrenal tissue. This study indicates that acclimation to FW results in significant changes in several plasma chemistries (including corticosteroids) within 24 hours. However, levels of plasma corticosteroids in animals remaining in FW for two weeks are not significantly different from animals in seawater.

2. MATERIALS AND METHODS

2.1. *Animals*

Sexually mature (>22 cm disk width; Tricas et al., 2000) *D. sabina* were captured using hand nets in shallow bays near Port Aransas, Texas. Thirty stingrays were used for this study (14 male, 16 female; disk width: 22 – 31.5 cm; mass: 0.4 – 1.0 kg). Animals were transferred to the laboratory, maintained in a 550 L tank with ambient flow-through SW (30 – 33 ppt) and fed chopped shrimp ad libitum. Animals were acclimated to captivity for at least two weeks prior to experimentation and were sacrificed by cervical dislocation using methods approved by the University of Texas Animal Care and Use Committee (IACUC protocol #06030101).

2.2. *Fresh water acclimation*

For FW acclimation, four animals at a time were transferred by net to a tank with recirculating, filtered SW (170 gallons, 30 ppt salinity) and allowed to acclimate for 48 h. Animals were not fed once moved to the acclimation tank and for at least 24 h after transfer to FW. The acclimation tank was surrounded by an opaque curtain and equipped with external

valves so that salinity adjustments could be made without disturbing the animals, eliminating the effects of additional handling stress. Two biological filter boxes were connected to the tank to allow for the recirculation of either SW or FW. Following the 48 h acclimation period, recirculation through the SW filter box was closed and dechlorinated, buffered FW (pH 8.2 – 8.4; Malawi/Victoria Buffer; Seachem) was transferred from a 600-gallon tank into the experimental tank using a submersible pump. FW flow was monitored and adjusted to result in a controlled salinity change from 30 ppt to 0 ppt over 12 hours. All FW acclimations were conducted from 9 PM to 9 AM to further reduce external stressors such as human activity. Following salinity change to FW, valves were then adjusted to allow recirculation through the FW filter box. Matched controls followed the above methods, except that 30 ppt SW was used in place of FW. Animals were terminally sampled during separate experimental runs at the following time points: 48 hrs after introduction to the experimental tank (pre-acclimation, n = 10), 24 hrs after salinity change to FW (n = 8), 24 hrs after salinity “change” to SW (n = 4), 14 days following acclimation to FW (n = 4) and 14 days in SW (n = 4). At each experimental time point, stingrays were removed individually from the tank by net, a blood sample (200 μ L) was quickly collected from the caudal vein using a 25-gauge needle and heparinized syringe (lithium heparin; Sigma) and animals were sacrificed by cervical dislocation. All stingrays from the tank were captured and sampled within a single 5 minute period to reduce variation in stress due to capture time, with blood samples placed on ice. Interrenal glands were then collected, rapidly frozen on dry ice and stored at -80 °C. Following hematocrit determination as described in section 2.3, blood samples were centrifuged for 5 min at 5000 x g to separate plasma from the cellular fraction, with plasma transferred to a new microcentrifuge tube and stored at -80 °C.

2.3. Plasma analyses

To examine the effects of acute and chronic salinity acclimation on plasma components, osmolality, chloride, urea and hematocrit were quantified. Plasma osmolality (mOsm kg^{-1}) was quantified in duplicate using a Wescor 5130B vapor pressure osmometer (Wescor Incorporated, Logan, UT). Chloride and urea (mM) were quantified by colorimetric improved Fried (Yokoi, 2002) and Jung (Jung et al., 1975) methods, respectively, using commercial kits (QuantiChrom Chloride and Urea Assay Kits; BioAssay Systems) and a spectrophotometer (SpectraMAX 190; Molecular Devices). For osmolality (1:2) and chloride/urea (1:50) quantification, 5 μL of each sample was diluted in ddH_2O . Hematocrit was determined by drawing approximately 50 μL of blood into 75 mm ammonium-heparin hematocrit tubes, centrifuging these tubes in a microhematocrit centrifuge (IEC Micro-MB, Thermo Scientific) and determining the percentage of packed red blood cells using a microhematocrit capillary tube reader disk.

2.4. Corticosteroid Enzyme-Linked Immunoassay

There is currently no specific antibody against $1\alpha\text{-B}$, and therefore plasma corticosteroids were quantified using a commercial corticosterone ELISA (Cayman Chemical) previously validated for $1\alpha\text{-B}$ (Evans et al., 2010), with the exception that samples were not subjected to HPLC fractionation. It has previously been demonstrated that corticosterone is not produced by *D. sabina* interrenal tissue *in vitro* (Evans et al., 2010; Klesch and Sage, 1975; Nunez and Trant, 1999); however because we did not specifically isolate the $1\alpha\text{-B}$ fraction from these samples our data should be interpreted as total plasma corticosteroids. $1\alpha\text{-B}$ was used for the standard curve to most accurately reflect corticosteroid concentrations, as it is anticipated that other steroids (e.g. corticosterone) will be absent or present at minimal concentrations. For individual extraction recovery determinations, plasma samples were equilibrated with 2000 dpm ^3H -cortisol in ddH_2O (200 μL total volume) overnight at 4 $^\circ\text{C}$. Cortisol was selected for individual recovery

determinations because this corticosteroid has not been identified in elasmobranch plasma, and the reported cross-reactivity of the ELISA antibody with cortisol is only 0.17% (cross-reactivity with 1 α -B is 5.2%; Evans et al., 2010). Plasma samples were then extracted twice with 2 mL dichloromethane, dried under nitrogen gas at 37 °C and reconstituted overnight in 200 μ L EIA assay buffer (Cayman Chemical) at 4 °C. Samples were run in duplicate and adjusted for individual extraction efficiency (75 – 100%, average = 95%) and initial sample volume.

2.5. Real time quantitative PCR

The effects of FW acclimation on interrenal *StAR* and *P450_{scc}* mRNA expression were determined using RNA extraction, reverse transcription (RT) and probe-based quantitative RT-PCR methods, reagents and primers as described previously (Evans and Nunez, 2010). Briefly, total RNA was isolated using Trizol reagent (Invitrogen) followed by a 30 min DNase treatment (Promega DNase I) at 37°C and re-purification of RNA using Trizol. 1 μ g of DNase-treated RNA was then used as template in 20 μ L reverse transcription (RT) reactions using SuperScript III reverse transcriptase (Invitrogen) and random hexamer primers following the manufacturer's instructions. RT reactions were diluted with water to 50 μ L and then used as template for realtime quantitative PCR (qPCR). Five μ L of each RT reaction was included in a total volume of 25 μ L containing 12.5 μ L Brilliant II qRT-PCR 2x master mix (Stratagene), 500 nM gene-specific dual-labeled probe and 600 nM of each gene-specific primer (probe and primer sequences and reaction efficiencies shown in Table 1). qPCR reactions were run in an Eppendorf Realplex² Mastercycler using the following method: 95°C for 10 min followed by 45 cycles of 95°C for 30 s and 62°C for 1 min. Reactions were run in duplicate, with relative mRNA levels determined using 18S (reference gene) reactions and the equation described by Fink et al. (1998).

2.6. Statistical analysis

Statistical analyses for osmolality, chloride, urea and hematocrit were made using ANOVA followed by Tukey HSD post hoc tests to identify significant pairwise differences. Corticosteroid data did not fit a normal distribution and was therefore analyzed using a Kruskal-Wallis ANOVA and pairwise Wilcoxon tests to identify significant differences. In all cases p -values less than 0.05 were considered significant.

3. RESULTS

For each experimental time point, all animals in the holding tank appeared robust and healthy prior to sampling, and there was no mortality in any of the acclimation experiments. Twenty-four hours after transfer to FW, *D. sabina* had significantly ($p < 0.01$) decreased plasma osmolality (Fig. 1a), chloride (Fig. 1b) and urea (Fig. 1c) compared to pre-acclimation animals. These parameters were also significantly different between FW-acclimated animals and matched SW animals at each time point. However, there were no differences in hematocrit between FW-acclimated *D. sabina* and either pre-acclimation animals or matched SW animals (Fig. 1d). Fourteen days following FW transfer, plasma osmolality, chloride and urea were still reduced compared to pre-acclimation and 14 d SW animals. There was no significant difference in osmolality, chloride, urea or hematocrit between 24 h FW and 14 d FW individuals.

Twenty-four hours following FW transfer, plasma corticosteroids were significantly lower ($p < 0.05$) in FW animals vs. both pre-acclimation and matched SW animals (Fig. 2). However, there was no significant difference between 14 d FW and 14 d SW animals. Plasma corticosteroids did not change between pre-acclimation, 24 h SW and 14 d SW animals. As determined by qRT-PCR, there were no differences in mRNA levels for *StAR* or *P450scc* (Fig. 3) in either FW or SW-acclimated animals at any time point compared to pre-acclimation levels.

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210 4. DISCUSSION

211 Acclimation of SW-adapted *D. sabina* to FW in the current study resulted in significant
212 reductions in plasma osmolality, urea and chloride within 24 hours. It has previously been
213 demonstrated that acclimation of SW-adapted *D. sabina* to 26% SW is accompanied by plasma
214 clearance of sodium, chloride and urea (de Vlaming and Sage, 1973), while the opposite strategy,
215 i.e. increased plasma sodium, chloride and urea, is adopted by FW-adapted *D. sabina* during
216 acclimation to increased salinities (Piermarini and Evans, 1998). The reduction in plasma
217 osmolality in response to lower salinity reduces the osmotic gradient between the stingray and its
218 environment, and de Vlaming and Sage (1973) demonstrated that the initial stages of acclimation
219 (down to 35% seawater) are characterized by greater reductions in ions, e.g. sodium and
220 chloride, vs. the nitrogenous osmolyte urea.

221 In the current study, plasma corticosteroids in SW animals did not differ from pre-
222 acclimation values at any time point. While our understanding of the role corticosteroids play in
223 elasmobranch stress is limited, this suggests that *D. sabina* were not chronically stressed due to
224 the experimental design. In fact, acclimation of *D. sabina* to FW resulted in a transient decrease
225 in plasma corticosteroids. This is an appropriate response if, as hypothesized, the primary
226 elasmobranch corticosteroid 1 α -B possesses MC properties and therefore facilitates ion
227 retention. Due to the osmoregulatory strategy of euryhaline elasmobranchs, it would therefore be
228 inappropriate to maintain basal levels of a MC during initial acclimation to FW, a phase that is
229 characterized by significant reductions in plasma ions. However, after two weeks in FW, plasma
230 corticosteroids increased to levels that are no longer different from matched SW control animals.
231 Based on these results, we propose a model in which blood 1 α -B concentrations in euryhaline

elasmobranchs are transiently reduced during the initial stages of acclimation from SW to FW in response to a need to reduce plasma ions in the face of increased osmotic pressure. Once a new baseline is established for plasma osmolality, 1α -B concentrations then increase to facilitate the retention of ions such as sodium and chloride which are scarce in FW environments. Supporting this model, plasma 1α -B is significantly elevated in stenohaline *S. canicula* two weeks after gradual (10% every ten days) acclimation to 50% SW (Hazon and Henderson, 1984).

The observed changes in plasma corticosteroid concentrations are not accompanied by changes in interrenal *StAR* or *P450scc* mRNA. *StAR* and *P450scc* are rate-limiting proteins in any steroidogenic pathway and their intracellular concentrations and activities in adrenocortical tissue are therefore the targets of regulation by hormones such as ACTH, ANG II and the natriuretic peptides (Miller and Bose, 2011). *In vitro* treatment of *D. sabina* interrenal tissue with ACTH significantly increases levels of *StAR* and *P450scc* mRNA, whereas treatment with either ANG II or C-type natriuretic peptide significantly decreases levels of both mRNAs (Evans and Nunez, 2010; Evans et al., 2010). However, *StAR* and *P450scc* mRNAs do not change as a result of acclimation to increased salinity (10 ppt for 2 weeks) in the stenohaline freshwater stingray *Potamotrygon motoro* (Nunez et al., 2006). Similarly, there is no change in *StAR* mRNA following transfer of freshwater eels to SW despite increased plasma cortisol (Li et al., 2003). Therefore changes in plasma corticosteroid concentrations in fishes induced by changes in environmental salinity may potentially be mediated by other factors, including changes in steroid clearance rates or altered translation or activity of rate-limiting steroidogenic proteins. Alternatively, specific downstream steroidogenic enzymes (e.g. the putative 1α -hydroxylase) may be targets for chronic regulation of 1α -B synthesis.

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347

348 **FIGURE CAPTIONS**

349

350 Figure 1: Pre-acclimation (n = 10), 24 hours post-acclimation (n = 8 FW; n = 4 SW) and two
 351 weeks post-acclimation (n = 4 FW, n = 4 SW) plasma (A) osmolality, (B) chloride, (C) urea and
 352 (D) hematocrit of SW *D. sabina* and animals challenged with a 12 h salinity change from SW to
 353 FW. Error bars are +SEM. Asterisks indicate significant differences from pre-acclimation
 354 animals (**p < 0.01).

355

356 Figure 2: Pre-acclimation (n = 10), 24 hours post-acclimation (n = 8 FW; n = 4 SW) and two
 357 weeks post-acclimation (n = 4 FW, n = 4 SW) plasma corticosteroids of SW *D. sabina* and

358 animals challenged with a 12 h salinity change from SW to FW. Error bars are +SEM. Asterisks
359 indicate significant differences from pre-acclimation animals (*p < 0.05).

360

361 Figure 3: Relative interrenal *StAR* and *P450scc* mRNA levels for pre-acclimation (n = 10), 24
362 hours post-acclimation (n = 8 FW; n = 4 SW) and two weeks post-acclimation (n = 4 FW, n = 4
363 SW) *D. sabina*. Levels of mRNA are expressed as log transformed values obtained using the
364 equation of Fink et al. (1998). Error bars are +SEM.

Gene	Primer / Probe	Sequence	Efficiency
18S	Forward primer	5'-GTTAATTCCGATAACGAACGAGACTC-3'	94%
	Probe	5' (FAM)-TTCTTAGAGGGACAAGTGGCGTT-(BHQ1)3'	
	Reverse primer	5'-ACAGACCTGTTATTGCTCAATCTCGTG-3'	
P450scc	Forward primer	5'-GCCAGCACTTATGCACAAAGGG-3'	100%
	Probe	5' (FAM)-CGGAGCATGACTTCACGTTGTTC-(BHQ1)3'	
	Reverse primer	5'-GCCAGTTCCTTCTCCAGTTGCC-3'	
StAR	Forward primer	5'-GGCATTTCAGCAATGGCAGC-3'	92%
	Probe	5' (FAM)-TAAATCTGGTTCGGAGAAGGAACT-(BHQ1)3'	
	Reverse primer	5'-CCATCTCCGCCTCAGAATAAGG-3'	

Table 1: Real time quantitative PCR primers, probes and reaction efficiencies

Figure 1

Figure 2

Figure 3