Regulation of plasma glucose and sulfate excretion in Pacific hagfish, *Eptatretus stoutii* is not mediated by 11-deoxycortisol.

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**Abstract**

The goal of this study was to identify whether Pacific hagfish (*Eptatretus stoutii*) possess glucocorticoid and mineralocorticoid responses and to examine the potential role(s) of four key steroids in these responses. Pacific hagfish were injected with varying amounts of cortisol, corticosterone or 11-deoxycorticosterone (DOC) using coconut oil implants and plasma glucose and gill total-ATPase activity were monitored as indices of glucocorticoid and mineralocorticoid responses. Furthermore, we also monitored plasma glucose and 11-deoxycortisol (11-DOC) levels following exhaustive stress (30 minutes of agitation) or following repeated infusion with SO42-. There were no changes in gill total-ATPase following implantation with any steroid, with only very small statistical increases in plasma glucose noted in hagfish implanted with either DOC (at 20 and 200 mg kg-1 at 7 and 4 days post-injection, respectively) or corticosterone (at 100 mg kg-1 at 7 days post-injection). Following exhaustive stress, hagfish displayed a large and sustained increase in plasma glucose. Repeated infusion of SO42- into hagfish caused increases in both plasma glucose levels and SO42- excretion rate suggesting a regulated glucocorticoid and mineralocorticoid response. However, animals under either condition did not show any significant increases in plasma 11-DOC concentrations. Our results suggest that while there are active glucocorticoid and mineralocorticoid responses in hagfish, 11-DOC does not appear to be involved and the identity and primary function of the steroid in hagfish remains to be elucidated.

**1. Introduction**

There has long been debate on the true phylogenetic relationship between the two extant groups of cyclostomes, the lamprey and hagfish (Janvier, 2010). Past morphology-based approaches suggested paraphyly with the lamprey being more closely related to the jawed vertebrates, gnathostomes, than the hagfish (Janvier, 2010). Contrarily, current molecular evidence provides support for monophyly with both groups forming a single clade separating from the vertebrate lineage evolution (Heimberg et al., 2010) although, the strength of these assumptions have recently been questioned (Thomson et al., 2014). A monophyletic cyclostome clade would indicate that the ancestral vertebrates were more complex than originally thought with the lamprey retaining a number of features that are present in gnathostomes and the hagfish undergoing an unprecedented loss of vertebrate features. Alternatively, the lamprey would have had to develop key physiological traits in a convergent evolutionary context with the gnathostomes, but also possess the genetic features at divergence to allow for this parallel evolution, suggesting that hagfish and lamprey diverged early on following the emergence of the cylclostome clade around 525MYA (Near, 2009).

 Two examples of physiological and endocrinological processes that have either been lost, or appear more primitive in hagfish are ionoregulation and the hypothalamic-pituitary axis (HPA). When comparing ionoregulatory strategies of lamprey and hagfish, lamprey are euryhaline with blood ion concentrations similar to the rest of the vertebrate lineage at ~1/3 seawater (Smith, 1932), while hagfish are strictly marine in their evolutionary history (Bardack, 1991). Hagfish are particularly unique within the vertebrates as their blood plasma is similar to seawater with respect to Na+ and Cl- concentrations (Bellamy and Jones, 1961). Furthermore, hagfish are stenohaline and thus cannot regulate Na+ and Cl- when placed in media differing in salt composition from seawater (Robertson, 1954; Sardella et al., 2009). Hagfish do, however, regulate the divalent ions sulfate (SO42-), magnesium (Mg2+) and calcium (Ca2+), giving support to the hypothesis that these divalent cations were among the first ions to be actively regulated in vertebrates (Bellamy and Jones, 1961; Clifford et al., 2015a). It has been proposed that the early steroids and receptors used for this mineral regulation have been adopted for regulation of the monovalent ions (Na+, Cl-, K+; Sardella et al., 2009)

The emergence of the hypothalamic-pituitary axis (HPA) is specific to vertebrates and is a key event in the evolution of divergent physiological processes (*e.g.* reproduction, growth, metabolism, stress response and osmoregulation; Bury et al., 2015). Current evidence suggests that hagfish possess a far less morphologically distinct HPA compared to lamprey (Uchida et al., 2010) in that there is no apparent partitioning of the pars distalis and pars intermedia of the pituitary gland as observed for lamprey and all the gnathostomes (*e.g.* Nozaki et al., 2007). A number of the corticotropins, melanotropins and gonadotropins that are released from the pituitary to stimulate steroid hormones synthesis have been described in lamprey (Sower et al., 2006; Takahashi et al., 1995) but have yet to be definitively identified in hagfishes (Uchida et al., 2010).

The HPA controls the synthesis of a number of steroid hormones that circulate the body and bind to nuclear hormone receptors to act as ligand inducible transcription factors, examples of which are the sex [estrogen (ER), androgen (AR) and progesterone receptors (PR)] and adrenal hormone receptors [the two corticoid receptors (CR); glucocorticoid (GR) and mineralocorticoid receptors (MR)] of the gnathostomes. The origins of these groups of receptors can be traced back to the estrogen “like” receptor (ERR) present in some groups of Protosomes, e.g. the molluscs and annelids (Keay et al., 2006; Keay and Thornton, 2009). These early ERR receptors are constitutively active and their mode of regulation remains unclear (Keay et al., 2006; Keay and Thornton, 2009). In the cephalochordate *Branchiostoma floridae*, an ERR (Paris et al., 2008), ER and steroid receptor (SR) have been reported (Bertrand et al., 2011). The SR is expressed in the gonads (Callard et al., 2011) and is activated by estrogens (Katsu et al., 2013), and negatively regulated by the ER (Callard et al., 2011), this suggests that the ancestral role of the SR may be to regulate reproduction.

 Whole genome duplication (WGD) events in the early vertebrate lineage has lead to the emergence of 3 distinct hormone receptors the CR, ER and PR in cyclostomes (Bridgham et al., 2006; Rossier et al., 2015). The cloning of these receptors and *in vitro* assays has identified the receptors hormone binding and transactivation profiles. The CRs of lamprey and Atlantic hagfish are promiscuous, being activated by a number of different corticoids including cortisol, 11-deoxycorticosterone (DOC), 11-deoxycortisol (11-DOC) and corticosterone (Bridgham et al., 2006). However, even though these *in vitro* assays have proven invaluable in identifying potential mechanisms of protein evolution at a molecular level (Bridgham et al., 2009) they do not provide information on the actual active hormones *in vivo* or the evolution of their physiological role (Close et al., 2010). For example, even though the lamprey CR is activated by a wide range of corticoids *in vitro,* Close et al. (2010), recently identified that only 11-DOC is elevated in the plasma of lamprey and that this hormone performs both a classical GR role, controlling gluconeogenesis, and a MR role, regulating ion homeostasis.

The clear divergence in ionoregulatory strategies between these two agnathan taxa and the knowledge that steroid hormones regulate ion balance in lamprey and more derived vertebrates provides the impetus to reevaluate steroid function in hagfish. In this study, we aim to identify the presence of classical corticosteroid hormone responses in the Pacific hagfish (*Eptatretus stoutii*) through either *in vivo* perturbations (handling or elevation of plasma sulfate *via* injection) or *via* hormonal implants followed by monitoring of classical glucocortoicoid (plasma glucose) and mineralocorticoid (gill ATPase activity and plasma sulfate regulation) responses. We utilized administration of cortisol, DOC, 11-DOC and corticosterone via coconut oil implants in order to investigate their potential to either directly cause a corticosteroid response or induce a response following steroidal biotransformation. Plasma 11-DOC levels were measured following *in vivo* perturbations. Furthermore, analysis of a hagfish transcriptome allowed us to identify the presence of specific enzymatic elements of the corticosteroid biosynthesis pathway.

**2. Materials and Methods**

*2.1 Experimental animals and holding*

Pacific hagfish (*Eptatretus stoutii;* 65-227 g) were captured near Bamfield, BC, Canada and held at Bamfield Marine Sciences Centre (BMSC) as previously described (Clifford et al., 2015b; Schultz et al., 2014). Fish remained unfed during captivity and were used for experimentation within 2 weeks of capture. All animals were used under the licenses of Department of Fisheries and Ocean Canada collection permits XR 214 2007, XR 214 2010, XR 214 2011 and XR 214 2013 and Bamfield Marine Science Centre Animal Care protocol numbers BMSC RS 10-42, RS 11-26, and RS-13-24.

*2.2 Chemicals*

Unless noted, reagents and enzymes were supplied by Sigma-Aldrich (St. Louis, MO). Cortisol, 11-DOC, DOC, and corticosterone were obtained from commercial suppliers (Stereloids, USA). Coconut oil was purchased from a local health food store. Tricaine methanesulfonate (TMS) was obtained from Syndel laboratories (Nanaimo, BC, Canada).

*2.3 Experiment 1* - *Exogenous elevation of plasma hormone concentrations.*

To induce elevated plasma hormone concentrations, hagfish were administered molten coconut oil (kept at 27 ˚C prior to injection) impregnated with cortisol, corticosterone or DOC at dose of 20, 100 or 200 mg kg-1 hagfish. Briefly, animals were lightly anaesthetized in seawater containing TMS (0.75 g L-1) buffered with 1.5 g L-1 sodium bicarbonate for 3-5 min. The animals were then removed from the water and were held vertically causing pooling of blood in the caudal subcutaneous sinus within 20 sec. A 200µL control blood sample was removed from the sinus with a heparinized 21G needle and 1mL disposable syringe, centrifuged briefly (30 sec, 14,000*g*) and the plasma was removed and rapidly frozen on liquid nitrogen (-80 ˚C) for later analysis. The animals were then laid on a flat surface and steroidal implants were placed in the body cavity approximately 3 inches caudal to the last branchial pore using a warm (27 ˚C) 18 g needle and 3 mL syringe. All implant concentrations were administered at a dose equivalent to 2% of body mass. Coconut oil injections without steroid served as a control for all experiments. Blood samples were also removed 4 and 7 days post-implantation. Confirmation of successful placement of the implant was evaluated visually upon termination and dissection. Plasma cortisol and glucose concentrations were measured 4 and 7-days post-implantation, whereas total gill ATPase activities were only measured 7-days post-implantation and these results have been reported elsewhere (Bury et al., 2015). To confirm the efficacy of the implant at achieving the desired nominal concentrations, we used a commercial cortisol RIA (MP Biomedical, Orangeburg, NY) as a surrogate for the efficacy of the DOC and corticosterone injections (see results Figure 2).

*2.4 Experiment 2 – Effects of handling stress on hagfish plasma glucose and 11-DOC levels.*

In order to determine the effects of handling stress on potential candidates of hagfish stress hormones, hagfish were subjected to an extended handling stress. Hagfish (*n* = 6) were acclimated in a darkened, aerated box with flowing ambient seawater overnight (>10 h) prior to any experimentation. Hagfish were then lightly anaesthetized (0.75 g L-1 TMS) in the dark without handling to allow for control blood samples (200 µL) to be taken. Following recovery from anesthesia (~ 1 h), hagfish were agitated by continual manual grasping for 30 min. They were then allowed to recover for 0.5, 1, 3, 6, 12, 24 and 48 h and blood samples collected as described above. Blood was centrifuged and then the plasma was snap frozen in liquid nitrogen (-80 ˚C) for later determination of plasma glucose and 11-DOC concentrations.

*2.5 Experiment 3 - Effect of sulfate loading.*

Sulfate is one of the three divalent ions that hagfish regulate (Bellamy and Jones, 1961) and we, therefore, hypothesized that a mineral stress may elicit a measurable hormonal response. Specifically, we sought to characterize the effects of sulfate loading on hagfish plasma glucose and 11-DOC levels. We also characterized recovery from sulfate loading by examining both glomerular filtration rate and sulfate excretion rate following sulfate plasma loading.

Hagfish were anaesthetized and individually weighed (range 115-192 g, *n* = 6 for each treatment). To chronically elevate plasma SO42-, hagfish were administered a daily load of 2 µL g-1 of a stock 200 mmol L-1 NaSO42- /300 mmol L-1 NaCl solution (400 µmol SO4- kg-1 body mass) for 3 days. Assuming a blood volume of 18% (Forster, 1989; McCarthy and Conte, 1966), this dose was designed to elevate plasma SO42- by a nominal amount of ~3 mmol L-1 injection-1. Controls consisted of an equivalent injected volume (2 µL g-1) of 500 mmol L-1 NaCl. On day 4, both sulfate-loaded and saline-loaded hagfish were then injected with 200 µL of either 200 mmol L-1 NaSO42- /300 mmol L-1 NaCl or 200 µL of 500 mmol L-1 NaCl solution as appropriate. Each solution on day 4 also contained 5 µCi of radiolabelled sulfate (35SO42-: Perkin Elmer as Na35SO42-: 3000 µCi mmol-1) and 1 µCi of 3H- inulin as a GFR marker Perkin Elmer as 3H-inulin: 3000 µCi mmol-1; Munger et al., 1991). Given the high specific activity of the injected 35SO42-, this would only amount to a nominal dose (~0.0017 mmol) of SO42- in the control hagfish and not be expected to increase plasma [SO42-]. Hagfish were then placed into individual flux chambers and 3H-inulin and 35SO42- activity allowed to equilibrate in the plasma for 6 h. Preliminary experiments determined that plasma concentrations were fully mixed and stable in the 6-12 h post-injection period. At the start of the experimental period, blood samples were withdrawn from lightly anesthetized hagfish at 6 h post-radiolabeled sulfate/inulin injection. To determine specific activity (SA) of plasma, 25 µl (0.025 mL) of plasma was added to 4 mL of ACS scintillation fluid (Fisher chemical) and 3H and 35S radioactivity determined using a Beckman LS-6000 with appropriate energy windows used for counting of each isotope independently. The remaining plasma was then snap frozen in liquid nitrogen (-80 ˚C) for later analysis of plasma sulfate, glucose and 11-DOC. The flux chambers were weighed at the beginning and end of the flux period to determine the flux volume (minus the weight of the fish). To begin the flux period, water samples (4 mL) were collected from the flux chamber (T1) and each flux was terminated after 2 h (8 h post injection) by withdrawing final water samples (T2). The T1 and T2 water samples were mixed with 8 mL of ACS fluor and 3H and 35S radioactivity measured using a Beckman LS-6000 beta counter. At the completion of each flux period, plasma samples were collected from the hagfish and analyzed as described above.

*2.6 Calculation of GFR and sulfate excretion rate*

Glomerular filtration rate was calculated based on 3H excretion rate using the following equation:

$GFR=[(CPM\_{water}^{T\_{2}}-CPM\_{water}^{T\_{1}})∙Weight^{-1}∙Time^{-1}]∙Volume\_{Flux}∙SA$ (1)

where specific activity (SA) was calculated as:

$SA=\frac{\left(\frac{volume}{CPM\_{plasma}^{T\_{2}}}+\frac{volume}{CPM\_{plasma}^{T\_{1}}}\right)}{2}$ (2)

Sulfate excretion rate in both saline loaded (control) and sulfate-loaded animals was calculated according to the following equation:

$J\_{SO\_{4}^{2-}}=[(CPM\_{water}^{T\_{2}}-CPM\_{water}^{T\_{1}})∙Weight^{-1}∙Time^{-1}]∙Volume\_{Flux}∙SA$ (3)

 where SA was calculated as:

$SA=\frac{\left(\frac{µmol}{CPM\_{plasma}^{T\_{2}}}+\frac{µmol}{CPM\_{plasma}^{T\_{1}}}\right)}{2}$ (4)

and plasma [SO42-] (mol mL-1) was measured at 600 nm on a microplate spectrophotometer (Spectramax 190 Molecular Devices, Sunnyvale, CA) using a commercially available assay kit (Quantichrom Sulfate Assay Kit, DSFT-200, BioAssay Systems, Hayward, CA).

*2.7 Plasma sample analysis*

Plasma glucose concentrations were measured at 340nm using a microplate spectrophotometer (Spectramax 190 Molecular Devices, Sunnyvale, CA) with a hexokinase assay utilizing glucose-6-phosphate as a coupling enzyme (Bergmeyer, 1983).

Plasma cortisol concentrations were measured using a commercially available RIA (MP Biomedical, Orangeburg, NY). Cross-reactivity from the manufacturer is listed as cortisol 100%; prednisolone 45.6%, 11-desoxycortisol 12.3, corticosterone 5.5%, prednisone, 2.7, cortisone, 2.1, 11-alpha-hydroprogesterone 1, progesterone, 0.25, dexamethasone <0.1, testosterone < 0.1. Tests for parallelism using control unstripped plasma found ~98% recovery of exogenously added cortisol with ~6.8% interassay variation. Plasma 11-DOC concentrations were analyzed with commercially available RIA kits (11-DOC RIA, 38-DESHU-R96, Alpco Diagnostics, Salem, NH; 11-DOC RIA, KIPI20000, DIAsource Immunoassays S.A., Louvain-la-Nueve. Belgium). Cross-reactivity from the manufacturer is listed as desoxycortisol 100%, 17-alpha-hydroprogesterone 5.6%, desoxycorticosterone 0.46%, progesterone, 0.59, cortisol 0.09%, estradiol-17-beta 0.03%. Recovery was 82-96% and interassay variation < 15%.

*2.8 Production and Analysis of hagfish transcriptome.*

A combined hagfish gill/slime gland transcriptome was commercially produced by BGI (Shenzhen, China) with tissues excised from control animals and animals exposed to stress by acid or base injection, or stress *via* handling as above(*n*= 3 for each condition). For the acid and base stress, hagfish were lightly anaesthetized in seawater containing 0.75 g l-1 TMS buffered with 1.5 g L-1 sodium bicarbonate for 3-5 minutes, weighed and then held vertically causing pooling of the blood in the caudal subcutaneous sinus. The animals were then injected (3 mL syringe and 23g needle) with either acid saline (250 mmol L-1 HCl [pH=0.60], 250 mmol L-1 NaCl) or alkaline saline (250 mmol L-1 NaHCO3, 250 mmol L-1 NaCl, [pH~8.43]) at a standard volume of 24 µL g-1 to induce an acid/alkaline/saline load of 6000 µmol kg-1; similar to injection protocols that have been employed in previous studies (Clifford et al., 2014; McDonald et al., 1991; Parks et al., 2007; Tresguerres et al., 2007). Following a 3 h recovery period, animals were then euthanized with an overdose of TMS (2 g L-1) buffered with 1.5 g L-1 sodium bicarbonate and the gills and slime glands were rapidly excised and freeze-clamped in liquid nitrogen (-80 ˚C, within 30 s). Total RNA was obtained from tissues (~100 mg) using a TRIzol extraction protocol. RNA samples were then cleaned of genomic contents using DNAse I (Ambion/Life technologies, Carlsbad, CA) after which, isolated RNA from the 24 samples (12 gill +12 slime gland) were mixed into one sample to specifications set forth by BGI. With this combined sample, a transcriptome was generated using Illumina technologies by BGI. Transcriptome annotation was performed by BGI via BLAST cross-referencing with NCBI non-redundant, Swiss-Prot, Kyoto Encyclopedia of Gene and Genome (KEGG) and COG (Clusters of Orthologous Groups) databases. We utilized KEGG pathway analysis provided by BGI to determine the presence of transcripts for specific enzymes involved in steroid biosynthesis.

*2.9 Statistical analysis*

 All data are presented as the mean ± SEM. Differences in plasma concentrations of glucose, sulfate and 11-DOC were analyzed using one-way analysis of variance (ANOVA). When significant differences were observed, differences between the means were quantified using a Dunnet’s post-hoc test at the *p* < 0.05 level. When the assumptions for ANOVA were not satisfied, a nonparametric ANOVA, followed by Dunn’s test by ranks was used where appropriate. All statistical analyses were completed using Prism 6 for Mac (Graphpad Software Inc, La Jolla, CA).

**3. Results**

*3.1 Identified elements of Hagfish corticosteroid biosynthesis pathway*

Analysis of a hagfish gill/slime gland transcriptome revealed the presence of homologous enzymes required to transform cholesterol sulfate to progesterone including steryl sulfatase/alcohol sulfotransferase, CYP11A1, and 3-HSD. However, transcripts for important enzymes necessary for the synthesis of 17-OH-progesterone, androstenedione and testosterone, CYP17; DOC, CYP21, cortisol CYP11B1 or estradiol CYP19 were not found in our transcriptome.

*3.2 Efficacy of steroid delivery via coconut oil implants*

We used cortisol implants and a cortisol RIA to determine the efficacy of the coconut oil implants at delivering the nominal doses of each steroid; assuming both equivalency of release and equivalency of degradation for each of the steroids from the coconut oil implant. To confirm that the RIA could detect cortisol in hagfish plasma, we initially conducted an RIA using hagfish control plasma spiked with varying concentrations of steroid to determine recovery and interassay variation (see methods). We acknowledge that we do not know the rates of adsorbtion, uptake, metabolism or excretion for each of these steroids and this could potentially alter our interpretations in the unlikely scenario whereby the rates of degradation exceed the rate of steroid release from the implant. However, as a first approximation, we believe our assumption that each steroid will be significantly elevated from normal unperturbed values is valid.

Plasma cortisol concentrations were elevated in fish that received the coconut oil implanted with cortisol, but were unaffected in those that received the sham injection or an implant with either DOC or corticosterone (Figure 2). Moreover, the target concentrations in the cortisol treated animals 4 days after injection were ~10, 100 and 150 µg dL-1 for the nominal 20, 100 and 200 dose mg kg-1 groups, respectively, demonstrating that the implants were relatively effective at delivering their intended dose.

*3.3 Effects of steroidal implants*

As published previously (Table 1, adapted from Bury et al. 2015), a significant increase in plasma glucose levels from 0.74 ± 0.17 mmol L-1 (control) to 1.88 ± 0.45 mmol L-1 was observed in hagfish 4 days post-implantation with 200 mg kg-1 DOC (table 1). There were also small but statistically significant increases at day 7 for the 20 mg kg-1 DOC (0.70 ± 0.09 mmol l-1, n=6) and 100 mg kg-1 corticosterone (0.84 ± 0.13 mmol l-1) implanted hagfish compared to hormone-free implanted controls (0.28 ± 0.07 mmol l-1 values. We did not detect any changes in total gill-ATPase activity 7 days post-implantation with any of the tested hormone implants at any dosage (table 1).

*3.4 Effects of handling stress on hagfish physiology*

Thirty minutes of continuous handling stress induced a significantly gluconeogenic response in the hagfish. At the 6 h post-handling stress plasma glucose levels significant increased from 0.27 ± 0.16 mmol L-1 (control) to 1.65 ± 0.46 mmol L-1 (Figure 3a). Plasma glucose levels were reduced by 12 h (1.59 ± 0.44 mmol L-1) and returned to control levels at 24 h post-handling (0.96 ± 0.36 mmol L-1). Hagfish plasma 11-DOC levels were also measured post-handling stress. Under control conditions, 11-DOC concentrations were extremely low, near the detection limit for the assay (from. 0.19 ± 0.004 ng mL-1 during control conditions to0.22 ± 0.004 at 30 minute post-handling (Figure 3b) and remaining unchanged from control values for the remainder of the 48 h recovery period (Figure 3b,c).

*3.5 Effects of sulfate loading on hagfish*

To test for the presence of a glucogenic response as a result of divalent ion plasma loading, we acutely injected 2 µL g-1 body mass of a 0.2 M NaSO4 solution, which was a dosage designed to elevate plasma SO42- by ~ 3 mmol L-1 over the resting plasma value of ~2-3 mmol L-1. However, acute elevation of plasma SO42- did not result in any increase in plasma glucose at either 6 or 8 h post-injection (data not shown).

Plasma SO42- levels in hagfish following chronic (daily injection for 4 days) treatment of NaCl did not deviate from the pre-injected controls (4.01± 0.47 mmol L-1), whereas, plasma SO42- levels significantly increased greater than two-fold in chronically SO42--loaded hagfish at both 6 and 8 h post-injection (9.65 ± 1.38 mmol L-1 and 9.25 ± 1.11 mmol L-1, respectively; Figure 4a).

Plasma glucose levels in NaCl-injected hagfish (control), remained stable at 6 and 8 h post-4th injection (1.71 ± 0.35 mmol L-1 and 2.04 ± 0.58 mmol L-1, respectively) compared to control levels (1.09 ± 0.19 mmol L-1,Figure 4b). In contrast, chronic-injection of NaSO4 resulted in a significant 3 to 5-fold increase in hagfish plasma glucose levels at both 6 h (3.16 ± 0.19 mmol L-1) and 8h post-injection (5.08 ± 1.12 mmol L-1; Figure 4b).

To investigate if 11-DOC played a role in the hagfish gluconeogenic stress response, we also measured plasma 11-DOC concentrations after sulfate loading. Interestingly, no changes in plasma 11-DOC were detected and hagfish plasma 11-DOC levels were approximately an order of magnitude lower than reported in Figure 3b and were at or below detectable limits of the assay (Figure 4c).

We calculated the SO42- excretion rates for both chronic NaCl and SO42- loaded hagfish and determined that SO42- loading resulted in a significantly greater SO42- excretion rate (1.29 ± 0.39 µmol kg-1 hr-1) compared to NaCl injected controls (0.29 ± 0.13 µmol kg-1 hr-1; Figure 5a). To ensure that the increased SO42- excretion rate was not associated with volume loading and a subsequent increase in glomerular filtration rate (GFR), 3H-inulin excretion rate was used as an indicator of GFR. Hagfish GFR was similar in chronic NaCl injected animals (0.15 ± 0.03 mL kg-1 hr-1) compared to the SO42- injected animals (0.14 ± 0.05 mL kg-1 hr-1; Figure 5b) demonstrating that no changes in GFR occurred during the SO42- loading experiments.

**4. Discussion:**

Our findings demonstrate that Pacific hagfish are able to generate both a glucocorticoid response (as demonstrated by an increased plasma glucose following hagfish handling and mineral/SO42- loading) and a mineralocorticoid response (as demonstrated by increased active secretion of SO42- following SO42- loading). These responses are not mediated by either cortisol or corticosterone, as implants failed to elevate these responses above basal values while DOC did illicit small but statistically significant increases in plasma glucose at both 4 days (200 mg kg-1) and 7 days (20 mg kg-1). However, ATPase activity remained unchanged regardless of steroidal implant type, dose or time. 11-DOC levels remained near or below detection limits following either exhaustive handling stress or chronic sulfate loading, suggesting 11-DOC, thought to be the active steroid in lamprey (Close et al., 2010), is not active in Pacific hagfish. While there have been numerous studies examining the steroidogenesis of the sex steroids (*e.g.* Kime and Hews, 1980; Kime et al., 1980; Nozaki et al., 2007; Weisbart et al., 1980), to our knowledge, this is the first study to demonstrate that both glucogenic and mineralocorticoid responses are present in hagfish and to further investigate the steroids responsible for eliciting of these physiological responses.

Weisbart an Idler (1970), using RIA suggested that cortisol in plasma of Pacific hagfish was near or below the detectability limit of their assay. We were also not able detect cortisol in our assay of control Pacific hagfish plasma as levels were below the limits of detection of our commercial RIA kit. This suggests that the cortisol is also not a candidate for hagfish steroidal control of either mineralocorticoid or glucocorticold responses. Our injection of cortisol did provide validation of our injection and dosing technique by a demonstrated elevation of plasma cortisol to values close to our nominal target dose. However, since the elevation in plasma cortisol did not increase either plasma glucose (at either 4 or 7 d post-injection) or gill ATPase activity (at 7 d post-injection), the absence of a steroidal role for cortisol in either response was confirmed. This lack of effect of cortisol then allows cortisol to be used as a tracer for the efficacy of the implant protocol for other steroids since cortisol is readily measured by RIA. Our method demonstrated that for cortisol, we achieved ~ 100 ng mL-1 plasma cortisol at 4 days post-implantation for the 100 mg kg-1 nominal group and ~ 150 ng mL-1 for the 200 mg kg-1 nominal group. We are therefore confident that hagfish were likely similarly dosed with either corticosterone or DOC.

Transcriptome analysis revealed the presence of the key steroidogenic enzymes CYP11A1 and 3β- HSD necessary for the conversion of cholesterol to progestoreone. Other studies have reported the presence of the sex steroids in the plasma of hagfish and thus, the enzymes CYP17 and 17β-HSD and CYP19 must be present in hagfishes (Nishiyama et al., 2013). However, we were unable to identify the enzymes (CYP21 and CYP11B1) necessary for the synthesis of 11-DOC and conversion of 11-DOC to cortisol, respectively. An absence of these enzymes would explain our findings of the inability of Pacific hagfish to convert steroid precursors to cortisol even with long-term (4-7 days) supraphysiological doses in the plasma.

Idler and Burton (1976) identified “presumptive interrenal” cells in the pronephron of the Atlantic hagfish (*M. glutinosa*) and presumed them analogous to the cortisol synthesizing tissues of the teleosteii, however, the capability of synthesizing cortisol in these tissues has never been definitely addressed and therefore the location of primary corticosteroid tissue(s) in hagfishes remains to be determined. Supporting our hypothesis that hagfish lack key biosynthesis enzymes are our findings that implants of corticosterone, 11-dexoxycorticosterone or cortisol failed to induce consistent and dose responsive changes in plasma glucose or total ATPase activity. Elevations of plasma glucose and ATPase activity are common indicators of glucocorticoid or mineralocorticoid responses (Close et al., 2010). Implants of 11-dexoxycorticosterone did result in small but inconsistent increases in plasma glucose at 20 and 200 ng L-1 on day 7 and day 4, respectively, while corticosterone was associated with elevated plasma glucose only on day 4 at the 100 ng L-1 dose. It is possible that Na+/K+ ATPase (NKA) activity itself was indeed altered by any or all of the steroidal implants. However, given that hagfish NKA is completely refractory to inhibition by the common NKA inhibitor Ouabain (up to 500 mM), we could only measure total ATPase activity rather NKA activity. Thus, our ability to detect NKA specific ATPase activity resulting from SO42- loading was impaired and we cannot conclusively eliminate any of these steroids from mediating a mineralocorticoid effect.The reasons for the lack of ouabain inhibition are unknown but similar reduced sensitivities have been observed in the goldfish brain (Chasiotis and Kelly, 2008).

In lampreys, the other extant vertebrate in the agnathan lineage, cortisol is not detectable in plasma and thus not considered to be a functional steroid. In lamprey, 11-DOC has been demonstrated to be the active steroid for both mineralocorticoid and glucocorticoid responses (Close et al., 2010). Progesterone has been demonstrated to be present in hagfish plasma (Nishiyama et al., 2013) and it has been suggested that 11-DOC can be produced at very low conversion efficiency by bathing hagfish ovarian tissue with pregnenolone (Hirose et al., 1975), suggesting the presence of CYP21 activity in the ovary. But, circulatory 11-DOC concentrations (Figure 3b,c) are exceptionally low and are arguably at levels that are unlikely to be biologically active.

We were also able to identify the presence of necessary enzymes (sterol sulfate and cholesterol monoxygenase and 3β hydroxyl delta 5 steroid dehydrogenase) to allow for conversion of cholesterol sulfate to pregnenolone and progesterone. However, despite extensive efforts, we were unable to identify the necessary biosynthesis enzymes (cyp21a, cyp17a) required for conversion of progesterone or pregnenolone to downstream steroids (see Fig 1). In *M. glutinosa*, progesterone was able to be converted to testosterone at a nominal rate (3% conversion efficiency; Kime et al., 1980) suggesting cyp17a is present in *Myxin*e species, however, further conversions requiring cyp 21 did not occur. Bearing in mind that our transcriptomes were obtained from gill and slime gland tissues, it is possible that our transcriptome does not contain transcripts for Cyp21a and cyp17a since we have not identified the tissues responsible for glucocorticoid and mineralocorticoid regulation. Steroidogenic tissues responsible for sex steroid metabolism have been identified in the gonads (Nozaki and Sower, 2015) but corticosteroidogenic tissues have not, as yet, been identified in hagfishes.

Elevations in plasma glucose are indicative of a glucogenic stress response, typically resulting from activation of GR receptors. We have demonstrated that hagfish can indeed generate a strong glucogenic response to either handling stress or repeated SO42- injections. Hagfish possess only single CR that has been demonstrated to bind DOC, cortisol, 11-DOC and corticosterone by stimulate reporter activity (at 100 nmol L‑1) in a heterologous expression system (Bridgeham et al, 2006). However, assuming our measured cortisol concentrations yield similar dosing for other steroidal implants, our plasma steroids would range from ~300 nmol L-1 in the 20 mg kg-1 dose to ~4500 nmol L-1 in the 200 mg kg-1 dose. Since we only measured small increases in plasma glucose following implant with 200 mg kg-1 DOC or 100 mg kg-1 corticosterone, even at the highest dose, this suggests that either DOC or corticosterone are not the endogenous glucocorticoids.

Hagfish are considered ionoconformers for plasma Na+ and Cl- (Smith, 1930). However, they are capable of regulating the divalent ions Ca2+, Mg2+ and SO42-. We elicited an ionoregulatory challenge through injection of NaSO4 to elevate plasma [SO42-]. This allows both the use of radiotracers (35S) and also avoids the known physiological impairments that would result from manipulation of either plasma Ca2+ or Mg2+. We demonstrate that hagfish are clearly able to increase SO42- excretion rates in response long-term elevations in plasma [SO42-]. This was accomplished without concomitant increases in GFR suggesting upregulation of active secretion mechanisms. This can be considered evidence of an active mineralocorticoid response. Slc26a1 is a known SO42- transporter in teleost fish (Katoh et al., 2006). We identified a slc26a1-like homologue in our hagfish transcriptome but were unable to demonstrate any changes in expression in kidney tissue response to plasma SO42- elevation (results not shown) so the molecular mechanism for sulfate excretion remains to be identified. This is the first measurement of whole animal GFR in hagfishes although previous estimates of single nephron GFR have been made (Riegel, 1978). Interestingly, GFR in hagfishes is relatively high (~0.150 mL kg-1 h-1) approx. ½ of that measured in more derived vertebrates) which agrees with a previously measured urine flow rate of 0.227 mL kg-1 h-1 in *M. glutinosa* (Morris, 1965) suggesting little resorpative capacity of the hagfish kidney.

**5. Conclusions**

In summary, we show that hagfish are able to mount plasma glucogenic responses and increased mineral excretory capacity in response to specific stimuli. However, this study rules out cortisol, DOC, 11-deoxycortisol and corticosterone as candidate endogenous CR ligands. The steroid responsible for eliciting these responses remains elusive and bears further investigation. Finally, given the relatively conserved nature of steroids in evolutionary history, understanding hagfish stress endocrinology will be important in resolving the relationships within the agnathans (hagfishes and lampreys) and also between the agnathans and the other clades in the vertebrate lineage.

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**Figure legends:**

**Figure 1.** Proposed steroid synthesis pathway in hagfish, *Eptatretus stoutii.* Next-Gen Illumina sequencing of hagfish gill and slime gland tissues followed by KEGG analysis revealed complete elements of the steroid biosynthesis pathways leading to pregnenolone and progesterone. Sequence information for several other important enzymes necessary for steroid biosynthesis (highlighted in grey) were not detected in the hagfish gill and slime gland transcriptome.

**Figure 2.** Plasma cortisol concentrations (µg dL-1) measured in hagfish 4 and 7 days post-implantation with coconut oil (control), cortisol, 11-deoxycorticosterone (DOC), and corticosterone. Cortisol, DOC, or corticosterone were dissolved in warm coconut oil and injected into hagfish to achieve nominal plasma concentrations of 0 (control), 20, 100 and 200 mgkg-1. Measurement of cortisol at 4 and 7 days post-implantation were used to confirm loading rates. Data is reported as mean ± SEM (*n*). Unless otherwise noted *n* = 6.

**Figure 3.** Hagfish plasma glucose concentrations (mmol l-1; a) and 11-deoxycortisol (11-DOC) concentrations (ng mL-1) pre- (0 h control) and 6, 12, 24, and 48 h post-desliming stress (b) and pre- (0 h control) and 0.5, 1, 3 and 6 h (c) post-desliming stress. Data is reported as mean ± SEM (*n* = 6). Asterisk (\*) denotes significant difference compared to control (*p* ≤ 0.05, ANOVA).

**Figure 4.** Plasma sulfate concentrations (mmol l-1; a), glucose concentrations (mmol l-1; b) and 11-DOC concentrations (ng mL-1; c) in non-injected hagfish (white bars; control), and hagfish 6 and 8 h post-injection with NaCl (grey bars) or NaCl/NaSO4 (black bars). Hagfish were injected daily for 3 days with 0.5 M NaCl (3000 µmol kg–1) or 0.5 M NaSO4 (3000 µmol kg–1) and were then injected with 0.5 M NaCl (3000 µmol kg–1) or 0.5 M NaCl/NaSO4 (6000 µmol kg–1) on day 4. Data is reported as mean ± SEM (*n* = 6 unless otherwise noted above bars). In (a) and (b), an asterisk (\*) denotes a significant difference between groups (*p* ≤ 0.05, ANOVA), and a hash tag (#) denotes a significant difference compared to control (*p* ≤ 0.05, ANOVA). Note, in (c) plasma 11-DOC concentrations were not measured in control hagfish samples.

**Figure 5.** Sulfate excretion rates (µmol kg–1 h-1) and glomerular filtration rates (mL kg–1 h-1) of hagfish 8 h post-injection with 0.5 M NaCl (white bars) or 0.5 M NaSO4 (black bars). Data is reported as mean ± SEM (*n* = 6 unless otherwise noted above bars). Different lower case letters denotes a significant difference between groups (*p* ≤ 0.05, ANOVA).

**Table 1:** Hagfish plasma glucose concentrations (mmol l-1) at 4 and 7 days post-implantation and total gill ATPase activity (µmol mg protein-1 hr-1) at 7 days post-implantation with coconut oil (control) or coconut oil infused with steroid (cortisol, DOC, or corticosterone) at a dose of 20, 100, and 200 mg kg-1. Data is reported as mean ± SEM (*n* = 6 unless otherwise noted). Asterisk (\*) denotes significant difference compared to control (*p* ≤ 0.05, ANOVA).

|  |  |  |  |
| --- | --- | --- | --- |
| Injected steroid | Dose(mg kg-1) | Plasma Glucose (mmol L-1) | Total gill-ATPase activity(µmol ADP mg protein-1 h-1) |
| Day 4 | Day 7 | Day 7 |
| Control | - | 0.74 ± 0.17 | 0.28 ± 0.07 | 10.44 ± 1.40 |
| Cortisol | 20 | 0.93 ± 0.16 | 0.51 ± 0.12 | 7.87 ± 0.81 |
| 100 | 0.64 ± 0.10 | 0.64 ± 0.08 | 7.81 ± 0.79 |
| 200 | 0.64 ± 0.08 | 0.60 ± 0.09 | 7.84 ± 0.40 |
| DOC | 20 | 0.83 ± 0.12 (5) | 0.70 ± 0.09**\*** | 11.45 ± 1.59 |
| 100 | 0.95 ± 0.45 | 0.63 ± 0.08 | 9.15 ± 1.09 (5) |
| 200 | 1.88 ± 0.64**\*** | 0.48 ± 0.12 (5) | 10.45 ± 1.94 (5) |
| Corticosterone | 20 | 0.52 ± 0.08 | 0.64 ± 0.12 (4) | 9.70 ± 1.46 (4) |
| 100 | 0.86 ± 0.16 | 0.84 ± 0.13**\*** | 10.64 ± 0.79 |
| 200 | 0.52 ± 0.09 | 0.60 ± 0.05 | 10.85 ± 1.56 (5) |

*Adapted from Bury et al. 2015*