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RESEARCH ARTICLE

11-Deoxycortisol is a stress responsive and gluconeogenic hormone in a jawless vertebrate, the sea lamprey (*Petromyzon marinus*)

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ABSTRACT

Although corticosteroid-mediated hepatic gluconeogenic activity in response to stress has been extensively studied in fishes and other vertebrates, there is little information on the stress response in basal vertebrates. In sea lamprey (*Petromyzon marinus*), a representative member of the most basal extant vertebrate group Agnatha, 11-deoxycortisol and deoxycorticosterone are the major circulating corticosteroids. The present study examined changes in circulating glucose and 11-deoxycortisol concentrations in response to a physical stressor. Furthermore, the gluconeogenic actions of 11-deoxycortisol and deoxycorticosterone were examined. Within 6 h of exposure of larval and juvenile sea lamprey to an acute handling stress, plasma 11-deoxycortisol levels increased 15- and 6-fold, respectively, and plasma glucose increased 3- and 4-fold, respectively. Radiometric receptor binding studies revealed that a corticosteroid receptor (CR) is present in the liver at lower abundance than in other tissues (gill and anterior intestine) and that the binding affinity of the liver CR was similar for 11-deoxycortisol and deoxycorticosterone. Transcriptional tissue profiles indicate a wide distribution of *cr* transcription, kidney-specific transcription of steroidogenic acute regulatory protein (*star*) and liver-specific transcription of phosphoenolpyruvate carboxykinase (*pepck*). *Ex vivo* incubation of liver tissue with 11-deoxycortisol resulted in dose-dependent increases in *pepck* mRNA levels. Finally, intraperitoneal administration of 11-deoxycortisol and deoxycorticosterone demonstrated that only 11-deoxycortisol resulted in an increase in plasma glucose. Together, these results provide the first direct evidence for the gluconeogenic activity of 11-deoxycortisol in an agnathan, indicating that corticosteroid regulation of plasma glucose is a basal trait among vertebrates.

KEY WORDS: Corticosteroid, Agnatha, Steroidogenesis, Evolution

INTRODUCTION

Lampreys (Petromyzontiformes) are a basal order of vertebrates within the superclass of jawless vertebrates, Agnatha, that includes the extant representatives hagfishes and lampreys. The sea lamprey (*Petromyzon marinus*) is an anadromous fish (exhibiting freshwater and seawater life stages) with a native geographic range broadly distributed along eastern and western coasts of the Atlantic Ocean, including recently established populations in the Great Lakes of North America. As an extant representative of the basal vertebrates, the sea lamprey is an excellent model organism to gain insight into the evolution of physiological and endocrine traits in vertebrates. The present study aimed to investigate aspects of lamprey corticosteroid regulation and gluconeogenic action during stress.

The mobilization of catecholamines, corticosteroids and glucose in response to stress is a fundamental and classically described component of a 'fight or flight' response to stress in vertebrates (Cannon, 1929; Romero and Butler, 2007). In mammals, the catecholamines adrenaline (epinephrine) and noradrenaline (norepinephrine) are produced in the adrenal medulla after detection of a 'stressor', defined as an external stimulus that promotes a stress response, and rapidly released into the circulation. Corticosteroid biosynthesis and release in the adrenal cortex involve the serial enzymatic conversion of cholesterol, beginning with the rate-limiting transfer of cholesterol within the mitochondria by the steroidogenic acute regulatory protein (StAR) and the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme (CYP11A1) (Stocco, 2001). Glucose can be produced in the liver either by the breakdown of glycogen stores (glycogenolysis) or by the synthesis of glucose from lactate, amino acids and glycerol (gluconeogenesis) (Polakof et al., 2012). Glycogenolysis is the enzymatic conversion of glycogen to glucose by phosphatases (including glucose 6-phosphatase, G6Pase). Gluconeogenesis occurs through the enzymatic metabolism of non-carbohydrate substrates to produce glucose (see review by Suarez and Mommsen, 1987). Of the enzymatic steps of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) is responsible for irreversibly catalyzing the first rate-limiting step, the decarboxylation of oxaloacetate to phosphoenolpyruvate and carbon dioxide. Although gluconeogenesis can occur in a variety of tissues in fishes, activity measurements of key enzymes indicate that the liver is the predominant site of gluconeogenesis in most species (Suarez and Mommsen, 1987).

The terms 'mineralocorticoid' and 'glucocorticoid' are widely used to describe the predominant action of corticosteroids produced in the adrenal cortex of mammals. The distinction between the terms mineralocorticoid and glucocorticoid generally serves to describe the endocrinology of mammals, in which the predominant mineralocorticoid, aldosterone, regulates electrolyte and water balance by controlling epithelial sodium and potassium transport, and the predominant glucocorticoids, cortisol and corticosterone, regulate glucose metabolism. In mammals, these hormones function by activating mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), respectively. In fishes, the distinction between mineralocorticoid and glucocorticoid is less applicable – aldosterone is not functionally relevant in fishes (Prunet et al.,

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2006). Accordingly, it is widely held that a single corticosteroid, cortisol, carries out both mineralocorticoid- and glucocorticoid-like actions in teleosts, mediated predominantly through GRs (Mommsen et al., 1999; Takahashi and Sakamoto, 2013). The role of corticosteroid signaling in the mobilization of glucose from the liver in teleosts is well described (Faught and Vijayan, 2016; Suarez and Mommsen, 1987).

Both lamprey and hagfish appear to have a truncated steroid biosynthesis pathway, resulting in terminal corticosteroids that are biosynthetic precursors in more derived vertebrates. In lamprey, the putative stress-responsive, terminal corticosteroid is 11-deoxycortisol acting through a single corticosteroid receptor (CR) (Bridgham et al., 2006; Close et al., 2010; Rai et al., 2015; Roberts et al., 2014). 11-Deoxycortisol has recently been described to have mineralocorticoid-like actions in lamprey by acting through the CR to control osmoregulation (Shaughnessy et al., 2020). Although it has been shown that lampreys subjected to stress respond with increases in circulating 11-deoxycortisol (Close et al., 2010; Rai et al., 2015) and glucose (Larsen, 1976; Wilkie et al., 2007), direct evidence linking 11-deoxycortisol to gluconeogenesis is lacking. Importantly, although a gluconeogenic action of corticosteroids has been shown in basal bony fishes (McCormick et al., 2020), it has not been shown in any elasmobranch or agnathan. Thus, it is not known whether corticosteroid control of glucose homeostasis is a basal vertebrate trait. Our study sought to test the hypothesis that 11-deoxycortisol is both a stress-responsive and gluconeogenic hormone in sea lamprey, a basal vertebrate.

MATERIALS AND METHODS

Study animals

Animal care and use followed procedures previously approved by the Internal Animal Care and Use Committee at the University of Massachusetts and US Geological Survey. Sea lamprey, *Petromyzon marinus* Linnaeus 1758, were collected from the Connecticut River and brought to the Conte Anadromous Fish Research Center (US Geological Survey) in Turners Falls, MA, USA, from July to December. Experimentation took place from October to January. Prior to experimentation, lamprey were maintained in 1.5 m diameter tanks supplied with flow-through Connecticut River water (ambient temperature and photoperiod). Acute stress, steroid administration and tissue profile experiments were carried out in larval, metamorphic (early stage 7; lamprey have a total of 7 metamorphic stages between larvae and juvenile stages; Youson and Potter, 1979) or juvenile (fully metamorphosed) sea lamprey. The sizes of animal subjects were as follows: larvae: mean length 13.6 cm (range 12.0–16.0 cm), mean body mass 3.2 g (range 2.1–5.4 g); metamorphic: mean length 14.7 cm (range 12.6–16.7 cm), mean body mass 4.4 g (range 3.2–5.9 g); and juvenile: mean length 15.1 cm (range 13.4–16.6 cm), mean body mass 4.6 g (range 3.0–6.1 g). Prior to any experimentation, fish were acclimated for 1 week to 60 l glass aquarium tanks supplied with recirculating, dechlorinated and aerated municipal fresh water at 15°C equipped with mechanical, chemical and biological filtration.

Experimentation and sampling

For all experiments, individuals were haphazardly assigned to treatment groups. For the acute stress treatment carried out in mid-January, unstressed larval and juvenile sea lamprey were first sampled as a pre-stress control. The protocol for acute stress treatment consisted of 1 min netting (approximately 10 fish per 60 cm^2 net), followed by 10 min exposure to a low water level (approximately one-half depth of body). Following the stressor, water was restored to the tank and fish were left to recover for sampling 3 and 6 h post-stress (in separate tanks for the different recovery times).

For steroid administration carried out in mid-October, metamorphic lamprey were anesthetized with MS-222 (100 mg l^{-1} buffered with $NaHCO_3$, pH 7.4). Steroids were suspended in a molten 1:1 mixture of oil:shortening (vehicle) by sonication and administered by intraperitoneal injection. Metamorphic lamprey were administered either 11-deoxycortisol or deoxycorticosterone at doses of 5 or 40 µg g^{-1} body mass (20 µl g^{-1} body mass), then sampled 12 days after injection.

Prior to sampling, lamprey were killed with a lethal dose of MS-222 (200 mg l^{-1} buffered with $NaHCO_3$, pH 7.4) and measured for body length and mass. Blood was collected from the caudal vasculature into heparinized capillary tubes and plasma was separated from blood by centrifugation at 2000 ***g*** for 5 min. Tissue (brain, pituitary, gill, heart, liver, kidney, intestine, muscle) was collected, flash frozen, then stored at −80°C.

For *ex vivo* experimentation, freshly excised liver tissue from juvenile lamprey was dissected by scalpel under a microscope into 2 mm cubes and incubated in 24-well culture plates for 24 h at 15°C in media (DMEM containing 5 mmol l^{-1} glucose and 100 U ml^{-1} penstrep) containing 11-deoxycortisol (0.05–5.0 µg ml^{-1}) or a media-only control. After 24 h incubation, all liver cultures appeared to be in good condition based on the following criteria: (i) no loss of tissue color and no change in color of media; and (ii) no decrease in house-keeping gene (*ef1a*) mRNA levels. After 24 h, liver tissue was removed from media, flash frozen and stored at −80°C. The culture media formulation and 24 h time point were chosen based on previously reported *ex vivo* studies with hormones in the liver and gills of sea lamprey (Kawauchi et al., 2002; Shaughnessy et al., 2020).

Plasma glucose and 11-deoxycortisol determination

Plasma glucose was determined against a standard curve in an assay utilizing the enzymatic coupling of hexokinase and glucose 6-phosphate dehydrogenase (Stein, 1963). Plasma 11-deoxycortisol was determined by a previously described radioimmunoassay protocol (Shaughnessy et al., 2020), using a commercial 11-deoxycortisol antibody (CET-M8, Absolute Antibodies Inc., Oxford, UK) and radiolabeled 11-deoxycortisol ([1,2-3H]11-deoxycortisol, American Radiolabeled Chemicals, Inc.; hereafter [^{3}H]11-deoxycortisol). In glass culture tubes, 10 µl plasma was combined with 100 µl PBS assay buffer (50 mmol l^{-1} NaH_2PO_4, 137 mmol l^{-1} NaCl, 0.4 mmol l^{-1} EDTA, BSA 0.2% w/v, pH 7.4, 5000 cpm [^{3}H]11-deoxycortisol) and 50 µl of CET-M8 antibody (diluted 1:5000). The tubes were incubated at 37°C for 1 h then overnight at 4°C to allow the reaction to reach equilibrium. To remove unbound 11-deoxycortisol, 500 µl of ice-cold dextran-coated charcoal (PBS, 0.25% w/v dextran, 2.5% w/v activated charcoal) was added to each tube and incubated on ice for 15 min, then centrifuged at 2000 ***g*** (4°C) for 15 min. Finally, 325 µl of supernatant was added to 2.5 ml of scintillation fluid (ECONO-SAFE, Research Products International Corp.) and counted on a liquid scintillation counter (LS-6500, Beckman Coulter). Plasma 11-deoxycortisol concentration was determined against a standard curve ranging from 0.5 to 32 ng ml^{-1} and the assay validated as previously described (Shaughnessy et al., 2020).

Receptor binding

Radioreceptor binding studies were performed as previously described (Shaughnessy et al., 2020). Frozen liver was

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Table 1. Details for primer pairs used in qPCR

Gene	Probe	Sequence (5′–3′)	T_m (°C)	Gene ID	Product length	Reaction efficiency (%)
star	Forward	GTGAATCTCCGCCACTCGAT	59.9	PMZ_0033036	101	96
	Reverse	AAGTCGAGCTGCATTCGTGA	60			
cyp11a1	Forward	ACGAGCTGTTCAAGTTCGCT	60.3	PMZ_0047646	123	108
	Reverse	ACATGACAGAGATGGCGTCG	60.2			
pepck	Forward	ACCATGTACGTGATGGCGTT	60	PMZ_0059901	132	102
	Reverse	TGAGCGATCAGCGTCTTCTC	56.9			
g6pase	Forward	TGTGGTACGTGATGGTGACG	57.1	PMZ_0050741	123	100
	Reverse	GTGGCAACGAACACCCTAGA	57.2			

T_m, annealing temperature (°C). Gene ID refers to the unique sequence identifier in the gene assembly (https://genomes.stowers.org/organism/Petromyzon/marinus).

homogenized on ice in Hepes assay buffer (25 mmol l^{-1} Hepes, 10 mmol l^{-1} NaCl, 1 mmol l^{-1} monothioglycerol, pH 7.4) using a ground glass homogenizer, then centrifuged at 2000 ***g*** (4°C) for 10 min. The supernatant was determined for protein content using a bicinchoninic acid protein assay (Pierce, ThermoScientific, Waltham, MA, USA) and used in the binding assay at 4–6 mg ml^{-1}. The receptor binding assay was carried out on a non-binding 96-well microplate. For each reaction, 25 µl of supernatant was incubated for 2 h on ice with 25 µl of assay buffer containing either [^{3}H]11-deoxycortisol alone (total binding) or [^{3}H]11-deoxycortisol with 500-fold excess of unlabeled 11-deoxycortisol (non-specific binding). Each 50 µl reaction contained 2.5, 5, 10 or 20 nmol l^{-1} [^{3}H]11-deoxycortisol. After incubation, any unbound steroids were removed by adding 150 µl of ice-cold dextran-coated charcoal (0.25% w/v dextran and 2.5% w/v activated charcoal in assay buffer), incubating on ice for 10 min, then centrifuging for 10 min at 2000 ***g*** (4°C). Finally, 100 µl of supernatant (containing receptor-bound steroids) was combined with 1 ml of scintillation fluid and counted using a liquid scintillation counter. Specificity of the 11-deoxycortisol or deoxycorticosterone receptor was determined using an assay identical to that described above, except that 1 nmol l^{-1} [^{3}H]11-deoxycortisol was incubated with 1, 10, 100 or 1000 nmol l^{-1} of unlabeled 11-deoxycortisol or unlabeled DOC.

mRNA expression analysis

Total RNA was isolated from frozen tissue using the TRIzol method following the manufacturer's protocol (Molecular Research Center Inc.) and quantified and analyzed for purity using a Take3 micro-volume plate reader (BioTek Instruments, Inc.). Only high-purity samples (A_{260}/A_{280}>1.9) were used for cDNA synthesis and real-time PCR analyses. A subset of samples was also examined electrophoretically to confirm batch-level RNA integrity. First-strand cDNA was synthesized using a high-capacity reverse transcription kit following the manufacturer's protocol (Applied Biosystems Inc.). A reaction lacking the reverse transcriptase was included to assess possible DNA contamination. Real-time quantitative PCR was carried out in 10 µl reactions containing 2 ng cDNA, 150 nmol l^{-1} forward and reverse primers, and 1× SYBRselect master mix, following the manufacturer's protocol (ThermoFisher Inc.). The thermal profile of the reactions was 2 min at 50°C, 2 min at 95°C (holding and activation), 40 cycles of 15 s at 95°C, 1 min at 60°C, 30 s at 72°C (cycling); finally, a ramp from 60 to 95°C (melt curve analysis) was used to confirm a single product in each reaction. Relative mRNA abundance of *cr* and *star* was calculated using the comparative method ($\Delta\Delta C_T$; Pfaffl, 2001) using elongation factor 1 (*ef1α*) as a reference gene. Nucleotide sequences of primer pairs for *star*, *cyp11a1*, *pepck* and *g6pase* are provided in Table 1. Primer pairs for *cr* and *ef1α* in sea lamprey have been previously described (Ferreira-Martins et al., 2016; Kolosov et al., 2017).

Calculations and statistics

Specific receptor binding was calculated as: total binding−non-specific binding. Receptor abundance (B_{max}) and dissociation constant (K_d) were determined using Prism 6.0 (GraphPad Software Inc.). Normality and homogeneity of variance assumptions were tested using Shapiro–Wilk and Levene's tests, respectively. One-way and two-way ANOVA and *post hoc* analyses were performed using GraphPad Prism 6.0 and significance for all statistical tests was set at P<0.05. Statistical tests and n values are given in figure captions and/or text. All n values represent biological replicates (i.e. data obtained from unique individuals). For experiments with n<3 (i.e. tissue profiles and receptor binding experiments), individual biological replicates are presented in addition to summary statistics (means±s.e.m.).

RESULTS

Acute stress

Pre-stress levels of plasma 11-deoxycortisol were 2.0±0.8 ng ml^{-1} in larvae and 1.7±1.0 ng ml^{-1} in juveniles (Fig. 1A). In response to acute stress, plasma 11-deoxycortisol increased significantly in both larvae and juveniles (P_{time}<0.001; $P_{interaction}$=0.098; two-way ANOVA). At 6 h post-stress, plasma 11-deoxycortisol had increased to 16.7±4.5 and 6.4±1.7 ng ml^{-1} in larvae and juveniles, respectively. Life stage differences were observed ($P_{life\ stage}$<0.001), represented by an overall greater effect of stress on larvae. Pre-stress levels of plasma glucose were 1.0±0.1 mmol l^{-1} in larvae and 1.8±0.1 mmol l^{-1} in juveniles (Fig. 1B). In response to acute stress, plasma glucose significantly increased in both larvae and juveniles

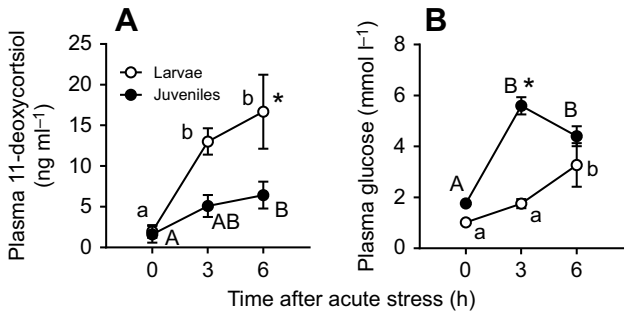

Fig. 1. Acute stress response in larval and juvenile sea lamprey. Plasma concentrations of (A) 11-deoxycortisol and (B) glucose in larval and juvenile sea lamprey before (time 0) and at 3 and 6 h after acute netting stress. Data represent means±s.e.m.; letters indicate significant differences within life stages and asterisks indicate significant differences between life stages (P<0.05, n=6 individuals, two-way ANOVA, Tukey's *post hoc*).

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(P_{time}<0.001) to greater than 4 mmol l^{-1}, and life stage differences in response to stress were observed ($P_{life\ stage}$<0.001; $P_{interaction}$=0.002), represented by an earlier upregulation in plasma glucose in larvae (at 3 h) compared with that in juveniles.

Tissue profiles of steroidogenic and gluconeogenic gene expression

Transcript abundance of *star* and *cyp11a1* was much higher in the kidney than in any other tissue: *star* mRNA expression was 12- to 1600-fold higher in the kidney than in the other tissues (Fig. 2A); *cyp11a1* mRNA expression was 7- to 70-fold higher in the kidney than in the other tissues (Fig. 2B). *pepck* was predominantly expressed in the liver followed by the kidney (12- and 5-fold higher mRNA abundance than in brain, respectively) (Fig. 2C). Transcript abundance of *g6pase* was highest in the brain, then kidney (approximately half of brain levels), then muscle and gill (approximately one-eighth of brain levels) (Fig. 2D).

In vivo and *ex vivo* corticosteroid treatments

Increasing doses of 11-deoxycortisol resulted in a dose-dependent increase in *pepck* mRNA abundance in *ex vivo* liver cultures (Fig. 3A). Transcript abundance of *pepck* at the highest doses (1.0 and 5.0 µg ml^{-1}) of 11-deoxycortisol was approximately 7- to 8-fold higher than for the media only control. In an *in vivo* corticosteroid treatment by interperitoneal injection, plasma glucose in lamprey that were administered vehicle alone was 2.2±0.2 mmol l^{-1} (Fig. 3B). There was a dose-dependent increase in plasma glucose with 11-deoxycortisol treatment (P=0.003). Lamprey that received a 40 µg g^{-1} dose of 11-deoxycortisol had a 50% increase in plasma glucose. There was no significant effect of *in vivo* treatment with deoxycorticosterone on plasma glucose.

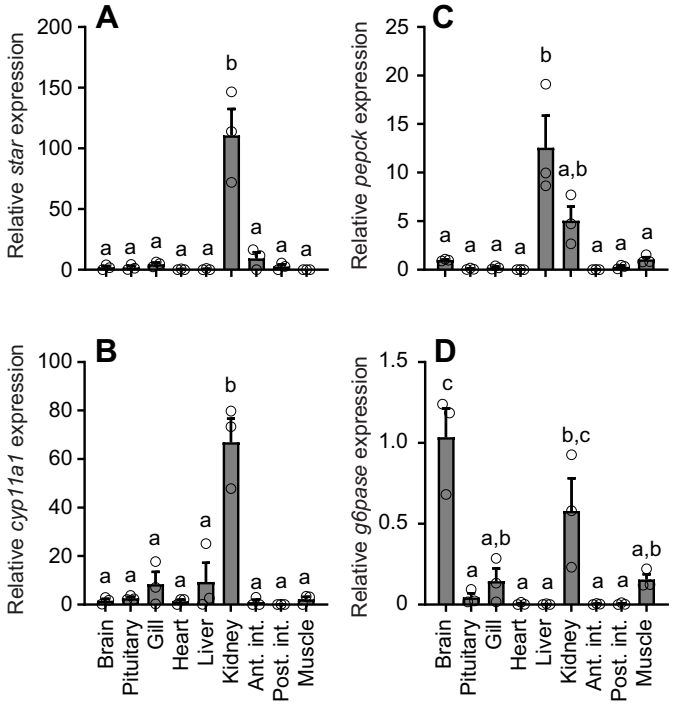

Fig. 2. Transcription profiles of steroidogenic and gluconeogenic enzymes. Tissue profile for mRNA levels of (A) steroidogenic acute regulatory protein (*star*), (B) cholesterol side-chain cleavage enzyme (*cyp11a1*), (C) phosphoenolpyruvate carboxykinase (*pepck*) and (D) glucose 6-phosphatase (*g6pase*) in juvenile sea lamprey. Relative mRNA expression was calculated by the comparative method ($\Delta\Delta C_T$) using elongation factor 1 (*ef1α*) as a reference gene. For each gene, mRNA levels were normalized to those in the brain (set to 1). Data represent means±s.e.m.; letters indicate significant differences (P<0.05, n=3 individuals, one-way ANOVA, Tukey's *post hoc*). Ant., anterior; Post., posterior; int., intestine.

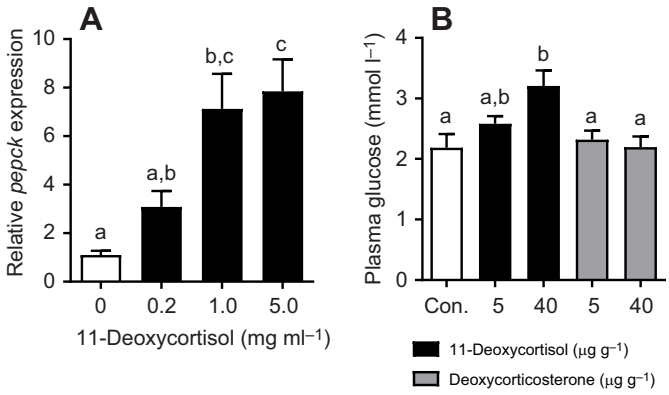

Fig. 3. Gluconeogenic action of corticosteroids in sea lamprey. (A) mRNA expression of *pepck* (relative to that of *ef1a*) in *ex vivo* sea lamprey liver incubated for 24 h with and without 11-deoxycortisol (n=6 individuals). (B) Sea lamprey plasma glucose concentrations sampled 12 days after intraperitoneal administration of 11-deoxycortisol, deoxycorticosterone or vehicle control (Con.) (n=10 individuals). Data represent means±s.e.m.; letters indicate significant differences (P<0.05, one-way ANOVA, Tukey's *post hoc*).

CR binding and expression

Liver CR abundance (B_{max}) was 91.6±3.1 fmol mg^{-1} and the dissociation constant (K_d) was 7.5±5.6 mmol l^{-1} (Fig. 4A). No difference was detected in binding affinity of the liver CR for 11-deoxycortisol (15.3±15.6 nmol l^{-1}) and deoxycorticosterone (26.3±28.1 nmol l^{-1}) (Fig. 4B). Transcript abundance of *cr* varied among tissues and was relatively high in the brain, gill, heart, kidney and anterior intestine, and relatively low in the pituitary, liver, posterior intestine and muscle (Fig. 4C). CR B_{max} varied among tissues (P=0.023, one-way ANOVA) with CR of the anterior intestine exhibiting the highest B_{max} at 344.5±81.9 fmol mg^{-1} (Fig. 4D).

DISCUSSION

This work is the first in lamprey, or any agnathan, to describe concurrent increases in plasma corticosteroids and glucose in response to an acute handling stress. Furthermore, this work provides the first direct evidence for the gluconeogenic action of 11-deoxycortisol in liver tissue *ex vivo* and the glucose stimulating action of 11-deoxycortisol treatment *in vivo*. We complement these novel findings with a characterization of 11-deoxycortisol binding in the liver and present transcriptional evidence localizing steroidogenesis and gluconeogenesis to the lamprey kidney and liver, respectively. The present study establishes a role for the corticosteroid 11-deoxycortisol in regulating plasma glucose levels during the stress response of sea lamprey. These findings confirm previous hypotheses and advance current understanding of agnathan stress physiology and endocrinology.

Increases in circulating 11-deoxycortisol and glucose in response to stress in the lamprey have been previously observed (Close et al., 2003, 2010; Rai et al., 2015; Wilkie et al., 2007), but not until the present study have concurrent increases in both physiological markers of stress been shown in the same study. We report that lamprey at both larvae and juvenile life stages exhibit an increase in plasma 11-deoxycortisol and glucose within 6 h of initiation of an

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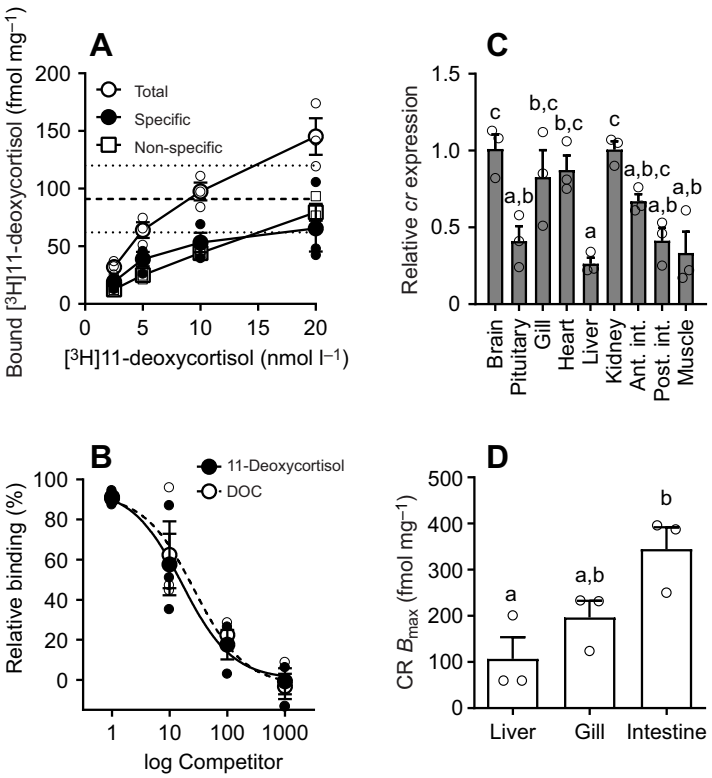

Fig. 4. Liver corticosteroid receptor binding characteristics and expression. (A) Specific binding of liver corticosteroid receptor (CR) to 11-deoxycortisol; total and non-specific binding of radiolabeled 11-deoxycortisol was used to calculate specific binding and receptor abundance (B_{max}; mean, dashed line; s.e.m., dotted lines). (B) Relative binding affinity for 11-deoxycortisol and deoxycorticosterone (DOC) (both nmol l^{-1}) in the liver. (C) Tissue profile for *cr* mRNA expression (relative to that of *ef1a*; see Fig. 2 for details). (D) Tissue comparison of CR abundance (B_{max}) in juvenile sea lamprey. Data points represent means±s.e.m.; letters indicate significant differences (P<0.05, n=3 individuals, one-way ANOVA, Tukey's *post hoc*).

acute handling stress. Post-stress larvae exhibited more immediate and greater changes in plasma 11-deoxycortisol compared with juveniles. In contrast, post-stress larvae exhibited less immediate and smaller changes in plasma glucose compared with juveniles. This may suggest that the glucose stimulating potential of 11-deoxycortisol is generally lower in larvae than in juveniles; that is, in larvae, a greater corticosteroid signal is needed to achieve a glucose response. Gluconeogenic action of stress-responsive catecholamines may also be contributing to the differences in stress responses between larvae and juveniles. It has been shown that in prespawning adult sea lamprey, administration of adrenaline significantly increased plasma glucose levels (Dashow and Epple, 1983), but the role of catecholamines in stimulating plasma glucose in larval and juvenile lamprey life stages has not been reported.

The levels of plasma 11-deoxycortisol in response to an acute stressor in larvae and juvenile sea lamprey (6 and 16 ng ml^{-1}, respectively) is greater than what has been previously observed in adult lamprey. Adult sea lamprey subjected to acute stressors (netting, air exposure and salt exposure) exhibited a significant rise in plasma 11-deoxycortisol from 0.75 to 1.75 ng ml^{-1} within 4 h (Close et al., 2010). Adult Pacific lamprey (*Entosphenus tridentatus*) subjected to acute netting and air exposure exhibited a significant elevation in plasma 11-deoxycortisol from 0.6 to 1.2 ng ml^{-1} within 30 min (Rai et al., 2015). These reports describe an approximate 2-fold increase in plasma 11-deoxycortisol in adult lamprey following an acute stressor. Although pre-stress levels of plasma 11-deoxycortisol in larvae and juvenile sea lamprey in the present study were similar to those previously reported for adults (~1–2 ng ml^{-1}), post-stress levels of plasma 11-deoxycortisol in larvae and juvenile lamprey were higher in absolute value (~17 and ~6 ng ml^{-1}, respectively) and magnitude of change (8- and 3-fold change, respectively). As experimental subjects, adult sea lamprey draw a sharp contrast to larvae and juvenile sea lamprey – adults are several orders of magnitude larger in mass than larvae and juveniles. Despite the large differences in size, the methods of applying an acute stressor used in the present study for larvae and juveniles were similar to the methods used by Close et al. (2010) and Rai et al. (2015) for adults. These previous studies netted adult lamprey from holding tanks and placed them into a dry bucket for 5 min before returning them to recovery tanks. In the present study, we netted larval and juvenile lamprey from rearing tanks and placed them in shallow water (one-half body depth) for 10 min, before returning them to recovery tank conditions. Considering the relative similarity in methods used between the present study and previous studies, life stage differences (and not experimental differences) likely explain the greater 11-deoxycortisol response to acute stress in the larval and juvenile sea lamprey compared with that in adults. It will be of interest to determine whether the higher 11-deoxycortisol response of larvae and juveniles is due to life stage differences in the hypothalamus–pituitary–interrenal (HPI) axis or the perception of stress. For instance, future studies might evaluate whether the response to stress differs between juveniles and adults with respect to production of hypothalamus-derived corticotropin releasing factor (CRF), pituitary-derived adrenocorticotropin hormone (ACTH) or interrenal-derived corticosteroids.

Plasma glucose levels of pre-stress sea lamprey in the present study (~1–2 mmol l^{-1}) were similar to those reported in previous studies for larval and adult lamprey (Larsen, 1976; Wilkie et al., 2007). These baseline levels of circulating glucose represent a generally lower homeostatic set point compared with that of later-derived fishes and tetrapods, which can range from ~2 to 20 mmol l^{-1} (Polakof et al., 2011, 2012). In larval sea lamprey, exposure to a toxic lampricide (3-trifluoromethyl-4-nitrophenol) increased plasma glucose from 1 to 2 mmol l^{-1} (Wilkie et al., 2007). In adult river lamprey (*Lampetra fluviatilis*), prolonged exposure to anesthesia (from 5 to 70 min) resulted in a time-dependent increase in blood glucose from 1 to 5 mmol l^{-1} (Larsen, 1976). In the same

study, intraperitoneal injection with a saline solution resulted in an increase in blood glucose from 2 to 7 mmol l^{-1} within 2 h before returning to pre-injection levels within 24 h (Larsen, 1976). In this context, we report levels and magnitudes of change (approximately 3-fold) in plasma glucose in larvae (1.0 to 3.3 mmol l^{-1}) and juvenile (1.8 to 5.6 mmol l^{-1}) sea lamprey following an acute stressor that are similar to those that have been previously reported in the literature. The lower levels of resting plasma glucose seen in juveniles may relate to the long-term (several months) fasting they endure during metamorphosis and prior to the onset of parasitic feeding.

Characterizing the site of steroid production in lamprey and its neuroendocrine regulation has been a topic of investigation for over half a century. Prior to the identification of the putative corticosteroids in lamprey, early histochemical work identified 'presumptive interrenal tissue' associated with the kidney, but experimental approaches to identify steroidogenic enzyme activity or morphological responses to acute stressor or ACTH stimuli were largely unsuccessful (Hardisty, 1972; Youson, 1973a,b). More recent *in vivo* and *ex vivo* work has identified the kidney as the likely site of 11-deoxycortisol production, although ACTH appears to lack steroidogenic action (Rai et al., 2015; Roberts et al., 2014). Interestingly, these studies also found that treatment with CRF results in the upregulation of plasma 11-deoxycortisol. Thus, it is still unresolved whether 11-deoxycortisol synthesis is regulated by a complete HPI axis, as is well described in more derived fishes. StAR has been used as a molecular marker for steroidogenic tissue in sturgeon (Kusakabe et al., 2009) and elasmobranchs (Evans and Nunez, 2010), and thus *star* and other gene-specific markers could help elucidate the molecular components involved in steroidogenesis in lamprey. Our initial work here showing kidney-specific expression of *star* mRNA provides further evidence that the kidney is the primary site of steroidogenesis in lamprey. This work also demonstrates the potential utility of gene-specific markers to resolve the unknown components of steroidogenesis and the neuroendocrine pathways which control steroidogenesis, whether by a complete HPI axis or by a more basal ACTH-independent endocrine program.

The role of the liver in controlling circulating glucose levels in lamprey is unclear. In more derived fishes, the liver is the predominant tissue for processes related to gluconeogenesis (Knox et al., 1980). In contrast, previous work in lamprey has demonstrated that the lamprey liver has relatively low stores of glycogen and glycerol and relatively low activity of enzymes that participate in gluconeogenesis compared with other tissues in lamprey such as the brain, heart, kidney and muscle. In lamprey, PEPCK activity is higher in the brain (meninges) and kidney than in the liver (Murat et al., 1979; Rovainen, 1970) and glycogen stores and G6Pase activity are higher in the brain and muscle than in the liver (Savina and Wojtczak, 1977). Importantly, it was shown that hepatectomized lamprey maintained normal levels of blood glucose (Larsen, 1978). In the present study, we took advantage of the recent release of the lamprey genome (Smith et al., 2018) to obtain gene-specific markers for enzymes involved in gluconeogenesis (*pepck*) and glycogenolysis (*g6pase*). In a tissue profile, we demonstrated that *pepck* mRNA was abundant in the liver and kidney and that *g6pase* mRNA was absent in the liver but abundant in the brain, kidney, gill and muscle. These tissue profiles are consistent with results from previous studies cited above using enzyme activity measurements. It seems possible that systemic glucose homeostasis in lamprey involves glucose production in both the liver and kidney, as well as other tissues. Specifically, while the liver may be important in gluconeogenesis, the liver, kidney and other metabolically active tissues (such as the brain, gill, heart and muscle) are likely to be involved in glycogenolysis.

It has been established that, in the absence of the steroidogenic enzymes needed to produce cortisol, corticosterone or aldosterone, the two terminal corticosteroids circulating at physiological levels in lamprey blood are 11-deoxycortisol and deoxycorticosterone (Bridgham et al., 2006; Close et al., 2010; Rai et al., 2015). Although it has been observed that both of these corticosteroids increase following an acute stressor (11-deoxycortisol much more so than deoxycorticosterone) (Close et al., 2010), no direct evidence for the role of either of these corticosteroids in gluconeogenesis has been presented prior to the present study. Classically, *ex vivo* and *in vitro* hepatic preparations have been used to study the control of intermediary metabolism of the liver in fishes (Moon et al., 1985). We employed a similar approach to identify transcriptional upregulation of *pepck*, a central gene in hepatic gluconeogenesis. The upregulation of *pepck* in excised liver incubated with 11-deoxycortisol is the first direct evidence that 11-deoxycortisol promotes gluconeogenic metabolism in lamprey. Future *ex vivo* studies which include the kidney, as well as the other endogenous corticosteroid, deoxycorticosterone, are needed to more completely understand the role of the liver in gluconeogenesis.

In teleosts, as well as mammals, cortisol treatment generally increases plasma glucose levels within hours to days (Mommsen et al., 1999), whereas more rapid changes (within minutes) in carbohydrate metabolism are attributed to the action of catecholamines such as adrenaline and noradrenaline (Fabbri et al., 1998). Such may also be the case with sea lamprey, in which treatment with exogenous adrenaline was shown to increase circulating glucose within 1 h (Bentley and Follett, 1965; Dashow and Epple, 1983). We have demonstrated that *in vivo* treatment with 11-deoxycortisol, but not deoxycorticosterone, resulted in an increase in plasma glucose above the vehicle control 12 days after initiation of treatment. Further studies will be needed to determine why treatment with 11-deoxycortisol (and not deoxycorticosterone) elicits a glucose response *in vivo*, considering that they appear to bind the liver CR with equal affinity *ex vivo*. Perhaps mechanisms of deoxycorticosterone inactivation, such as 5α-reductase or a hydroxysteroid dehydrogenase, are present in the sea lamprey liver, conferring specific activation of the liver CR by 11-deoxycortisol *in vivo* (Baker, 2004).

We confirm the findings of a previous report in adult sea lamprey (Close et al., 2010) that the abundance of CR (B_{max}) in the liver is lower than that in other tissues such as the gill and intestine. The gills and intestine of juvenile and adult lamprey are prominent osmoregulatory tissues (Barany et al., 2020; Ferreira-Martins et al., 2016; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020; Shaughnessy et al., 2020). As 11-deoxycortisol is presumed to serve both a glucocorticoid- and mineralocorticoid-like function acting through a single CR, the relatively high abundance of CR in the juvenile and adult sea lamprey gills and intestine is likely the result of the osmoregulatory action of 11-deoxycortisol in these tissues. In addition to our binding studies, we show that mRNA transcript abundance of *cr* is widely distributed but, importantly, also relatively low in the lamprey liver. Curiously, whereas the lamprey gill exhibits specificity for binding of 11-deoxycortisol over deoxycorticosterone (Close et al., 2010; Shaughnessy et al., 2020), the lamprey liver appears to bind the two corticosteroids equally (Fig. 4C). Post-translational modifications to CRs can alter ligand specificity, translocation of the receptor or receptor–ligand complex, or transcriptional activity of the receptor–ligand complex

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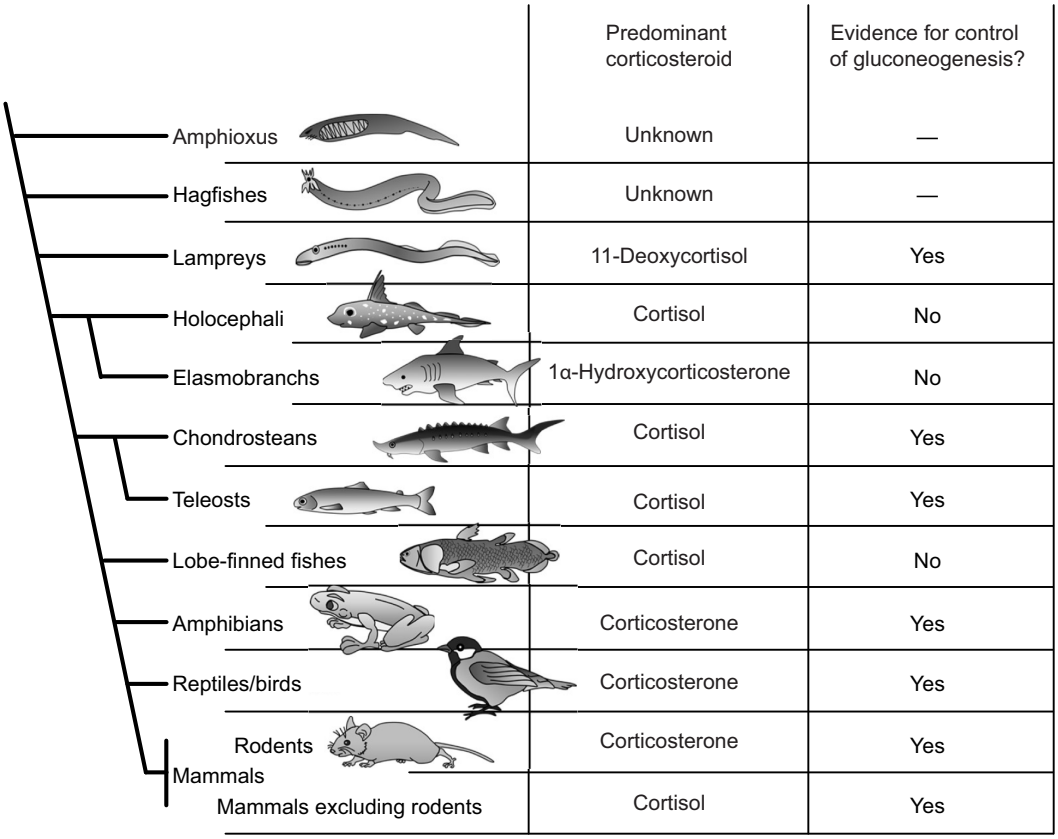

Fig. 5. Summary of glucocorticoid activity in a generalized vertebrate phylogeny. The predominant glucocorticoid and whether evidence exists regarding its gluconeogenic activity are presented for major vertebrate taxa, with particular attention given to basal vertebrates. References: hagfishes, Clifford et al. (2017); lampreys, present study; chondrosteans, McCormick et al. (2020); all others, see Bentley (1998).

in binding DNA (Faus and Haendler, 2006). In addition, reductases and hydroxysteroid dehydrogenases are known to regulate tissue-specific metabolism of endogenous corticosteroids (Baker, 2003). More *ex vivo* studies using isolated lamprey liver tissue to evaluate the gluconeogenic effects of corticosteroids and catecholamines and their interaction would greatly advance the understanding of how glucose production in the lamprey liver is regulated.

Our findings mark an important contribution to what is known regarding the identity and gluconeogenic activity of predominant glucocorticoids across and within major vertebrate taxa (Fig. 5). There is some consistency regarding glucocorticoids among the derived vertebrate groups, wherein corticosterone and cortisol serve as the major glucocorticoids in response to stress (Cockrem, 2013). Corticosterone is the predominant glucocorticoid in most amphibians, reptiles (including birds) and rodents. Cortisol is the predominant glucocorticoid in bony fishes and mammals (excluding rodents). Basal vertebrate groups, in contrast, exhibit conspicuous deviations from the stress endocrinology of more derived vertebrates and each other. In sturgeon (basal actinopterygians), cortisol plays a role in regulating plasma glucose (McCormick et al., 2020). In cartilaginous fishes, a novel corticosteroid, 1α-hydroxycorticosterone, appears to be the predominant corticosteroid (Anderson, 2012) and plasma 1α-hydroxycorticosterone is to be correlated with plasma glucose during stress (Ruiz-Jarabo et al., 2019). However, direct evidence that 1α-hydroxycorticosterone is a gluconeogenic hormone in elasmobranchs is still lacking. The predominant corticosteroid in hagfishes is still unknown, but it appears that plasma glucose is not regulated by 11-deoxycortisol (Clifford et al., 2017) as it is in lamprey (present study).

The present study therefore provides the first direct evidence that the corticosteroid control of gluconeogenesis occurred before the divergence of actinopterygia and sarcopterygia. The present study offers further insight into the evolution of corticosteroid function, and indicates that corticosteroid regulation of glucose and other aspects of intermediary metabolism are basal traits among vertebrates. Hopefully, this work will inspire renewed efforts to understand the evolution of the essential endocrine programs of HPI regulation of steroidogenesis and steroidogenic control of gluconeogenesis in other basal vertebrates such as hagfishes, holocephali and elasmobranchs.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.A.S., S.D.M.; Methodology: C.A.S., S.D.M.; Validation: C.A.S., S.D.M.; Formal analysis: C.A.S.; Investigation: C.A.S., S.D.M.; Resources: S.D.M.; Data curation: C.A.S.; Writing - original draft: C.A.S.; Writing - review & editing: C.A.S., S.D.M.; Supervision: S.D.M.; Project administration: S.D.M.; Funding acquisition: S.D.M.

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