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RESEARCH ARTICLE

Studies on cortisol, corticosterone, and 17β-estradiol indicate these steroids have no role in stress or reproduction in the common octopus (*Octopus vulgaris*)

Benjamin H. Maskrey,[1]* Carolina Costas,[2]* Luís Méndez-Martínez,[2] Laura Guerrero-Peña,[2] Ricardo Tur,[3] Pablo García,[3] Pablo Touriñan,[3] David Chavarrias,[3] Adelino V. Canario,[4] Alex P. Scott,[1] and Josep Rotllant[2]

[1]*Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, Weymouth, United Kingdom;* [2]*Aquatic Biotechnology Laboratory, Instituto Investigaciones Marinas—CSIC, Vigo, Spain;* [3]*Pescanova Biomarine Center, Pontevedra, Spain; and* [4]*Centro de Ciências do Mar do Algarve (CCMAR/CIMAR), University of Algarve, Faro, Portugal*

Abstract

The common octopus (*Octopus vulgaris*) is a promising candidate for aquaculture diversification, particularly in Europe. As interest in octopus farming grows, animal welfare concerns arise. In bony vertebrates (teleosts and tetrapods), measurements of the levels of corticosterone or cortisol have been successfully used as indicators of stress and welfare. Here, it is explored whether octopuses also produce cortisol or corticosterone and, if so, whether they are released into the water in response to stress (as can be done in teleosts and amphibians). The ability of the octopus to absorb cortisol from the water is also investigated—with another steroid, the principle vertebrate estrogen, 17β-estradiol (E_2), being used as a positive uptake control. In this study, using liquid chromatography tandem mass spectrometry techniques, it was found that octopus hemolymph did not contain either cortisol, corticosterone, cortisone (a common metabolite of cortisol), or E_2. Nor were any of the corticosteroids consistently found in the water in which stressed octopuses were held. The results support the evolutionary argument that octopuses are unlikely to exhibit a stress response mediated by vertebrate-like corticosteroids. Octopus demonstrated a low ability to absorb cortisol from the water (<2% over 24 h) but showed a high ability to absorb E_2 from water (92% over 24 h). In this latter respect, the octopus is similar to other mollusks. The finding calls into doubt the origin of the E_2 measured in this species.

NEW & NOTEWORTHY This study demonstrates that common octopuses (*Octopus vulgaris* Cuvier 1797) do not produce cortisol, cortisone, or corticosterone in response to stress. Using liquid chromatography tandem mass spectrometry, it was also shown that octopuses have a low absorption rate of cortisol from water but a high absorption rate of 17β-estradiol (E2). The findings support the evolutionary argument that octopuses are unlikely to exhibit a stress response mediated by vertebrate-like corticosteroids.

estradiol; corticosteroids; octopus; stress

INTRODUCTION

The aquaculture potential of the common octopus (*Octopus vulgaris*) has been investigated over the past 30 years (1). However, it is only in the past 3 years it has become clear that despite its sensitivity to environmental factors and its need for specific nutritional requirements, its biological and market potential makes the common octopus an ideal candidate to support the diversification of aquaculture and reduce pressure on wild populations. The once-difficult obstacle of high mortality rates during the rearing phase has been overcome, enabling the industrial-scale cultivation of these animals. As interest in octopus aquaculture grows, concerns arise regarding the potential impacts of large-scale production and intensified farming practices on the welfare of the animals. Assessing farm animal welfare typically involves evaluating physical health, immune response, behavior, and physiological indicators, with a particular focus on identifying stress levels. Therefore, understanding how to recognize signs of stress, establish reliable stress biomarkers, and effectively manage stress levels is essential for the protection and management of these creatures.

In bony vertebrates, glucocorticoids (GCs) release is a critical step in the stress response—with key steroids being cortisol and corticosterone. They regulate energy balance (2) and dampen the immune response in mammals (3). Their levels in the blood are widely used as an indicator of stress in mammals (4), birds (5), reptiles (6), amphibians (7), and teleosts

*B. H. Maskrey and C. Costas contributed equally to this work.
Correspondence: J. Rotllant (rotllant@iim.csic.es).
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(8). The first question that needs to be asked about these steroids is why should one expect them to also be stress steroids in cephalopods? From what is known about the evolution of vertebrates and mollusks (9–11), it seems highly unlikely. First, neither of these steroids function as stress steroids in the two most primitive vertebrate classes (elasmobranchs and cyclostomes), suggesting that their synthesis and subsequent use as stress steroids evolved relatively late in vertebrate evolution. Second, although nuclear receptors for adrenal steroids arose by a series of gene duplications from an ancestral nuclear receptor in a primitive vertebrate ∼540 million years ago (9, 10), the mollusk phylum (which includes cephalopods) diverged from the vertebrates ∼670 million years ago (11). Indeed, searches for genes resembling the vertebrate corticosteroid receptor in genomes of the common octopus (12), the snail, *Lymnaea stagnalis* (13), and the Mediterranean mussel, *Mytilus galloprovincialis* (14), have yielded negative results.

Despite the improbability that cortisol and corticosterone are stress steroids in mollusks, this has not stopped attempts to measure them in hemolymph (equivalent to blood in vertebrates), feces, crude tissue extracts, and skin mucus (15–24). In the present study, the presence of corticosteroids was investigated in octopus hemolymph, tissue extracts, and (a new medium) the holding water. This last was done because it has been shown that teleost fish excrete abundant amounts of cortisol into the water, where it is very easy to measure (25, 26). It has been shown that the cortisol is released into the water via the gills (27). Furthermore, a single study has shown that very little cortisol is reabsorbed by a bony fish, the tench, *Tinca tinca*, so that the cortisol effectively builds up in the water overtime (28).

Another aspect that was also investigated was the ability of the octopus to absorb [$^{13}C_3$]-labeled cortisol from the water. This was important for knowing how to interpret the presence of cortisol in mollusks. There is only one other study that has looked at cortisol uptake in a mollusk, and that was on a bivalve, the blue mussel, *Mytilus edulis*, which was, like the teleost mentioned earlier, shown to be very poor at absorbing cortisol (29). Since this finding contrasted with the strong ability of *M. edulis* to absorb other vertebrate steroids, including 17β-estradiol (E_2) (30), the uptake of this steroid was also investigated in the present study. Although this was initially done as a positive uptake control for the cortisol, the results were of sufficient interest to warrant further investigation and discussion. Mass spectrometry was used to identify and measure steroid concentrations in water and hemolymph, which is considered a more specific analytical method compared with immunoassay (which has been used to measure cortisol or corticosterone in all but one of the other studies on cephalopods).

MATERIAL AND METHODS

Animals

A total of 22 1-yr-old fifth-generation *O. vulgaris*, of equal number of females and males, weighing ∼0.8–1 kg, reared and maintained at the facilities of the Pescanova Biomarine Center (O Grove, Spain, 42°29′N 8°51′W), were used in the three experiments. All animals were kept in 12-m^3 square tanks connected to a semiopen water recirculation system at a temperature of (15 ± 1°C), salinity of (35 ppt), photoperiod of (10:14-h light/dark), and density of (10 $kg \cdot m^{-3}$) (31). They were fed an undisclosed proprietary diet, comprising frozen discard products from the fish and crustacean fishing fleet. The animals were provided with food twice daily, at a rate of 8–10% of their body weight. The tanks contained stones and various structures (cylindrical tubes) for cognitive enrichment. There was at least one shelter per animal in each tank. Hemolymph sampling procedures were conducted consistently under anesthetic conditions using a mixture of $MgCl_2$ (1.5%) and ethanol (1%) dissolved in seawater (1.5% $MgCl_2$ + 1% ethanol; <15 min). Subsequent to the extraction of hemolymph and before the dissection of the various organs, all animals were euthanized with an excess of anesthetic (3.5% $MgCl_2$ + 3.5% ethanol; <30 min). Following euthanasia, the animals were rinsed with clean salt water before the dissection of the various organs. The anesthesia and euthanasia procedures were conducted in accordance with the published guidelines for the care and welfare of cephalopods in research (32). All animals were fasted for 24 h before sampling.

The animals were housed and handled in accordance with animal welfare principles. Particular attention was paid to the 3Rs strategy (reduce, refine, reuse), reducing usage to the smallest number of animals required for statistical robustness. Procedures for care, handling, euthanasia, and necropsy were carried out in accordance with the EU Animal Welfare Directive (Directive 2010/63/EU) and according to the authorization file of the animal experiment projects ES360570202001/19/EDUC.FORM.07/JRM and ES360570202001/23/FUN.01/BIOL.AN08/JRM02.

Chemicals and Reagents

Cortisol (H4001), cortisone (C2755), 17β-estradiol (E8875), $^{13}C_3$-cortisol (2,3,4-13C3) (803146), and $^{13}C_3$-17β-estradiol (2,3,4-13C3) (719552) were purchased from Sigma-Aldrich (Spain). Corticosterone (235135) was purchased from Calbiochem, Sigma-Aldrich (Spain). Cortisol-D_4 (9,11,12,12-D4) (C-113) was purchased from Supelco-Merck Life Science (Spain). The seawater used in the steroid experiments was filtered (50-μm sieve). Reverse osmosis water was used for all other purposes. For mass spectrometry, solvents were purchased from Thermo Fisher Scientific (Loughborough, UK) and were of LC-MS grade, whereas LC-MS grade water was prepared using an in-house purification system (Merck Millipore).

Experiment 1: Collection of Hemolymph and Determination of Endogenous Steroids

To determine the presence or absence of the three main glucocorticoids governing energy balance and stress response in bony vertebrates (cortisol, cortisone, and corticosterone) and the sex steroid 17β-estradiol (E_2) in *O. vulgaris* hemolymph samples, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used. *O. vulgaris* specimens (five male and five females) with an average weight of 1 kg were taken from two 12 m^3 square holding tanks and transferred to a 500 L conical tank with static water conditions and exposed to stressful conditions [chasing with a net for 3 min (19)]. Hemolymph was collected

from each animal 60 min after exposure to the stress. All animals were rapidly anesthetized (1.5% $MgCl_2$ + 1% ethanol; <15 min) and bled from the posterior vein. Plasma was recovered by centrifugation of the hemolymph (4,500 rpm, 5 min), separated into aliquots, and stored at −80°C. Steroid extraction from plasma samples was performed using Sep-Pak C18 SPE cartridges preconditioned with methanol. Hemolymph (2.5 mL) was passed through the cartridges, washed with 5 mL of distilled water, and eluted with 5 mL of ethanol. The ethanol was evaporated under nitrogen flow, and the solid residue was solubilized with 1 mL of methanol for LC-MS/MS analysis. Samples were processed with and without the addition of 60 ng each of the specific standards before extraction. Animals were stressed and sampled in pairs in five batches.

Experiment 2: Steroid Release Studies

The methodology for the steroid release study consisted of placing the animals ($n = 4$, two males and two females) individually in a cylindrical tank with a capacity of 100 L, sealed and lined with a polypropylene plastic cover, containing filtered seawater with continuous oxygenation. Water samples (100 mL) were taken over 24 h at various intervals (0, 2, 6, 10, and 24 h) after a 3-min handling stress (Table 2). A negative control was also used with the same tank system but without animals.

Experiment 3: Steroid Uptake Studies

Exposure to steroids.

The methodology of the steroid uptake studies consisted of placing the animals ($n = 8$, four males and four females) individually in a cylindrical tank with a capacity of 100 L, sealed and lined with a polypropylene plastic cover, containing filtered seawater with continuous oxygenation with unlabeled "cold" standard corticosteroids (25 ng·mL^{-1}) ($n = 4$, two males and two females) (*Experiment 3A*) or isotopically labeled [$^{13}C_3$]-cortisol and [$^{13}C_3$]-E_2 (25 ng·mL^{-1}) ($n = 4$, two males and two females) (*Experiment 3B*). Water samples (50 mL) were taken over 24 h at intervals of 0 h, 2 h, 6 h, 10 h, and 24 h to measure the amount of steroids remaining in the water [Fig. 2 (*Experiment 3A*) and Fig. 3 (*Experiment 3B*)] and the amount of [$^{13}C_3$]-cortisol and [$^{13}C_3$]-E_2 absorbed by the animal and specifically accumulated in five tissues, namely muscle, gonads, digestive gland, systemic heart, and gills [Fig. 4 (*Experiment 3B*)]. In all cases, two negative controls with the same tank system but without animals were also used.

Extraction and determination of steroids from water samples.

Steroids were extracted from 100 mL (*Experiment 2*) or 50 mL (*Experiment 3*) of water using 360 mg C18 cartridges (Waters Sep-Pak, WAT020515) preconditioned with 5 mL of methanol and 5 mL of water. The cartridges were washed with 5 mL of distilled water before the steroids were eluted with 5 mL of absolute ethanol. The extract was taken to dryness under nitrogen, resuspended in 1 mL of methanol, and stored at −20°C until analysis by LC-MS/MS.

Analysis of Data and Calculation of Absorbed Steroids

The amount of steroids accumulated for each animal was calculated as follows: *1*) the percentage of labeled and unlabeled steroids remaining in the water after each sampling time was corrected (if necessary) for steroid loss due to adsorption in the tanks and *2*) the tissue concentrations determined at each sampling time were subtracted from the initial concentration in the water at *time 0*, and the values were expressed as percentages, with the value at 0 h considered as 100%. In all cases, containers with and without animals were used with the same initial concentration.

Extraction and Hydrolysis of ^{13}C-Labeled Steroids from Tissues

After 24-h exposure, the animals (two males and two females) were rinsed with filtered seawater to remove any remaining steroids. Then, five tissues from each animal were dissected: muscle, gonads, digestive gland, systemic heart, and gills. The tissue extraction method was based on the procedure described by Gooding et al. (33). Approximately 3 g (wet weight) of each tissue was homogenized in 3 mL of methanol using a blender. Ethyl acetate (5 mL) was added to the homogenate, mixed for 5 min using a vortex, and centrifuged at 2,500 *g* for 10 min. The supernatant was decanted into a 15-mL polypropylene tube. Tissue extraction was repeated with 3 mL of ethyl acetate. The crude tissue extract (1 mL) was transferred to a polypropylene tube and dried. Alkaline hydrolysis of tissue extracts was performed by the addition of 12.5 μL of 2.5 M NaOH to 100 μL of tissue extract and heating at 80°C for 40 min, after which they were neutralized with 12.5 μL of 2.5 M HCl. Samples were analyzed by LC-MS/MS as described next, with concentrations adjusted for wet weight.

LC-MS/MS Analysis and Quantitation of Steroids

Steroids were analyzed using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, UK) operating in multiple reaction monitoring (MRM) mode with positive polarity coupled to an Acquity I-Class UPLC system. Chromatographic separation was achieved using an Acquity HSS T3 Premier column (2.1 × 100 mm; 1.8 μm; Waters, UK) maintained at 40°C, with a gradient starting at 40% B for 0.1 min, rising to 100% B at 10 min, held for 2 min, before dropping back to 40% B at 12.5 min with an overall cycle time of 15 min with mobile phase A being water and mobile phase B being methanol, both containing 0.1% formic acid. The injection volume was 2 μL. Instrument parameters such as collision energy and cone voltage for individual steroids were optimized by infusion of pure standards into the electrospray ionization (ESI) source. Individual steroids were identified based on a characteristic primary MRM transition, with the presence of at least one matching secondary transition for confirmation at a specific retention time, as detailed in Table 1. In *Experiment 3A*, preliminary studies using unlabeled "cold" corticosteroids were determined using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). An Agilent 1200 Series liquid chromatograph coupled with an Ultra Bruker Esquire HCT ion trap mass spectrometer with an APCI ion source operated in single ion monitoring mode to track positive polarity compounds. Separation used a PLP-S 100 A 5-μm 150 ×

Table 1. *Optimized tandem mass spectrometry (MS/MS) conditions for steroid analysis*

Compound	MW	Molecular Ion	Parent Ion (*m/z*)	Daughter Ion(s) (*m/z*)	Cone Voltage (V)	Collision Energy (V)
Cortisol	362.2	$[M + H]^+$	363.3	**121.1**; 144.9	40	24; 28
Cortisone	360.2	$[M + H]^+$	361.2	**163.1**; 120.9	40	26; 26
Corticosterone	346.2	$[M + H]^+$	347.2	**120.8**; 97.3	40	26; 26
17β-estradiol	272.2	$[M + H\text{-}H_2O]^+$	255.2	**159.2**; 133.1	40	16; 18
Cortisol-$^{13}C_3$	365.3	$[M + H]^+$	366.4	**124.1**; 270.1	40	20; 18
Estradiol-$^{13}C_3$	275.2	$[M + H\text{-}H_2O]^+$	258.3	**162.1**; 136.0	40	18; 16

Note: Daughter ions denoted in bold indicate quantitative transition. MW, molecular weight; V, volt.

2.1-mm column, with individual steroids identified by their specific parent ion (Table 1).

For quantitation, standard curves were prepared in methanol with samples bracketed by standards, with a blank (methanol) sample injected between samples to ensure no carryover occurred. Linearity was assessed by r^2 with values $\geq$0.99. Limit of quantification (LOQ) cutoff was determined as the lowest standard concentration to generate a signal: noise ratio for the quantitative MRM transition $\geq$10 over three injections. Data were acquired using MassLynx and processed with TargetLynx.

LC-HRMS Analysis of Sulfated E_2 and Esterified Steroids

Analysis of sulfated E_2 and identification of fatty acid esters was performed using liquid chromatography high-resolution mass spectrometry (LC-HRMS) using an Orbitrap Exploris 120 coupled to a Vanquish UHPLC system (Thermo Fisher Scientific, Hemel Hempstead). For sulfated E_2, the water extracts were separated on a HSS T3 1.8 μm column (2.1 × 100 mm; Waters, UK) at a flow rate of 0.4 mL·min^{-1}, and a temperature of 40°C, with mobile phase A being water and B acetonitrile with both containing 0.1% formic acid. The gradient started at 5% B for 1 min before increasing to 95% B at 10 min, held for 2 min before returning to 5% B at 12.5 min with a total cycle time of 15 min. The injection volume was 5 μL. The orbitrap was operated in negative ionization mode at a voltage of −2,500 V, scanning from 100 to 1,000 amu at a resolution of 120,000 with LC effluent directed into the H-ESI source from 0.5 to 12 min with an ion transfer tube temperature of 325°C, a vaporizer temperature of 350°C, and sheath, aux, and sweep gas values of 50, 10, and 1 (arbitrary values), respectively. For analysis of fatty acid esters, samples were acquired similarly except for the use of an Acquity UPLC BEH C8 1.7-μm (2.1 × 50 mm) column (Waters, UK). The gradient started at 25% and increased to 100%B at 10 min. The injection volume was 5 μL for unhydrolyzed samples and 6.25 μL for hydrolyzed samples to account for the dilution factor applied during hydrolysis. Orbitrap was operated in positive ionization mode, scanning a mass range from 200 to 800 amu at a resolution of 120,000. Data were acquired using Xcalibur v4.5 and analyzed using FreeStyle v1.8.

Statistical Analyses

Results were analyzed with R Commander. The limited sample size in some groups makes it challenging to ascertain a normal distribution, which may impact the reliability of the results. In light of this, a nonparametric Kruskal–Wallis one-way analysis of variance by ranks test (or H test) was conducted. A nonparametric Wilcoxon statistical test was also applied to compare paired groups. The level for accepted statistical significance was $P < 0.05$. Significant differences among groups at particular time points and within a tissue are indicated by symbols and significant differences among tissues are indicated by different letters in the figures. Results are presented as means ± standard deviation.

RESULTS

Experiments 1 and 2—Presence and Release of Endogenous Steroids

All steroids (unlabeled and isotopically labeled) standards were detected under positive ion ESI-LC-MS/MS conditions. They formed strong protonated $[M + H]^+$ ions, which upon collision-induced dissociation fragmented to unique daughter ions, except for E_2, which formed the singly dehydrated $[M + H\text{-}H_2O]^+$ parent ion via the loss of H_2O in the source (Table 1). The LOQs ranged from 0.5 to 2 ng·mL^{-1} (equivalent to 1–4 pg on the column). Cortisol had the lowest LOQ at 0.5 ng·mL^{-1}, with corticosterone having an LOQ of 2 ng·mL^{-1} and cortisone and E_2 of 1 ng·mL^{-1}. As shown in Fig. 1*B*, the application of this method to hemolymph extracts failed to detect the presence of endogenous steroids, cortisol, cortisone, corticosterone, and E_2 in octopus hemolymph. However, hemolymph samples fortified with cortisol, cortisone, corticosterone, and E_2 before extraction show clear peaks at 3.87, 3.53, 4.73, and 5.48 min, respectively (Fig. 1*C*), and no interferences of other peaks were observed in this region. Table 2 shows the results of experiments designed to evaluate the capacity of octopuses to secrete the three corticosteroids. No peaks were detected above LOQ in any octopus water samples (100 mL), indicating that these animals do not release detectable amounts of the three corticosteroids analyzed into the water. Low concentrations (1.6–3 ng/100 mL) of the three glucocorticoids were detected in the control samples at 24 h.

Experiment 3—Uptake and Metabolism of Steroids (Esterified Estradiol in the Tissue Samples)

No differences between treatment and control were found in standard steroid concentrations (Fig. 2). Similar results were found using [$^{13}C_3$]-cortisol (Fig. 3*A*). However, *O. vulgaris* strongly absorbed [$^{13}C_3$]-E_2, with a rapid decrease in the concentration in water to 8% (2.52 ± 1.2 ng·mL^{-1}) after 2 h, with complete removal after 10 h (Fig. 3*B*). To examine the tissue distribution of E_2, extracts from muscle, digestive gland, gonad, heart, and gills were analyzed for the presence of [$^{13}C_3$]-E_2. Hemolymph was not analyzed. As shown in Fig. 4, low concentrations of "free" [$^{13}C_3$]-E_2 were detected in

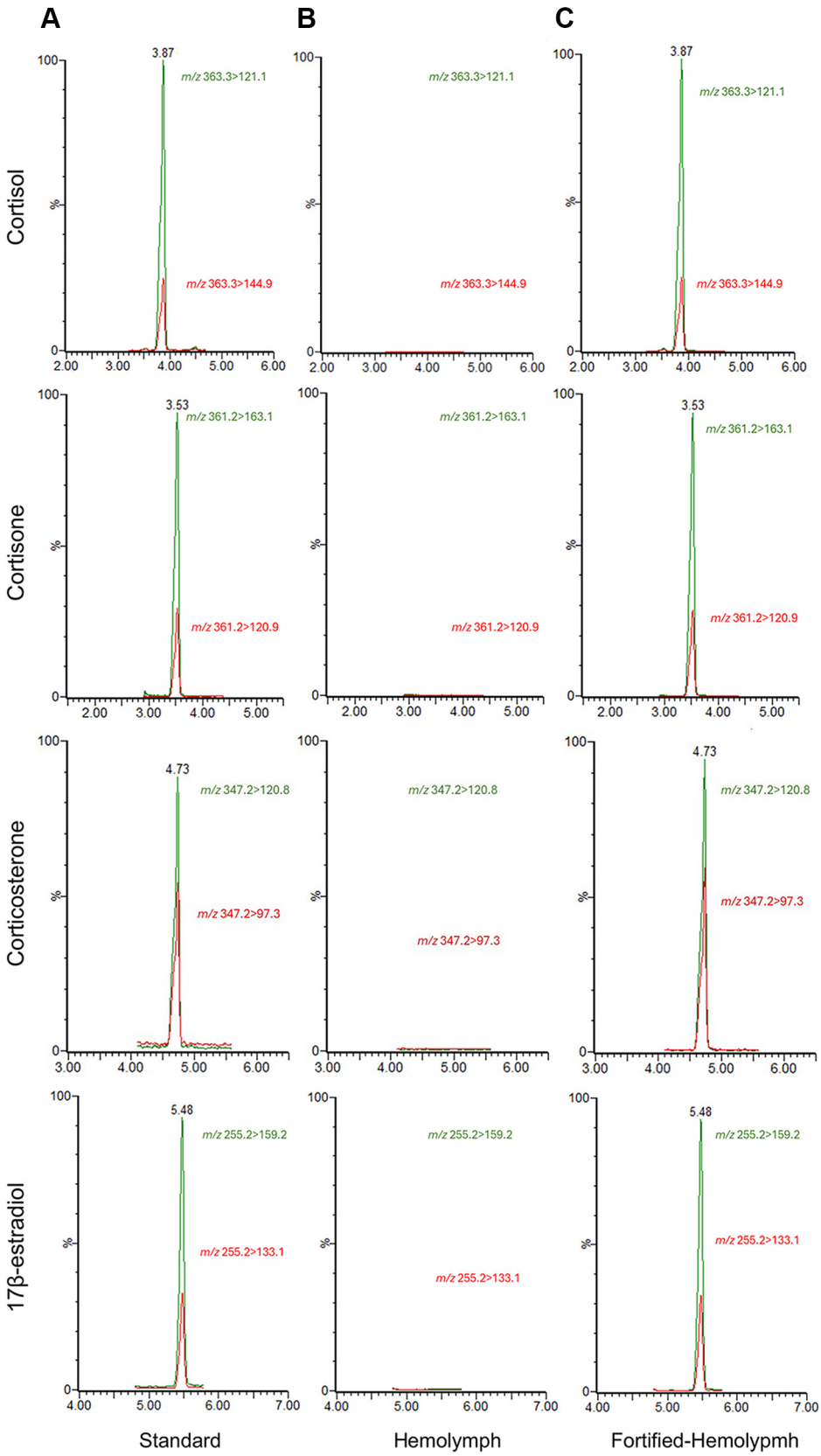

Figure 1. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis of cortisol, cortisone, corticosterone, and 17β-estradiol of standards (*A*), octopus hemolymph (*B*), and fortified (60 ng) hemolymph (*C*). Green traces denote quantitative MRM transitions, and red traces qualitative MRM transitions. Note: fortified hemolymph and hemolymph are presented on the same scale. The vertical axis denotes relative intensity of the peak (%) and the horizontal axis retention time (min). MRM, multiple reaction monitoring.

Table 2. *Amounts of glucocorticoids found in water samples from the 24-h immersion experiment of previously stressed animals (see MATERIALS AND METHODS for details)*

Steroid	Time	3 h	6 h	12 h	24 h
Cortisol	F1				
	F2				
	M1				
	M2		<LOQ		<LOQ
	Control				1.6
Cortisone	F1				
	F2				
	M1		<LOQ	<LOQ	
	M2		<LOQ		
	Control				3.1
Corticosterone	F1			<LOQ	
	F2				
	M1				
	M2				<LOQ
	Control				1.9

There were five separate 100-L conical tank systems (that all contained 20 L of water), two of which contained females (F), two contained males (M), and one contained water only (control). Values are shown as nanograms per sample (ng per 100 mL of seawater). A blank entry indicates that no steroid was detected in that sample with <LOQ denoting the presence of a peak below the limit of quantitation. LOQ, limit of quantification.

the gill extracts (10.1 ± 4.2 ng·g^{-1} tissue) but not in any other tissues. Tissue extracts were then subjected to alkaline hydrolysis to cleave any E_2 covalently bonded to fatty acids and reanalyzed by LC-MS/MS to provide "total levels" of [$^{13}C_3$]-E_2 (sum of free and esterified E_2). As shown in Fig. 4, total concentrations of [$^{13}C_3$]-E_2 were significantly higher in the gills, reaching a mean of 371.5 ± 135.9 ng·g^{-1} tissue. Low levels were detected in muscle, gonad, and heart tissue equivalent to 1.5, 0.35, and 1.8%, respectively.

To identify the fatty acid conjugates to which the labeled E_2 might have been esterified, all gill extracts were directly analyzed by LC-HRMS. Formulae and theoretical masses of all possible fatty acids esterified to $^{13}C_3$-E_2 were calculated as detailed in Table 3, and samples were interrogated for the presence of chromatographic peaks consistent with those masses. Due to the absence of analytical standards for these compounds, identification is only tentative, but the peaks had to demonstrate a mass error of <5 parts per million (ppm) and either to be absent or have <15% peak area in the hydrolyzed samples to suggest that they decreased following alkaline hydrolysis. As shown in Table 3, identifications consistent with compounds containing polyunsaturated fatty acids of C18, C20, and C22 were identified. For many compounds, two peaks were detected at similar masses and retention times, but without synthesis of authentic standards, identity cannot be assigned.

In the nonhydrolyzed tissue extracts, peaks of $^{13}C_3$-cortisol were detected in the hearts of one male and one female (0.5 ng and 0.7 ng·g^{-1}), the gills of one male and female (0.2 and 0.3 ng·g^{-1}), and the digestive gland of one male (0.2 ng·g^{-1}). There was no detection of natural free cortisol in any of the extracts, but there was a trace of free nonlabeled E_2 in the gills of one female. The alkaline hydrolysis did not release any [$^{13}C_3$]-cortisol or unlabeled cortisol (suggesting that this steroid could not be esterified). There were nonquantified traces of nonlabeled E_2 in all the gills samples.

Finally, analysis of water samples by LC-HRMS for the presence of the 3-sulfated version of [$^{13}C_3$]-E_2 using a theoretical [M-H]$^-$ of *m/z* 354.1372 failed to detect the presence of [$^{13}C_3$]-E_2 3-sulfate at any of the time points (data not shown). Analysis of standard nonlabeled E_2 3-sulfate revealed a strong peak at 5.73 min with a [M-H]$^-$ of *m/z* 351.1265 and a mass error of Δ −1.99 ppm from the theoretical [M-H]$^-$ of *m/z* 351.1272 (Supplemental Fig. S1).

DISCUSSION

In the present study, no cortisol, corticosterone, cortisone, or E_2 were found in the hemolymph of *O vulgaris*. Although traces of the corticosteroids were sporadically found in the water where individuals were suspended for 24 h, any physiological significance of such measurements was negated by the fact that they were also found in a control water sample. Their presence was most likely due to contamination at some stage when handling the sample. There was no evidence of a consistent build-up of cortisol in holding water over time, as found in fishes (25, 26). There was also no evidence that cortisol was present in the analyzed tissue extracts. This finding contrasts with all other studies that claim to have measured cortisol or corticosterone in mollusks. The first was a pilot study by Larson and Anderson (15), who found that levels of immunoreactive corticosterone in the feces of a single Giant Pacific octopus, *Enteroctopus dofleini*, varied with time and were higher after certain events, such as the injection of vertebrate corticotrophin. A later, more thorough, investigation was carried out by O'Brien et al. (16), who also found immunoreactive corticosterone in dried feces of cuttlefish *Sepia officinalis*, but with no significant difference between stressed and unstressed animals. Hu et al. (24) found significant differences of immunoreactive cortisol in aqueous extracts of the combined visceral masses of the juvenile webfoot octopus, *Amphioctopus fangsiao*. These correlated with the amount of shelter that was available to the animals. Zhang et al. (17) found concentrations of immunoreactive cortisol of up to 200 ng·g^{-1} in aqueous extracts of golden cuttlefish, *Sepia esculenta*, muscle tissue, which correlated with the social status of the animals. Chancellor et al. (18, 19) measured cortisol and corticosterone by immunoassay in the skin mucus of three types of cephalopod. They found what appeared to be easily

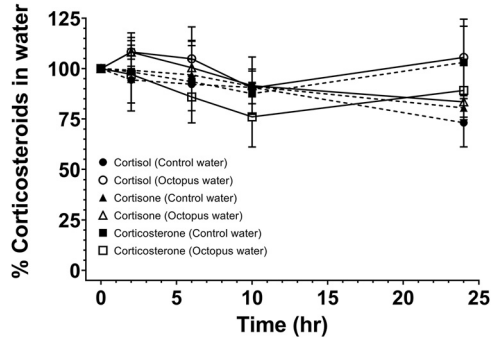

Figure 2. Ability of octopuses for external uptake of cold nonlabeled human steroids (25 ng·mL^{-1}), cortisol (○,●), corticosterone (□,■), and cortisone (△,▲) from water (see MATERIALS AND METHODS for details). Each treatment had four replicates.

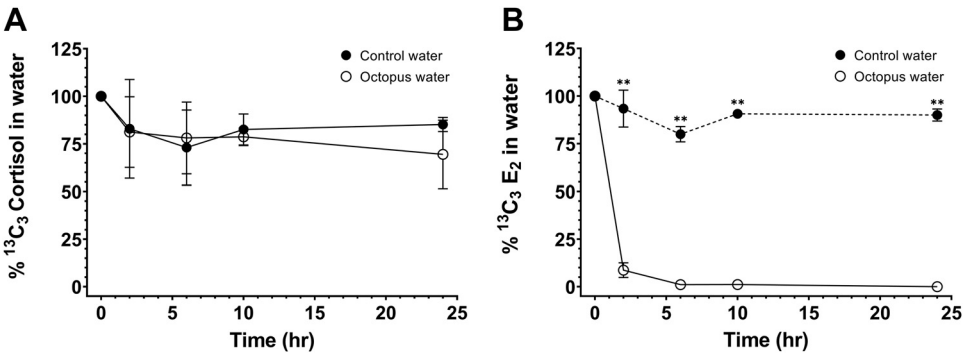

Figure 3. Ability of octopuses for external uptake of labeled steroids, cortisol-$^{13}C_3$ (*A*), and 17β-estradiol-2,3,4-$^{13}C_3$ (*B*) from water. The nonspecific loss of C^{13} was negligible (0.34% for cortisol-$^{13}C_3$ and 0.16% for 17β-estradiol-2,3,4-$^{13}C_3$) (see MATERIALS AND METHODS for details). Each treatment had four replicates. Significant differences among groups at particular time points and within a tissue are indicated. $^{**}P < 0.01$.

measurable amounts of cortisol [~5 ng·swab^{-1}] and arbitrarily chose a greater-than-twofold increase or decrease in concentrations as representing a "significant" variation. In experiments that looked at changes over time in response to stress, they found several such variations in individuals. However, the results were highly inconsistent between animals, as were the timing of such "peaks," such that they were unable to demonstrate a statistically significant response to stress in the population. They did find, however, that levels in subadults were statistically significantly higher overall than in adults.

Regarding other mollusks, a study by Binder et al. (20) examined the effects of copper, starvation, and sodium chloride on the freshwater duck mussel, *Anodonta anatina*. The researchers detected relatively low concentrations of immunoreactive cortisol (0.05 to 0.25 ng·g^{-1}) in various tissues, with the highest levels found in the hepatopancreas. However, anti-intuitively, the concentrations in the hepatopancreas appeared to decrease in response to the stressors. In the bivalve mollusk, the Pacific oyster, *Crassostrea gigas* (21), ~300 ng·g^{-1} of immunoreactive cortisol was detected in hepatopancreas tissue. The authors found only a 1.2-fold increase in temperature-stressed animals that was nevertheless statistically significant. Shi et al. (23) reported levels between 350 and 450 ng·g^{-1} (although, elsewhere in their article, they imply this might have been ng·mg^{-1}) of protein in the hemolymph of the bivalve mollusk, *Tegillarca granosa*, which were unaffected by the presence of contaminants. In the clam *Macoma balthica*, suffering from contagious cancer, there were significantly higher tissue concentrations of cortisol, but not corticosterone, in the affected animals (3 ng·g^{-1} vs. 1 ng·g^{-1} in healthy animals) (22).

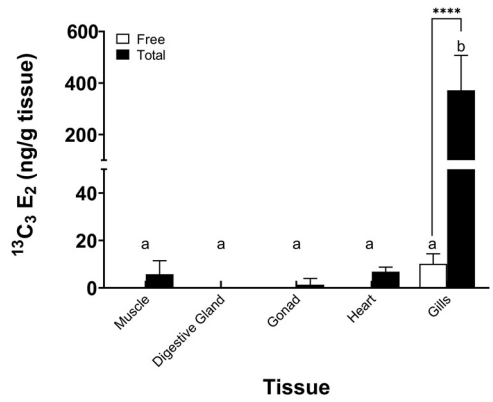

Figure 4. Tissue distribution of 17β-estradiol-2,3,4-$^{13}C_3$ into the octopus. Tissues extracts were subject to alkaline hydrolysis to cleave any such bond, followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) reanalysis to determine "total levels" of [$^{13}C_3$]-E_2 (sum of free and esterified E_2). Each treatment had four replicates. Significant differences among groups at particular time points and within a tissue are indicated. $^{****}P < 0.0001$. ^{a,b}Significant differences among tissues.

All the aforementioned studies have been carried out assuming that corticosteroids are endogenously synthesized, implying that mollusks have the full suite of enzymes for transforming cholesterol into cortisol. Although there is some evidence that they might convert cholesterol as far as 17-hydroxyprogesterone (34), there is no evidence yet (13) for the presence in mollusk genomes of the cytochrome P450 enzymes that vertebrates use to insert oxygen atoms onto the carbon atoms at position 11 and 21 (as occurs during the synthesis of corticosterone and cortisol). Wang et al. (21) in *C. gigas* and Juarez et al. (35) in *Octopus maya* found evidence for a gene homologous to the vertebrate enzyme 11β-hydroxysteroid dehydrogenase, that, in vertebrates, is able to convert cortisol to cortisone by simple removal of two hydrogen atoms. However, this enzyme is a metabolic, not synthetic, enzyme.

If the corticosteroids are not endogenously synthesized in mollusks, then what is the reason for their apparent presence in the studies listed above? One possibility is that the animals take them up via the water or diet. For instance, in the two earliest studies where corticosterone was measured in feces, the steroid might well have come from the diet. Besides, in relation to contamination via the water, although it was shown that the cortisol absorption rate from the water in octopus is low compared with E_2 [as also found in *M. edulis* (29) and a teleost fish (28)], it is not zero—as shown by the presence of small amounts of [$^{13}C_3$]-cortisol in some tissues in the present study. A low rate of uptake of cortisol could account for the low levels of cortisol reported in the tissues of *A. anatina* [0.05–0.25 ng·g^{-1}; see Ref. (20)] and perhaps diseased *M. balthica* [3 ng·g^{-1}; see Ref. (22)]. Contamination might possibly also explain some of the cortisol and corticosterone found in cephalopod mucus samples by Chancellor et al. (18, 19), because, as shown in the present study, corticosteroids were even found in the final control water sample, even though gloves were worn to take samples. High cortisol levels (up to 120 ng on the surface of a single forearm) are found on human skin (34), and it is not inconceivable that water and samples can be contaminated in this way.

Table 3. *LC-HRMS identification of $^{13}C_3$-estradiol fatty acid conjugates in octopus gill tissue*

Fatty Acid	Formula	Calculated $[M + H]^+$	Measured $[M + H]^+$	Mass Error, ppm	RT, min
14:0	$^{13}C_3C_{29}H_{50}O_3$	486.39334	ND		
16:0	$^{13}C_3C_{31}H_{54}O_3$	514.42464	ND		
16:1	$^{13}C_3C_{31}H_{52}O_3$	512.40899	ND		
16:2	$^{13}C_3C_{31}H_{50}O_3$	510.39334	ND		
18:0	$^{13}C_3C_{33}H_{58}O_3$	542.45594	ND		
18:1	$^{13}C_3C_{33}H_{56}O_3$	540.44029	ND		
18:2	$^{13}C_3C_{33}H_{54}O_3$	538.42464	538.4232; 538.4230	−2.67; −3.05	7.09; 7.46
18:3	$^{13}C_3C_{33}H_{52}O_3$	536.40899	ND		
18:4	$^{13}C_3C_{33}H_{50}O_3$	534.39334	534.3914; 534.3914	−3.63; −3.63	6.49; 7.00
20:0	$^{13}C_3C_{35}H_{62}O_3$	570.48724	ND		
20:1	$^{13}C_3C_{35}H_{60}O_3$	568.47159	ND		
20:2	$^{13}C_3C_{35}H_{58}O_3$	566.45594	566.4543	−2.90	8.18
20:3	$^{13}C_3C_{35}H_{56}O_3$	564.44029	564.4387; 564.4385	−2.82; −3.17	7.60; 7.92
20:4	$^{13}C_3C_{35}H_{54}O_3$	562.42464	562.4231	−2.74	7.37
20:5	$^{13}C_3C_{35}H_{52}O_3$	560.40899	ND		
22:0	$^{13}C_3C_{37}H_{66}O_3$	598.51854	ND		
22:1	$^{13}C_3C_{37}H_{64}O_3$	596.50289	ND		
22:2	$^{13}C_3C_{37}H_{62}O_3$	594.48724	ND		
22:3	$^{13}C_3C_{37}H_{60}O_3$	592.47159	592.4698; 592.4698	−3.02; −3.02	8.28; 8.64
22:4	$^{13}C_3C_{37}H_{58}O_3$	590.45594	590.4564	0.78	8.03
22:5	$^{13}C_3C_{37}H_{56}O_3$	588.44029	ND		
22:6	$^{13}C_3C_{37}H_{54}O_3$	586.42464	ND		
24:0	$^{13}C_3C_{39}H_{70}O_3$	626.54984	ND		
26:0	$^{13}C_3C_{41}H_{74}O_3$	654.58114	ND		

LC-HRMS, liquid chromatography high-resolution mass spectrometry; ND, not detected; RT, retention time.

Contamination is unlikely to explain the high levels (up to 400 ng·g^{-1}) in tissues and hemolymph in some of the other studies. Therefore, an alternative explanation should be considered. It is respectfully suggested that the researchers may not have been measuring these steroids but instead been reporting "false positive" data generated by the way in which the immunoassays were carried out. Ellis et al. (36) have shown how easy it is for mistakes to be made when using immunoassay kits and how likely it is that such mistakes adversely affect an estimated 30% of studies that have been published on fish steroids. One likely cause of error in most of the mollusk studies is that the researchers have relied on simple aqueous extraction to solubilize the cortisol instead of extracting it with an organic solvent (such as diethyl ether or ethyl acetate). Only one such study did so (20), and it is likely not a coincidence that this study reported the lowest overall cortisol concentrations of all the studies. The point of extracting the cortisol with an organic solvent is that it separates it from salts and proteins that are known to interfere with antibody binding and potentially generate false-positive readings in immunoassays. Even having extracted the steroids with an organic solvent, it is considered good practice to characterize the immunoactivity before publishing (for example, by running some of the extracts on HPLC to show that the cortisol immunoactivity elutes in the same position as standard cortisol). However, this was not done in any of the immunoassay studies on mollusks.

The final thing to discuss regarding the likely presence of corticosteroids in mollusks is that the release of cortisol and corticosterone in vertebrates is regulated via a relatively complex chain of events involving the hypothalamo-pituitary-adrenal axis (8). There is no equivalent of the hypothalamus, pituitary gland, or adrenals in the octopus. Nor is there any firm evidence for the existence of the pituitary hormone, corticotropin. Although there is immunological evidence for the existence of a peptide that resembles the vertebrate corticotropin-releasing hormone (CRH) (37) and genetic evidence for a protein equivalent to the corticotropin-releasing hormone receptor 2 (CRHR2) in cephalopods (35), there is, in the absence of any corticotropin, no evidence that either of these proteins has anything to do with the control of corticosteroid production. Any belief that they do so is based purely upon their names and is just one more example of "nominative determinism" that has caused much confusion in the field of invertebrate endocrinology (38, 39).

E_2 Uptake

As stated in the INTRODUCTION, when designing the cortisol uptake experiment, E_2 was initially included as a positive control. Previous studies on *M. edulis* showed that this species not only had a strong ability to absorb E_2 from water (30), but also conjugated it to fatty acids to form highly lipophilic esters and that E_2 could be retained by the animals in this conjugated form for several weeks. As expected, like *M. edulis* and other mollusks (38), the octopus has the same ability to absorb and esterify E_2 from the water. It was calculated that each octopus effectively had an E_2 clearance rate from the water of 9.2 L·h^{-1}·kg^{-1}, similar to that of the blue mussel at 10 L·h^{-1}·kg^{-1} (30). It should be stressed that these numbers are independent of the concentration of steroids in the water. A simple way to understand them is to imagine an animal living in water with a constant concentration of E_2 of 1 ng·L^{-1}. After a period of 1 h, the animal will have absorbed 10 ng from 10 L, and after 24 h, a total of 240 ng. If the initial concentrations were 2 ng·L^{-1}, then this final value would be 480 ng.

The high rate of E_2 absorption turns out to be an interesting finding in itself, even though it has no link to stress or the presence/absence of corticosteroids. Concentrations of E_2 have been measured numerous times in mollusk tissues

in the belief that it regulates ovarian development. However, this belief has been challenged by Scott (13, 34, 38) who argued that although the evidence for its endogenous synthesis and bioactivity in mollusks is very poor, the evidence (at least in bivalves and gastropods) that it can be absorbed from water and, importantly, stored intact for weeks, or even months, covalently attached to fatty acids, is incontrovertible. This fact should be considered in conjunction with the fact that freshwater and seawater sources are known to be widely contaminated with E_2 (40, 41). It could be argued that esterification, especially since it appears to be localized in the gills, is a mechanism for keeping exogenous E_2 from interfering with the role of endogenous E_2. However, this is highly speculative. There is theoretically nothing to stop this esterified E_2 from being "deesterified" (i.e., hydrolyzed and released back into the animal as intact "free" steroid) in exactly the same manner as "everyday" storage fats are turned back into glycerol and free fatty acids. This article shows that cephalopods have the same mechanism for E_2 uptake and sequestration as bivalves and gastropods and calls into question the significance of previous reports on the presence of E_2 in the tissues of *O. vulgaris* (42, 43).

It is interesting that the bulk of the esterified E_2 was found in gill tissue. It is suggested that this is because the gills are the main point of uptake of the labeled steroid and that this is probably where the esterification takes place. However, as the study was only done over 24 h, the long-term fate of this esterified E_2 is unknown, that is, how rapidly it gets incorporated into additional tissues over time or for how long the esters remain in the octopus. That the E_2 is indeed esterified to fatty acids has been shown by the existence of compounds with the exact predicted masses of [^{13}C]-E_2 esterified to known long-chain fatty acids in gill tissue. Compounds putatively formed by esterification of testosterone, 5α-dihydrotestosterone and E_2 to relatively short-chain fatty acids (C16 and C18) were previously identified in *Mytilus* spp. in the same way (44, 45). The difference between the studies perhaps indicates differences in the types of fatty acids that are available in the tissues of the two species. One further observation in the present study was that, unlike *M. edulis* (30) but like *L. stagnalis* (13), *O. vulgaris* did not appear to convert any of the E_2 in the water into E_2-3-sulfate. This was shown to be a strong conversion pathway in *M. edulis* (30).

Perspectives and Significance

In conclusion, the evolutionary argument that *O. vulgaris* is highly unlikely to have a stress response based on corticosteroids found in bony vertebrates has been borne out by the present findings. Neither cortisol nor corticosterone was found in hemolymph or water (or in the albeit small number of tissue extracts that were examined). A different approach is likely to be needed to provide the sort of scientific evidence that is urgently needed to answer questions on cephalopod welfare and sentience (46). *O. vulgaris* was found to be just like bivalve mollusks and gastropods in that it showed not only a poor ability to absorb cortisol from the water but a very strong ability to absorb E_2. It also showed a strong ability to esterify the E_2 to long-chain fatty acids. The purpose, if any, of the esterification process is still unknown. Based on the results in this article, nothing can be concluded about the potential role of E_2 as a reproductive steroid in *O. vulgaris*, except for it to be pointed out that its presence in the tissues of *O. vulgaris* is not a firm evidence that it has been endogenously synthesized.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.27966927.v1.

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DISCLOSURES

Four of the authors of this study (R.T., P.G., P.T., and D.C.) have an affiliation with PESCANOVA BIOMARINE CENTER, an organization in the aquaculture sector. However, this affiliation has not influenced the design, execution, analysis, and conclusions of this study, which have been conducted in an objective and independent manner. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

A.P.S. and J.R. conceived and designed research; C.C., L.M.-M., L.G.-P., R.T., P.G., P.T., D.C., and J.R. performed experiments; B.H.M., C.C., L.G.-P., A.P.S., and J.R. analyzed data; B.H.M., A.V.C., A.P.S., and J.R. interpreted results of experiments; B.H.M. and J.R. prepared figures; A.P.S. and J.R. drafted manuscript; B.H.M., A.V.C., A.P.S., and J.R. edited and revised manuscript; B.H.M., C.C., L.M.-M., L.G.-P., R.T., P.G., P.T., D.C., A.V.C., A.P.S., and J.R. approved final version of manuscript.

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