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RESEARCH ARTICLE

The influence of 17β-estradiol on intestinal calcium carbonate precipitation and osmoregulation in seawater-acclimated rainbow trout (*Oncorhynchus mykiss*)

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SUMMARY

The intestine of marine teleosts produces carbonate precipitates from ingested calcium as part of their osmoregulatory strategy in seawater. The potential for estrogens to control the production of intestinal calcium carbonate and so influence osmoregulation was investigated in seawater-acclimated rainbow trout following intraperitoneal implantation of 17β-estradiol (E2) at two doses (0.1 and 10 μg E2 g^{-1}). Levels of plasma vitellogenin provided an indicator of estrogenic effect, increasing significantly by three and four orders of magnitude at the low and high doses, respectively. Plasma osmolality and muscle water content were unaffected, whereas E2-treated fish maintained lower plasma [Na^+] and [Cl^-]. Plasma [Ca^{2+}] and [Mg^{2+}] and muscle [Ca^{2+}] increased with vitellogenin induction, whereas the intestinal excretion of calcium carbonate was reduced. This suggests that elevated levels of circulating E2 may enhance Ca^{2+} uptake *via* the gut and simultaneously reduce $CaCO_3$ formation, which normally limits intestinal availability of Ca^{2+}. Increasing E2 caused an elevation of [Na^+] and [Cl^-] and a reduction of [HCO_3^-] in intestinal fluid. We speculate that E2 may influence a number of intestinal ion transport processes that ultimately may influence water absorption: (1) reduced NaCl cotransport, (2) reduced Cl^- uptake *via* Cl^-/HCO_3^- exchange and (3) reduced precipitation of Ca^{2+} and Mg^{2+} carbonates. Despite these effects on intestinal ion and water transport, overall osmoregulatory status was not compromised in E2-treated fish, suggesting the possibility of compensation by other organs.

Key words: calcification, intestine, estrogen, marine teleost, endocrine.

INTRODUCTION

Marine teleost fish are osmoregulators, maintaining their body fluids (~300 mOsm kg^{-1}) hypo-osmotic to seawater (~1000 mOsm kg^{-1}), and thus face the problem of osmotic water loss and the passive gain of ions (Evans et al., 2005; Marshall and Grosell, 2006). To overcome this potential dehydration, they continuously drink the surrounding seawater at rates of 1–5 ml kg^{-1} h^{-1} (Smith, 1930; Evans, 1993; Fuentes et al., 1996; Marshall and Grosell, 2006) and excrete the excess salt load across the gills (Na^+ and Cl^-) and in the rectal fluid and urine (Ca^{2+}, Mg^{2+} and SO_4^{2-}) (Marshall and Grosell, 2006). Therefore, the gastrointestinal tract has a key role in processing ingested seawater and absorbing water (Shehadeh and Gordon, 1969; Marshall and Grosell, 2006).

The most intensively studied driving force for water absorption in the intestine of seawater teleosts is the simultaneous absorption of Na^+ and Cl^- (Smith, 1930; Skadhauge, 1974; Usher et al., 1991; Marshall and Grosell, 2006), driven by a range of membrane transporters including basolateral Na^+,K^+-ATPase and two Na^+ and Cl^- cotransporters, Na^+:Cl^- and Na^+:K^+:2Cl^- (Musch et al., 1982; Grosell, 2006; Grosell, 2010). A second direct driving force for fluid absorption involves the apical anion exchanger Cl^-/HCO_3^-, which may contribute as much as 70% of the total Cl^- and water absorption by this epithelium under non-fed conditions (Grosell et al., 2005; Grosell, 2006; Grosell, 2010). A third indirect driving force for water absorption is the precipitation of divalent cations (Ca^{2+} and Mg^{2+} from ingested seawater). The presence of elevated concentrations of HCO_3^- (30–110 mmol l^{-1}), driven by the above anion exchanger, and high luminal pH (up to 9.2) in the intestinal fluid provide favourable conditions for the precipitation of calcium and magnesium carbonates. In the absence of carbonate precipitation, the poorly absorbed Ca^{2+} and Mg^{2+} would rise to very high levels (67 and 353 mmol l^{-1}, respectively), which would clearly retard osmotic water absorption from the intestine (Wilson et al., 2002). Removal of most of the ingested Ca^{2+} and a smaller proportion of the ingested Mg^{2+} by alkaline precipitation conveniently avoids an excessive accumulation of these ions, reducing the luminal osmolality and thereby maximising the potential for water absorption by the intestine (Wilson et al., 2002; Marshall and Grosell, 2006; Whittamore et al., 2010).

Seawater ingestion provides the intestine with a large potential source of Ca^{2+} for absorption and, because systemic Ca^{2+} is very tightly regulated within the intracellular and extracellular fluids, the intestine has an important role in Ca^{2+} homeostasis. As the gills do not participate in the excretion of divalent ions (Flik and Verbost, 1993), any Ca^{2+} absorbed by the intestine and not sequestered by any of the internal pools (such as bones, otoliths, scales, gonads, etc.) will be removed by the kidneys. The intestinal precipitation process is thought to assist calcium homeostasis in marine fish by limiting the intestinal absorption of Ca^{2+} from ingested seawater. Indeed, *in vivo* the vast majority (>80%) of Ca^{2+} entering the intestine can be accounted for as excreted *via* the gut, with ~40–60% in the form of solid $CaCO_3$ (Wilson and Grosell, 2003). Any Ca^{2+} absorbed and in excess of growth requirements must be excreted, but such an excess enhances the risk of kidney stones because of high urinary concentrations of SO_4^{2-} and the insolubility of calcium sulphate (Wilson and Grosell, 2003), in addition to very low urine

flow rates in marine teleosts (Hickman and Trump, 1969; McDonald et al., 1982; Fletcher, 1990). However, other studies report a net absorption of Ca^{2+} across the intestine (Sundell and Björnsson, 1988; Flik et al., 1990; Flik and Verbost, 1993), and in particular, the intestine can contribute in times of extra demand for Ca^{2+}, such as during gonadal maturation. During gonadal maturation, the females require abundant Ca^{2+} to provide oocytes with a ready store for subsequent skeletal development of their offspring. In salmonids, estradiol can induce Ca^{2+} resorption from the scales and bones (Mugiya and Watabe, 1977; Carragher and Sumpter, 1991; Persson et al., 1994) and from the ambient water, at least under freshwater conditions (Persson et al., 1994). Because of the continual ingestion of Ca^{2+}-rich seawater, the intestine of marine fish has potential access to a readily available pool of Ca^{2+}, which can be used to supply developing oocytes during sexual maturation.

Sex steroid hormones include androgens and estrogens. The function of the latter is well conserved in vertebrates and includes the regulation of oocyte growth within the gonads and secondary sex characteristics and behaviours (Nagahama et al., 1995; Tyler and Sumpter, 1996). One estrogen, 17β-estradiol (E2), has been shown to play a role in the regulation of ionic and osmotic homeostasis, and the physiological processes involved in salt and water transport in fish (Mancera et al., 2004; Carrera et al., 2007). The effects reported include inhibition of key osmoregulatory processes (Mancera et al., 2004; McCormick et al., 2005; Lerner et al., 2007). For example, significant increases in total and ionic Ca^{2+} have been observed in the plasma of sea bream (*Sparus aurata*) following intraperitoneal implants containing 10 μg E2 g^{-1} body mass (Guerreiro et al., 2002; Guzman et al., 2004). Further indirect evidence supporting the involvement of E2 in osmoregulation is provided by the expression of estrogen receptors in the intestine in a number of fish species including, the sea bream (Socorro et al., 2000) and fathead minnows (*Pimephales promelas*) (Filby and Tyler, 2005). E2 exerts its effect by binding and activating the nuclear estrogen receptors (Edwards, 2005). In the rainbow trout, four estrogen receptors have been described and expression of all four genes was demonstrated in the digestive tract (Nagler et al., 2007). It has been suggested that E2 and its receptors, expressed in the gills of salmonids, may be involved in osmoregulation (Rogers et al., 2000).

A number of previous studies have examined the effect of estradiol on hypo-osmoregulation in teleosts (Madsen and Korsgaard, 1991; Vijayan et al., 2001; Guzman et al., 2004; Mancera et al., 2004). However, only one study has investigated the potential for calciotropic hormones [parathyroid hormone-related protein (PTHrP) and stanniocalcin 1] as endocrine factors to regulate bicarbonate and calcium transport by the marine teleost intestine *in vitro* (Fuentes et al., 2010). In that study, they reported antagonistic action in the control of Ca^{2+} and HCO_3^- movement in the intestine of marine teleost fish such as sea bream. PTHrP increased Ca^{2+} uptake and reduced HCO_3^- secretion, whereas stanniocalcin 1 had the opposite effect, reversing Ca^{2+} net flux from absorptive to secretory, and promoting intestinal HCO_3^- secretion (Fuentes et al., 2010). In their study, Fuentes et al. measured the HCO_3^- and Ca^{2+} transport rates but did not measure the $CaCO_3$ precipitation process itself. The present study raises the hypothesis that estrogens can influence intestinal handling of ingested Ca^{2+} in seawater teleost fish, and specifically the relative amount of ingested Ca^{2+} that is precipitated and excreted in the form of calcium carbonate, which in turn will also affect overall osmoregulation. We aimed to test this hypothesis using seawater-acclimated rainbow trout to represent marine teleosts generally, by using intraperitoneal implants to raise internal levels of the endogenous estrogen E2.

MATERIALS AND METHODS

Experimental animals and acclimation to seawater

Immature female rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) (N=30; 194.1±3.5 g, 24.8±0.2 cm fork length) were obtained from Houghton Springs fish farm (Blandford Forum, Dorset, UK) and kept in aerated, dechlorinated freshwater (pH 7.5, 11.2±0.3°C) at the University of Exeter. Fish were fed on commercial pellet feed (BioMar, 5 mm, Aqualife, Brande, Denmark) while in freshwater, but were not fed thereafter (i.e. during the salinity acclimation period or during the implant experiment itself). Fish were then transferred to a salinity of 10 ppt (approximately iso-osmotic) for 48 h. Salinity was then increased to 20 ppt for a further 24 h and then to full strength seawater (salinity of 33 ppt; Na^+=420; K^+=10; Ca^{2+}=10; Mg^{2+}=47; Cl^-=455; SO_4^{2-}=21 mmol l^{-1}), where the fish were left to acclimate for 10 days before starting the experiments. All fish experiments described in this study were conducted according to UK Home Office guidelines.

Implantation of E2

After acclimation to seawater, fish were anaesthetised in a 50 mg l^{-1} solution of MS-222 (Pharmaq Ltd, Fordingbridge, Hampshire, UK) buffered with 150 mg l^{-1} $NaHCO_3$, followed by prolonged aeration to restore normal CO_2 and pH levels. Estradiol was dissolved in coconut butter (Sigma-Aldrich, St Louis, MO, USA) to achieve the concentrations required for the experiment [control=coconut butter only (vehicle); low dose=0.1 mg E2 ml^{-1}; high dose=10 mg E2 ml^{-1}]. Fish were randomly allocated to one of the three treatments (10 fish per treatment) before intraperitoneal injection at a dose of 1 μl per gram of fish, to achieve the required doses of 0.1 mg and 10 mg of E2 per kilogram of fish body mass. After injection, the needle was withdrawn and ice was applied to the injection site to help solidify the implant and avoid losses of the injected coconut butter. Fish were then transferred to individual chambers (18 l capacity) containing aerated seawater.

In vivo experimental procedures and sampling

A semi-static system was used, whereby fish were maintained in their individual chambers for 2 weeks before terminal sampling. Intestinal precipitates of $CaCO_3$ excreted into the chamber were collected twice daily (10.00 and 17.00 h) from each fish, and 80% of the water in each chamber was renewed every 48 h.

Two weeks after E2 implantation, fish were administered an overdose of anaesthetic (300 mg l^{-1} buffered MS-222), followed by blood sampling (~1 ml) *via* caudal puncture. The blood sample was divided into two aliquots, one added to 10 μl of aprotinin (Sigma-Aldrich) to reduce enzymatic degradation of vitellogenin, and the second without aprotinin for ion measurements. Blood samples were then centrifuged (16,060 ***g*** for 5 min at 4°C; Heraeus by Biofuge Fresco, Kendro Laboratory Products, Hanau, Germany) and the plasma was separated from the blood cells. The undiluted plasma was used to measure osmolality and chloride, and the remainder was subsequently diluted for ion analysis. The abdominal cavity was then opened by a ventral midline incision and the entire intestine was ligated to collect fluid for analysis. A piece of epaxial white muscle, ventral and lateral to the dorsal fin, was removed (2.09±0.06 g) for ion and water content measurement. The peritoneal cavity was then examined to confirm the integrity of the injected implant of coconut butter.

Analytical techniques for plasma and muscle variables

Osmolality was measured on 10 μl samples using a vapour pressure osmometer (Wescor Vapro 5520, Logan, UT, USA). Cations (Na^+,

Ca^{2+} and Mg^{2+}) and anions (SO_4^{2-}) were measured by ion chromatography (Dionex ICS-1000, Sunnyvale, CA, USA) on samples of plasma, intestinal fluids and seawater, following appropriate dilution. Total CO_2 of intestinal fluids was measured using a carbon dioxide analyser (Mettler Toledo model 965D, Columbus, OH, USA). The pH of intestinal fluid was determined using an Accumet combined microelectrode (Fisher Scientific, Loughborough, UK) connected to Hanna HI 8314 pH meter (Hanna Instruments Ltd, Leighton Buzzard, Bedfordshire, UK).

White muscle samples were transferred to a pre-weighed Teflon tube and then to an oven (70°C), where they were dried to a constant mass. The dried samples were digested with five volumes of nitric acid (15.6 N; AnalaR, BDH Laboratory Supplies, Poole, Dorset, UK). Following complete digestion, two sets of dilutions were prepared (100× and 500×) and 10% (w/v) lanthanum chloride (Fisher Scientific) was added to achieve 0.1% (w/v) for measurement of divalent ions by atomic absorption spectrophotometry (Thermo Elemental SOLAAR AAS, Cambridge, UK). Muscle chloride was measured on 100-fold diluted digests using a colourimetric assay (Zall et al., 1956) and the absorbance was read on a microplate reader (Molecular Devices, Spectra MAX 340pc, Sunnyvale, CA, USA) at 480 nm.

Plasma vitellogenin was measured as an indicator of the exposure to E2 using a homologous ELISA, according to previously described protocols (Tyler et al., 2002). Plasma was diluted at least 1:10 prior to analysis of vitellogenin concentrations, resulting in a detection limit for this study of 30 ng ml^{-1}.

Determination of carbonate content of intestinal precipitates

The collected $CaCO_3$ precipitates were rinsed with deionised water before being oven dried (45°C) overnight. The next day, the dry weight was taken before adding 1 ml of 5% (w/v) sodium hypochlorite (Fisher Scientific) and left for 4 h to digest the organic mucus component. Samples were then rinsed three times with deionised water and centrifuged before being oven-dried for a further 24 h before the final dry mass measurement was taken. The cleaned, dried inorganic samples of the precipitates were then sonicated (Vibra-Cell, Sonics and Materials Inc., Newtown, CT, USA) in 20 ml of ultrapure water (Maxima Ultrapurewater, ELGA, Marlowe, Buckinghamshire, UK) for double end-point titration to determine the bicarbonate equivalent content.

The bicarbonate equivalent (HCO_3^-+$2CO_3^{2-}$) content of these precipitates was determined using the double titration method of Hills (Hills, 1974), as described by Wilson et al. (Wilson et al., 2002) using an autotitrator (TIM845 titration manager and SAC80 automated sample changer, Radiometer Analytical SAS, Villeurbanne, France). Samples were gassed with N_2 to remove all HCO_3^- and CO_3^{2-} as gaseous CO_2 during acidification and to ensure a stable pH measurement when returning to the starting pH (Wilson et al., 2002). Subsequently, samples were re-acidified to ensure complete dissolution of Ca^{2+} and Mg^{2+} that had been liberated during titration of the precipitates. These acidified samples were then diluted for analysis of Ca^{2+} and Mg^{2+} content by ion chromatography.

Calculations and data analysis

The rate of excretion of carbonate precipitates was calculated (μequiv. kg^{-1} h^{-1}) based on the titration of excreted precipitates to establish their total bicarbonate equivalent content ($[HCO_3^-+2CO_3^{2-}]$) as follows:

$$\text{CaCO}_3 \text{ excretion rate} = ([HCO_3^- + 2CO_3^{2-}]) / (M \times t), \quad (1)$$

where M is the fish mass (kg) and t is the duration (h) of the precipitate collection period.

Hickman (Hickman, 1968) and Genz et al. (Genz et al., 2008) have previously used SO_4^{2-} concentrations in rectal fluid as a surrogate of intestinal fluid absorption based on the assumption that intestinal epithelium is impermeable to $MgSO_4$, thus considering it as a potential endogenous marker for water absorption. In the present study, seawater and intestinal $[SO_4^{2-}]$ for each fish were measured and used to obtain an estimate of fractional water absorption (%) by the intestine by using the formula:

$$\text{Fractional water absorption} = 100 - \{([SO_4^{2-}]_{\text{Seawater}} / [SO_4^{2-}]_{\text{Intestinal}}) \times 100\}. \quad (2)$$

The concentration of bicarbonate equivalents (HCO_3^- + $2CO_3^{2-}$) in the intestinal fluid were calculated according to the Henderson–Hasselbalch equation using measurements of total CO_2 and pH:

$$[HCO_3^-] = [TCO_2] / (1+10^{(\text{pH} - 9.46)}). \quad (3)$$

The total CO_2 (mmol l^{-1}) content $[TCO_2]$ is the sum of [molecular CO_2] + $[HCO_3^-]$ + $[CO_3^{2-}]$. Therefore, the carbonate (CO_3^{2-}) fraction was calculated by the following equation:

$$[CO_3^{2-}] = [TCO_2] - [HCO_3^-]. \quad (4)$$

Hence, total HCO_3^- equivalent=$[HCO_3^-]$+2$[CO_3^{2-}]$.

Vitellogenin data were log transformed prior to analysis. Significant differences between treatments were determined using one-way ANOVA followed by the Student–Newman–Keuls method to determine significant differences between individual groups, when data were normally distributed and had approximate equal variance. For data that failed to meet these assumptions, significant differences between treatment groups were determined using the non-parametric Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's method to determine differences between individual groups. Differences between treatment groups were considered to be significant at P<0.05 and highly significant at P<0.001. As an additional indication of the effect of estrogenic stimulation on physiological variables, regression analyses were performed comparing these against plasma vitellogenin concentrations (log transformed) of individual fish. Correlation analysis was carried out by linear regression, and the Pearson correlation coefficient (r) was calculated. For all statistical analyses, P<0.05 was considered significant. All statistical analyses were conducted using SigmaStat 3.5 (Systat Software, Inc., San Jose, CA, USA).

RESULTS

Plasma vitellogenin and osmoregulatory variables

Seawater-acclimated rainbow trout implanted with E2 for 2 weeks showed highly significant inductions of plasma vitellogenin (Fig. 1), indicating strong and dose-dependent estrogenic response. The E2 implants did not affect plasma osmolality at either dose. However, increasing levels of circulating vitellogenin were associated with significant decreases in both plasma $[Na^+]$ and $[Cl^-]$ (Fig. 2). Estrogen exposure, as expected, caused a dose-dependent and highly significant increase in plasma $[Ca^{2+}]$, and plasma $[Mg^{2+}]$ also increased significantly, but in the high dose group only (Fig. 3). No significant differences were detected in the plasma $[SO_4^{2-}]$ (Table 1).

Concurrent with observations of plasma osmolality, E2 treatment did not affect muscle water content of the seawater-acclimated rainbow trout in any of the treatment groups (Table 1). Calcium concentrations in white muscle increased significantly by almost

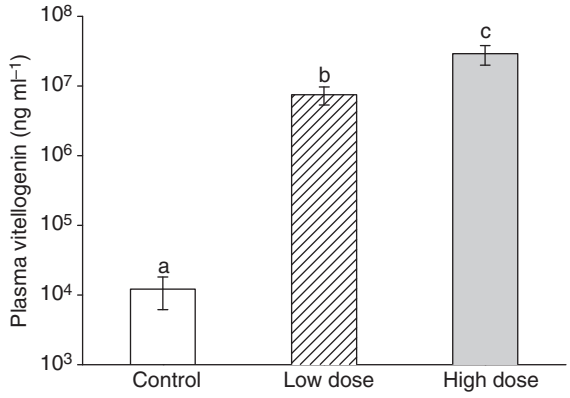

Fig. 1. Plasma vitellogenin concentration in seawater-acclimated rainbow trout after 2 weeks implantation with 17β-estradiol (E2; 0.1 and 10 μg E2 g^{-1}). Data are means ± s.e.m. Asymmetric error bars are due to the logarithmic scale of the vitellogenin data. Different letters above bars indicate significant differences between treatment groups within a given time point (P<0.001, Student–Newman–Keuls method).

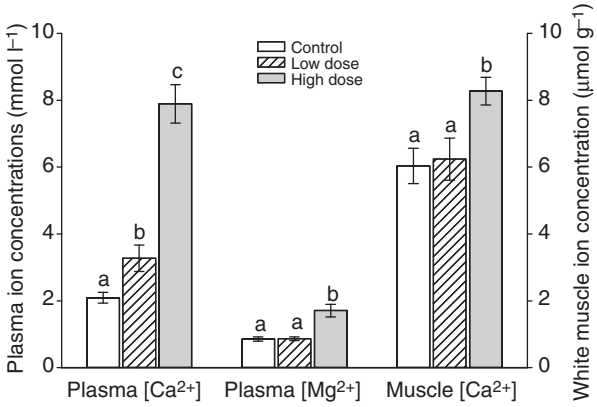

Fig. 3. Plasma [Ca^{2+}] and [Mg^{2+}] and white muscle [Ca^{2+}] in seawater-acclimated rainbow trout treated with E2 (0.1 and 10 μg E2 g^{-1}) for 2 weeks. Different letters above bars indicate significant differences between treatment groups. Data are means ± s.e.m.

40% in the high dose group in comparison to the control group (Fig. 3).

Intestinal fluid chemistry and fractional water absorption

E2 had no significant effect on intestinal fluid osmolality. Intestinal [Na^+] was significantly elevated by 33% in the high dose group in comparison to controls. No significant effect was detected in [Cl^-], although mean values followed the same upward trend as intestinal [Na^+] with increasing E2 dose (Table 2). Under control conditions, the intestinal fluid pH was distinctly alkaline (pH=8.41±0.06), with high concentrations of HCO_3- equivalents (95.7±5.8 mequiv. l^{-1}). pH and [HCO_3-] decreased significantly in the high dose group by 0.2 pH units and 22 mequiv. l^{-1}, respectively, in comparison to the control (Table 2). Magnesium and SO_4^{2-} concentrations were significantly lower in the high dose group than in the control group by 23 and 27%, respectively, with no differences observed in relation to Ca^{2+} (Table 2). The estimated fractional water absorption based on [SO_4^{2-}] was significantly reduced by 17% at the highest E2 dose group (Fig. 4).

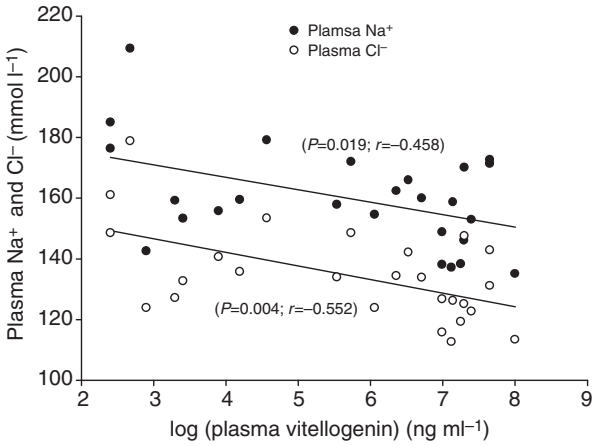

Fig. 2. Relationship between plasma vitellogenin and plasma Na^+ and Cl^-. Correlation analysis was carried out by linear regression, and the Pearson correlation coefficient (r) was calculated.

Intestinal excretion of $CaCO_3$ precipitates

The $CaCO_3$ excretion rate was significantly reduced following exposure to E2 (Fig. 5A), with a 50% reduction observed in the mean value of the high dose group in comparison to the control group during the second week after implantation. A similar relationship was observed in the first week (declining $CaCO_3$ excretion with increasing plasma vitellogenin), although this was not significant (Fig. 5B). During the second week, the rate of excretion of Ca^{2+} within the precipitates was also significantly reduced by 40% in the high dose group compared with the control (10.5±2.0 *versus* 17.6±2.1 μmol kg^{-1} h^{-1}, respectively). The rate of Mg^{2+} excretion within the precipitates showed a similar significant decrease, and this was apparent in both the low and high dose groups (2.0±0.3 and 2.2±0.2 μmol kg^{-1} h^{-1}, respectively) in comparison to the control (3.0±0.3 μmol kg^{-1} h^{-1}) during the first week, but only significantly reduced in the high dose group in comparison to the control (1.1±0.2 and 2.5±0.6 μmol kg^{-1} h^{-1}, respectively) during the second week.

DISCUSSION

The major aim of this study was to investigate the possible effect(s) of E2 on the handling of calcium by the intestine, in particular $CaCO_3$ formation, and the implications this may have in terms of osmoregulation. To achieve this, we experimentally elevated the circulating estrogen levels using intraperitoneal implants of E2. Most of the measured parameters within the present data set were compatible with our hypothesis in terms of the reduction in $CaCO_3$ formation and excretion, and the subsequent potential for enhanced absorption of ingested divalent ions (particularly Ca^{2+}).

Plasma vitellogenin as a marker of elevated circulating E2

Vitellogenin is a complex precursor protein for yolk production in oviparous vertebrates produced by the liver. It has been widely used as a biomarker for exposure to estrogens based on circulating levels in the plasma of immature female or male fish (Sumpter and Jobling, 1995). Plasma vitellogenin was therefore measured in the present study as an indicator of the biological response to the E2 implantation treatments and their estrogenic stimulation. The concentrations of vitellogenin in the control group corresponded to those expected in female rainbow trout during early gonadal development (Bon et al., 1997).

Table 1. Plasma osmoregulatory variables and muscle water content in seawater-acclimated rainbow trout implanted with either control or one of two doses of 17β-estradiol (0.1 and 10 μg E2 g^{-1}) for 2 weeks

	Control	Low dose	High dose
Muscle water content (%)	78.2±0.3	78.2±0.2	77.5±0.3
Plasma osmolality (mOsm kg^{-1})	322.8±8.0	315.9±5.1	326.2±8.3
Plasma [Na^+] (mmol l^{-1})	168.8±6.8[a]	157.8±4.0	153.2±4.9[b]
Plasma [Cl^-] (mmol l^{-1})	144.6±5.9[a]	132.7±4.2	126.5±3.5[b]
Plasma [SO_4^{2-}] (mmol l^{-1})	1.2±0.2	0.7±0.1	0.9±0.2

Different superscripted letters indicate significant differences between treatment groups.

Elevated levels of plasma vitellogenin were clearly observed in a dose-dependent manner for the low and high E2 treatments (Fig. 1). For females injected with the low dose and high dose of E2, the concentrations of vitellogenin measured in the plasma correspond with those of females undergoing early and late vitellogenesis during the reproductive cycle (Bon et al., 1997).

The role of E2 in promoting absorption of divalent cations *via* the intestine

Treatment with E2 was associated with enhanced uptake of Ca^{2+} into the body, indicated by very large elevations in calcium concentration in both the plasma and muscle (Fig. 3). The most obvious routes for enhanced uptake of Ca^{2+} from the marine environment are the gills and the intestine. We have no direct evidence of E2 directly enhancing Ca^{2+} uptake from the intestine. However, the potential scope for this being an important route is clear and our data provide some quantitative, though indirect, support for this. Over the 2 week experimental period, the cumulative excretion of calcium in the form of gut precipitates in the high dose E2 group was 1815 μmol less (per kilogram of fish) than in the control group. There was also no simultaneous accumulation of dissolved Ca^{2+} in the intestinal fluid (Table 2), so we have assumed that all of the Ca^{2+} that was not precipitated was therefore absorbed by the intestine. The corresponding increase in Ca^{2+} stored in the plasma and white muscle of the high dose E2 group, relative to the controls, accounted for 69% of the reduction in Ca^{2+} excreted as precipitated carbonates. This proportion was estimated based on a plasma volume of 3.05% and a white muscle volume of 49% of total body mass in rainbow trout (Bushnell et al., 1998; Taylor et al., 1996). The remaining 31% of the Ca^{2+} potentially absorbed by the intestine could be stored in other tissues that were not measured. The reduced intestinal carbonate precipitation in response to E2 would be a logical component of an estrogenic stimulation of Ca^{2+} absorption processes in the intestine, as we hypothesised. It is worth noting that although Guerreiro et al. (Guerreiro et al., 2002) concluded that intestinal uptake of Ca^{2+} was not affected by E2, they only considered measurements based on intestinal fluid, and therefore did not take into account any changes in Ca^{2+} present in carbonate precipitates.

Plasma divalent cations, [Ca^{2+}] and [Mg^{2+}], were both increased in a dose-dependent manner after treatment with E2 (Fig. 3), perhaps not surprisingly given that these two ions are known to increase in parallel with the elevated plasma vitellogenin in fish (Guerreiro et al., 2002). Indeed, increases in plasma Ca^{2+} and Mg^{2+} following estrogenic stimulation have previously been used as indirect indicators of effective vitellogenin induction (Gillespie and de Peyster, 2004). Highly elevated [Ca^{2+}] in white muscle of the high dose group suggests that extra Ca^{2+} was accumulating in more than one major compartment (i.e. plasma as well as muscle). A key question for the present study is the source of the extra calcium that builds up in plasma and white muscle in response to E2, and in particular whether some of this could be due to enhanced uptake by the intestine.

Calcium uptake in the intestinal tract follows a similar cellular route to that in the gills, and occurs *via* active transport mechanisms (Flik et al., 1990). The cytoplasm of enterocytes has very low free Ca^{2+} levels and is electrically negative relative to the intestinal lumen, which subsequently generates an electrochemical gradient to drive Ca^{2+} across the apical brush border membrane into the enterocytes. Enterocytes from the Atlantic cod (*Gadus morhua*) demonstrated a presence of voltage-gated L-type Ca^{2+} channels mainly located on the apical side of the cell, which suggests the involvement of these channels in the entry of Ca^{2+} into the enterocytes (Larsson et al., 1998). The subsequent basolateral extrusion of Ca^{2+} to the extracellular fluid occurs *via* transporters, which include Ca^{2+}-ATPase (Flik et al., 1996). Arjmandi et al. (Arjmandi et al., 1994) showed that E2 administration in rats promotes intestinal absorption of Ca^{2+} *in vivo*. This supports the idea that there is a route whereby Ca^{2+} can be absorbed from the intestine, which could potentially fulfil the increased need of Ca^{2+} in female fish, to support the vitellogenic growth of oocytes during gonadal maturation.

Table 2. Intestinal fluid osmolality and ion concentrations in seawater-acclimated rainbow trout implanted with either control or one of two doses of 17β-estradiol (0.1 and 10 μg E2 g^{-1}) for 2 weeks

Intestinal fluid	Control	Low dose	High dose
Osmolality (mOsm kg^{-1})	332.3±11.3	313.3±7.0	344.3±17.0
[Na^+] (mmol l^{-1})	43.4±3.2[a]	49.5±3.6	64.5±5.3[b]
[Cl^-] (mmol l^{-1})	74.9±6.0	81.5±5.3	87.2±6.1
[Ca^{2+}] (mmol l^{-1})	6.7±0.7	7.7±0.5	6.9±0.8
[Mg^{2+}] (mmol l^{-1})	152±8.3[a]	139.0±13.6	116.6±9.8[b]
[SO_4^{2-}] (mmol l^{-1})	68.8±3.8[a]	59.6±4.9	50.3±4.7[b]
[HCO_3^-] (mequiv. l^{-1})	95.7±5.8[a]	95.9±8.4	73.8±7.9[b]
pH	8.4±0.1[a]	8.4±0.1	8.2±0.1[b]

Different superscripted letters indicate significant differences between treatment groups.

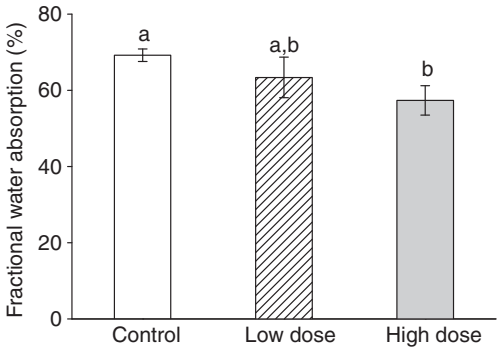

Fig. 4. Fractional water absorption in the intestine of seawater-acclimated rainbow trout after 2 weeks implantation with E2 (0.1 and 10 μg E2 g^{-1}). Different letters above bars indicate significant differences between treatment groups. Data are means ± s.e.m.

The present study draws attention to the potential for estrogens to influence the calcification process by the intestine of seawater-acclimated fish. Having shown this using implants of exogenous E2, this raises the question of whether natural elevation of endogenous estrogens (as occurs during sexual maturation of female fish) will have a similar effect. This may be important to know because estrogens in sexually maturing females may act directly on the intestine to control carbonate precipitation and the supply of Ca^{2+} for gonadal uptake, and ultimately affect osmoregulation.

Effect of E2 on calcium homeostasis in freshwater

The implantation with E2 revealed a significant increase in plasma Ca^{2+} levels. It is interesting to note that intraperitoneal injection of E2 in freshwater-acclimated fish, rather than seawater-acclimated fish such as in the present study, had no effect on Ca^{2+} absorption across the intestine of rainbow trout given 10 μg E2 g^{-1} body mass (Mugiya and Ichi, 1981). However, freshwater fish are constantly faced with an osmotic water influx and therefore have extremely low drinking rates (Flik and Verbost, 1993). In freshwater fish that are not feeding, the gut is therefore much less important than the gills in terms of uptake of exogenous Ca^{2+} (Perry and Wood, 1985). E2 treatment of freshwater rainbow trout increases the uptake of Ca^{2+} from the water (presumably *via* the gills) along with mobilization from scales (Carragher and Sumpter, 1991; Persson et al., 1994; Persson et al., 1995; Persson et al., 1998) during the induction of vitellogenin.

Effect of E2 on plasma and white muscle osmoregulatory variables

Several previous studies have presented a negative effect of E2 on the osmoregulatory performance and ion balance in freshwater, seawater and euryhaline fish following acute transfer between different salinities (Madsen and Korsgaard, 1991; Coimbra et al., 1993; Vijayan et al., 2001). Our study, however, showed no overall effect on the ability to maintain plasma osmolality or muscle water content, despite evidence in support of an inhibition of the ion transport processes in the intestine that are associated with water absorption. The reason for the differences between our data and that published in the literature are not entirely clear, but may be a consequence of the dose of E2 chosen or physiological differences in responsiveness between the fish used in each study, or differences between acute salinity challenges (previous studies) and fish maintained in a constant salinity (present study).

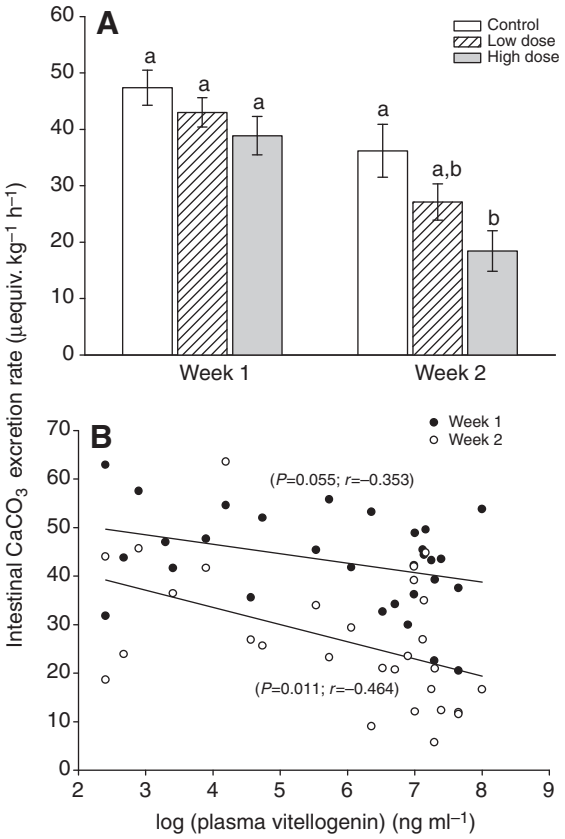

Fig. 5. (A) Precipitate carbonate excretion rate in seawater-acclimated rainbow trout treated with E2 (0.1 and 10 μg E2 g^{-1}) for 2 weeks. Different letters above bars indicate significant differences between treatment groups. Data are means ± s.e.m. (B) Relationship between the precipitate carbonate excretion rate and plasma vitellogenin. Correlation analysis was carried out by linear regression, and the Pearson correlation coefficient (*r*) was calculated.

Plasma $[Na^+]$ and $[Cl^-]$ showed a significant decrease at the high E2 dose (Table 1), which suggests that these fish were not compromised in their ability to excrete excess Na^+ and Cl^- from their plasma (performed by the gills) and no overall osmoregulatory stress was detected (plasma or muscle). Indeed, fish undergoing treatment with E2 appeared better able to hypo-regulate Na^+ and Cl^- (relative to their ambient seawater) compared with the control fish. The results suggest that the fish were able to compensate and hypo-osmoregulate despite the changes taking place in the intestine. The potential mechanisms by which estrogenic stimulation may improve hypo-regulation of these plasma ions remain to be investigated.

Effect of E2 on ion and water handling processes in the intestine

The intestine of seawater fish plays an essential role in compensating for whole-body osmotic water loss. We hypothesised that the treatment with E2 may induce a negative effect on bicarbonate secretion and $CaCO_3$ precipitation by the intestine and, in turn, the rate of intestinal water absorption. The present data support this hypothesis. Although the actual transport rates of Na^+, Cl^- and HCO_{3^-} by the intestine were not measured, the concentrations of these ions within the intestinal fluid observed across treatments were affected by E2 in a manner consistent with changes in transport

(Table 2). Similarly, the measured excretion of precipitated $CaCO_3$ was also substantially reduced (Fig. 5). Furthermore, the intestinal concentration of sulphate (used as a surrogate marker for fluid absorption) indicated that fractional absorption of water by the intestine may also have been reduced by E2 (Fig. 4). A potential caveat to this statement is that E2 could have increased the intestinal absorption of SO_4^{2-} and the apparent change in fluid absorption is an artefact of lowered intestinal [SO_4^{2-}] rather than fluid movements. However, assuming that sulphate transport was not affected, and was close to negligible as previously found (Hickman, 1968; Genz et al., 2008), a reduction in the intestinal water absorption can occur for the two reasons described below.

(1) Reduction in solute-linked water absorption driven by apical NaCl cotransport and Cl^-/HCO_3^- exchange in the intestine. There was a general pattern of increased intestinal Na^+ and Cl^- concentrations (Table 2) and decreased plasma Na^+ and Cl^- concentrations in response to E2 (Table 1). Together with the reduction in HCO_3^- levels in the intestinal fluid (Table 2), these data suggest a reduction in Na^+ and Cl^- absorbed by the intestine *via* both NaCl cotransport and Cl^-/HCO_3^- exchange. Both of these transport systems drive an important fraction of the net water absorption in the intestine of marine fish (Grosell, 2006; Grosell et al., 2009; Whittamore et al., 2010), so any inhibition of these processes would be expected to directly interfere with water absorption. Usually, the intestinal absorption of the Na^+ and Cl^- from the ingested seawater is followed by water absorption, leaving behind the poorly absorbed Mg^{2+} and SO_4^{2-} in elevated concentrations in the intestinal fluid (Smith, 1930; Grosell et al., 2001).

E2 has been found to reduce the Na^+/K^+-ATPase activity in the gills of salmonids (Madsen et al., 1997). Although the same basolateral transporter is the underlying driving force for apical NaCl co-transport in the intestine, we can only speculate at this stage as to whether E2 has a direct influence on any of the individual transporters involved in NaCl uptake or Cl^-/HCO_3^- exchange within the intestine of marine fish, which could include any of the various apical and basolateral transport components involved (Grosell et al., 2007; Whittamore et al., 2010). However, it is worth pointing out that using an *in vitro* preparation of the toadfish intestine, Tresguerres et al. (Tresguerres et al., 2010) found no effect of luminally applied E2 on the short-circuit current under symmetrical conditions. This suggests that E2 may not necessarily influence NaCl cotransport.

Estrogen was found to act through estrogen receptors to modulate fluid reabsorption in the adult mouse efferent ductules of the reproductive tract by regulating the expression of ion transporters involved in Na^+ and Cl^- movement (Lee et al., 2001). Furthermore, it has been found that E2 can inhibit Cl^- transport processes in the isolated distal colon of female rats (Condliffe et al., 2001), showing the potential E2-driven mechanisms for regulating ion transport processes in the intestine of other vertebrates.

(2) Reduced water absorption due to decreased precipitation of Ca^{2+} and Mg^{2+} carbonates. Another potential cause for reduced water absorption in the present study is the significantly decreased production of carbonate precipitates within the intestine. The divalent cations Ca^{2+} and Mg^{2+} present in the ingested seawater are poorly absorbed by the intestine and would become extremely concentrated in the intestine because of water absorption driven by the previously described mechanisms (Wilson et al., 2002). The prevention of excessive accumulation of these cations by precipitation as their insoluble carbonates normally aids in Ca^{2+} homeostasis (preventing excessive intestinal absorption) and in promoting further water absorption by reducing intestine fluid osmolality (Wilson et al., 2002; Wilson and Grosell, 2003). The reduction in $CaCO_3$ excretion associated with reduced intestinal fluid HCO_3^- levels in response to E2 would presumably mean that less water absorption is driven by this alkaline precipitation mechanism. However, although $CaCO_3$ precipitation is predicted to permit additional fluid absorption by the intestine, there is currently no direct experimental evidence that this process of $CaCO_3$ formation drives intestinal fluid absorption (Wilson et al., 2002; Whittamore et al., 2010).

CONCLUSIONS

Studies of Ca^{2+} balance have mostly focused on freshwater and euryhaline fish rather than marine fish, especially with regard to the intestine as an osmoregulatory organ. Our novel finding is that E2 induces a major Ca^{2+} accumulation in plasma and muscle that is in parallel with plasma vitellogenin increases, and reduces $CaCO_3$ production by the intestine in seawater-acclimated rainbow trout. The findings reported here have implications for understanding of at least three areas of marine teleost biology: (1) the physiology of intestinal $CaCO_3$ production and associated ion transport mechanisms in relation to natural reproductive maturation in female teleosts; (2) the potential for xenoestrogens to influence these processes; and (3) to help in refining estimates of the global contribution of marine fish to the inorganic carbon cycle *via* their gut excretion of $CaCO_3$ precipitates (Wilson et al., 2009). Currently such spatial models of carbonate production by fish populations do not differentiate between immature and sexually mature fish, apart from considering body size (Jennings and Wilson, 2009; Wilson et al., 2009).

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