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17β-Estradiol affects osmoregulation in *Fundulus heteroclitus*

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Abstract

The effect of 17β-estradiol (E_2) on osmoregulatory performance was examined in the euryhaline killifish, *Fundulus heteroclitus*. Fish were injected once with 1, 2 and 5 μg g^{-1} E_2 and, 6 h after injection, transferred from 1 ppt seawater (SW) to full strength SW (40 ppt) or from SW to 1 ppt SW. In another set of experiments, fish were injected four times on alternate days with 2 μg g^{-1} E_2 and then, 6 h after the last injection, transferred from 1 ppt SW to SW or from SW to 1 ppt SW. Fish were sampled 18 h after transfer (i.e., 24 h post-injection), and plasma osmolality, Na^+ and Cl^- concentration and gill K^+-*p*NPPase activity (a reflection of the sodium pump) were examined. Transfer from 1 ppt SW to SW resulted in significantly increased plasma osmolality, but did not affect gill K^+-*p*NPPase activity. A single dose of E_2 (1, 2 and 5 μg g^{-1}) prior to transfer from 1 ppt SW to SW increased plasma osmolality and decreased gill K^+-*p*NPPase activity in a dose-dependent manner. Prolonged treatment with E_2 increased plasma osmolality and decreased gill K^+-*p*NPPase activity in 1 ppt SW-adapted fish. Transfer of fish thus treated from 1 ppt SW to SW increased plasma osmolality and did not alter gill K^+-*p*NPPase activity. Transfer from SW to 1 ppt SW had no significant effect on plasma osmolality or gill K^+-*p*NPPase activity. Only the highest single dose of E_2 (5 μg g^{-1}) prior to transfer from SW to 1 ppt SW decreased gill K^+-*p*NPPase activity. Prolonged treatment with 2 μg g^{-1} E_2 decreased gill K^+-*p*NPPase activity only following transfer from SW to 1 ppt SW. The results substantiate an inhibitory action of E_2 on hypoosmoregulatory capacity in this euryhaline teleost.

Keywords: 17β-Estradiol; Osmoregulation; Salinity adaptation; K^+-*p*NPPase activity; Gill; Killifish

1. Introduction

For salmonids, evidence is available for a negative relationship between sexual maturation and seawater (SW) adaptability (McCormick and Naiman, 1985; Lundqvist et al., 1989; Staurnes et al., 1994; Le François et al., 1997; Le François and Blier, 2000). This negative effect on hypoosmoregulatory capacity can be mimicked by exogenous administration of gonadal steroids (17α methyltestosterone and 17β-estradiol (E_2); McCormick, 1995). E_2 treatment reduces the chloride cell density as well as Na^+,K^+-ATPase activity in gills in a variety of salmonid species (Miwa and Inui, 1986; Ikuta et al., 1987; Madsen and Korsgaard, 1989, 1991; Madsen et al., 1997). In medaka (*Oryzias latipes*) exposed to acid water, E_2 treatment prevents in male (but not female) fish a decrease in plasma sodium level without apparent effects on branchial Na^+,K^+-ATPase activity (Yada and Ito, 1999). Recently, Vijayan et al. (2001) reported that E_2 impairs hypoosmoregulatory capacity in *Oreochromis mossambicus* through a decrease in branchial Na^+,K^+-ATPase activity as well as in the metabolic capacity of liver and gills.

The negative effects of E_2 on osmoregulatory performance may result from direct as well as indirect effects, e.g., through other endocrines. Prolactin (PRL) is known to

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decrease gill Na^+,K^+-ATPase activity, which forms part of its well-known action to promote adaptation of euryhaline fish to fresh water (Hirano, 1986; McCormick, 1995; Manzon, 2002). There is, indeed, (ultra-) structural evidence that E_2 stimulates pituitary PRL cells in tilapia *O. mossambicus*, European eel *Anguilla anguilla* and the long-jawed mudsucker *Gillichthys mirabilis* (Nagahama et al., 1975; Olivereau and Olivereau, 1979; Olivereau et al., 1986). Moreover, in vitro studies showed that E_2 stimulates PRL release from the pituitary gland of *O. mossambicus* (Wigham et al., 1977; Barry and Grau, 1986), a direct effect of the steroid on an established osmoregulatory hormone.

Fundulus heteroclitus is an extremely euryhaline teleost and appreciated model in studies on osmoregulation in fish (e.g., Wood and Marshall, 1994; Marshall and Bryson, 1998). A series of recent papers analysed the involvement of various hormones (cortisol, growth hormone, insulin-like growth factor and 3,3′,5-triiodo-L-thyronine) on its osmoregulatory capacity (Mancera and McCormick, 1998, 1999) and salinity adaptation (Marshall et al., 1999; Mancera and McCormick, 2000; Katoh et al., 2001). To the best of our knowledge, there is no report on osmoregulatory effects of E_2 in this species. The aim of the present study was, therefore, to establish effects of E_2 treatments on hypo-osmoregulatory and hyperosmoregulatory capacity in *F. heteroclitus*, realised by transfers between 1 ppt sea water and seawater and vice versa. The results are discussed in relation to the osmoregulatory role of this hormone in other teleosts.

2. Material and methods

2.1. Fish

Killifish, *F. heteroclitus* (4–8 g body mass), were collected in the salt ponds around Cádiz Bay and transferred to the Faculty of Marine Science, Puerto Real, Cádiz. Female fish in their pre-vitellogenic phase were used in the experiment (April–May 2000). They were acclimatised to full-strength SW (40 ppt salinity, 1200 mOsm kg^{-1} H_2O) in 60-l aquaria for at least 2 weeks in an open system or to 1 ppt SW (30 mOsm kg^{-1} H_2O) in a closed system with recirculation and filtration. SW (1 ppt) was obtained by mixing SW with dechlorinated tap water to the desired salinity. Water salinity was checked every day and adjusted as necessary. Half of the 1 ppt SW was replaced with fresh solution every 3 days during the experiments. In the recirculation systems, the common water quality criteria (hardness, levels of oxygen, carbon dioxide, hydrogen sulphide, nitrite, nitrate, ammonia, calcium, chloride and suspended solids) were continuously monitored and no major changes were observed. The fish were maintained under natural photoperiod at a constant 18 °C and fed once daily with commercial fish food (Tetra Standard Mix, Tetrawerke, Germany). The fish were not fed 24 h before hormone treatment and throughout the experiment.

2.2. Injection protocol

Fish were caught by netting, lightly anaesthetised with 2-phenoxyethanol (0.05% v/v; Sigma, St. Louis, MO, USA), weighed, injected and returned to their aquarium. Hormone was dissolved in vegetable oil and injected intraperitoneally (10 μl g^{-1} body mass); carrier injections served as controls. Injections were given between 09:00 and 10:00 h.; no mortality was observed during the experiments.

2.3. Experimental design

2.3.1. Experiment 1

Fish adapted to 1 ppt SW received a single injection of vegetable oil (control group) or vegetable oil containing E_2 (1, 2 or 5 μg g^{-1} body mass) and were transferred to SW 6 h after the injection. Eighteen hours after the transfer (24 h post-injection), the fish were sampled (see below). A reference group of fish stayed in 1 ppt SW and was injected with oil and handled similarly to controls and E_2-treated groups.

2.3.2. Experiment 2

SW (1 ppt)-adapted fish were injected four times with E_2 (2 μg g^{-1} body mass) every other day while the control group received the same volume of vegetable oil. Six hours after the last injection, half of the oil and E_2-treated fish were transferred to SW (experimental group) and half remained in 1 ppt SW (control group). Sampling was done 18 h following transfer (24 h post-injection).

2.3.3. Experiment 3

A similar protocol to Experiment 1 but with SW fish and transfer from SW to 1 ppt SW.

2.3.4. Experiment 4

A similar protocol to Experiment 2, but with SW fish and transfer from SW to 1 ppt SW.

2.4. Sampling

Fish were euthanised with 2-phenoxyethanol (0.1% v/v) and weighed. Blood was obtained by severing the tail and collecting the blood in ammonia heparinized microcapillary tubes. Capillary tubes were centrifuged at 3000×*g* for 5 min and plasma stored at −80 °C. Gill tissue was dissected and placed in 100 μl ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80 °C.

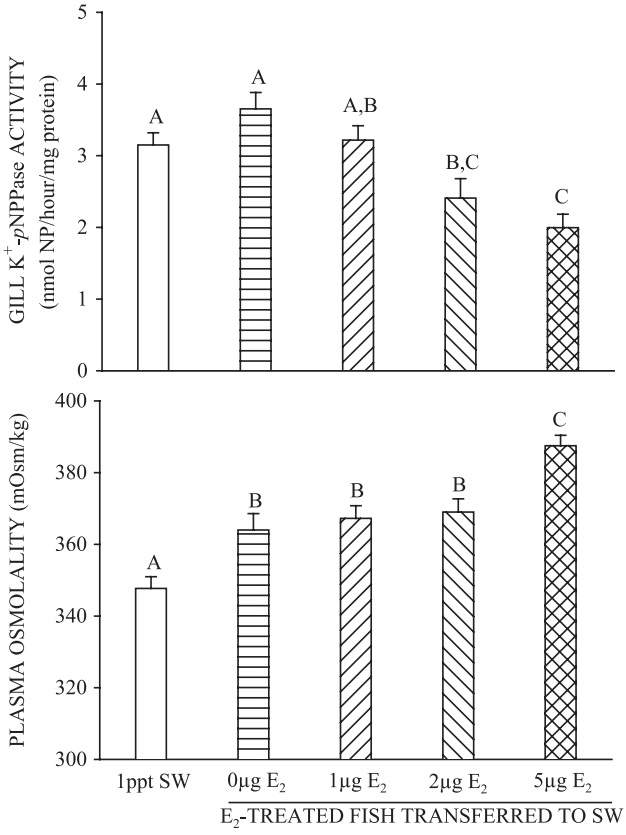

Fig. 1. Effects of a single injection of different doses of E_2 (0, 1, 2, 5 µg g^{-1} body mass) on gill K^+-*p*NPPase activity (top) and plasma osmolality (bottom) of 1 ppt SW killifish (*F. heteroclitus*) and subsequent transfer from 1 ppt SW to SW. Following injection, fish were kept in 1 ppt SW for 6 h and then transferred to SW for 18 h. Values are the means±S.E.M. (n=7–8). Same letters indicate no difference among groups (P<0.05, one-way ANOVA).

2.5. Analytical techniques

K^+-dependent *p*-nitrophenol phosphatase (K^+-*p*NPPase) activity, which reflects the dephosphorylation step (i.e., the phosphatase activity) of the Na^+,K^+-ATPase, was determined using the method of Bury et al. (1996, 1998). Gill tissue was homogenised in 125 µl of SEI buffer with 0.1% deoxycholate, then centrifuged at 2000×g for 30 s. Duplicate 10 µl homogenate samples were mixed with 100 µl of either medium A or medium E and incubated for 15 min at 26 °C. Medium A contained 10 mmol l^{-1} KCl, 7.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} *trans*-1,2,-diaminocyclohexane-*N*,*N*,*N*′ ,*N*′ -tetra-acetic acid (CDTA) and 5 mmol l^{-1} *p*-nitrophenolphosphate (*p*NPP), pH 7.4; medium E consisted of medium A to which 1 mmol l^{-1} ouabain had been added while KCl was omitted. The reaction was stopped by the addition of 1 ml ice-cold 1 mol l^{-1} NaOH. The K^+-dependent, ouabain-sensitive *p*NPPase activity was defined as the difference in the amount of *p*-nitrophenol (*p*NP) released in media A and E, measured at 420 nm and expressed as nmol NP min^{-1} mg^{-1} protein. The Pierce BCA Protein kit (Pierce, Rockford, IL, USA) was used with bovine albumin as standard. Both assays were run on a microplate reader (EL340i, Bio-Tek Instruments, Winooski, VT, USA) using Delta Soft3 software for Macintosh.

Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA) and expressed as mOsm kg^{-1}. Plasma Na^+ was measured using an atomic absorption spectrophotometer, plasma Cl^- levels with the Chloride Sigma kit (no. 461) and total plasma Ca^{2+} with the Calcium Sigma kit (no. 587).

2.6. Statistics

For Experiments 1 and 3, differences among groups were assessed by one-way ANOVA, followed by the Student–Newman–Keuls multiple comparison test (SNK) to determine the level of significance. Results were considered significantly different when P<0.05.

For Experiments 2 and 4, differences between sham and E_2-treated fish were assessed with a Student t test (significance level P<0.05). The differences observed between different groups assessed after E_2 treatment followed by subsequent transfer to different salinity were analyzed using a two-way ANOVA with treatment (oil and E_2) and salinity (SW and 1 ppt SW) as main factors. When significant differences were indicated by ANOVA, multiple comparisons were carried out using the SNK-test. Significance was accepted when P<0.05.

3. Results

3.1. 1 ppt SW–SW transfer

Transfer from 1 ppt SW to SW significantly increased plasma osmolality (P<0.001, one-way ANOVA), but did not affect gill K^+-*p*NPPase activity (P=0.098, one-way ANOVA) in fish without hormone treatment. A single dose of E_2 (1, 2 and 5 µg g^{-1} body mass, Experiment 1) before transfer from 1 ppt SW to SW decreased gill K^+-*p*NPPase activity in a dose-dependent manner. This reduction was statistically significant at the higher doses of 2 µg g^{-1} (P=0.006, one-way ANOVA) and 5 µg g^{-1} body mass (P<0.001, one-way ANOVA) (Fig. 1, top). The highest

Table 1
Effects of a single dose of E_2 on plasma Na^+ and Cl^- values following 1 ppt SW to SW transfer

	1 ppt SW	0 µg g^{-1}	1 µg g^{-1}	2 µg g^{-1}	5 µg g^{-1}
Na^+ (mM)	167±2[a]	196±3[b]	198±7[b]	199±5[b]	216±2[c]
Cl^- (mM)	132±4[a]	157±4[b]	160±3[b]	165±4[b]	172±3[b]

Fish were kept in 1 ppt SW 6 h following treatment and then transferred to SW for 18 h.
Values are the means±S.E.M. (n=7–8). Same letters indicate no differences among groups (P<0.05, one-way ANOVA).

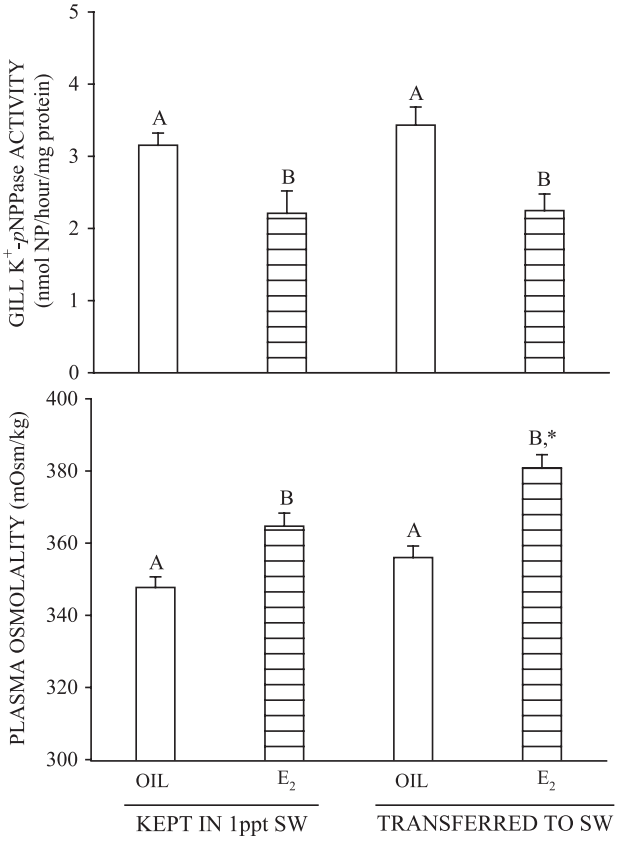

Fig. 2. Effect of four injections of E_2 (2 μg g^{-1} body mass) on alternate days on gill K^+-*p*NPPase activity (top) and plasma osmolality (bottom) followed by transfer from 1 ppt SW to SW. Fish were kept in 1 ppt SW for 6 h after last injection before transfer to SW for 18 h. Values are the means±S.E.M. (n=7–8). Same letters indicate no difference among groups before or after transfer (P<0.05, one-way ANOVA). Asterisks indicate significant difference compared to the same fish before transfer (P<0.05, two-way ANOVA).

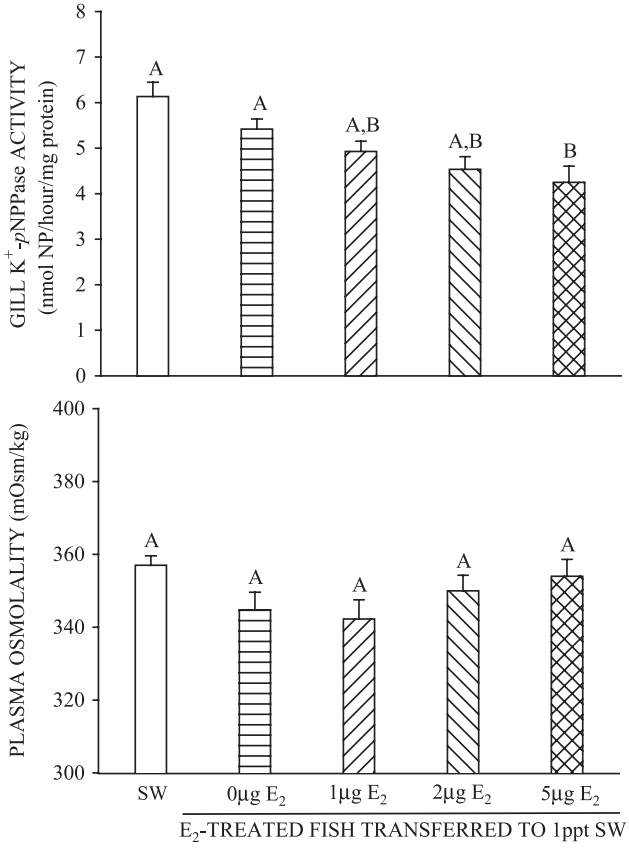

Fig. 3. Effect of a single injection of different doses of E_2 (0, 1, 2, 5 μg g^{-1} body mass) on gill K^+-*p*NPPase activity (top) and plasma osmolality (bottom) of SW killifish and subsequent transfer from SW to 1 ppt SW. Following injection, fish were kept in SW during 6 h and then transferred to 1 ppt SW for 18 h. Values are the means±S.E.M. (n=7–8). Same letters indicate no difference among groups (P<0.05, one-way ANOVA).

dose used, 5 μg g^{-1} body mass, further increased the rise in plasma osmolality following transfer (P=0.001, one-way ANOVA) (Fig. 1, bottom). Values for plasma Na^+ and Cl^- levels followed the pattern of change observed for plasma osmolality (Table 1).

Prolonged treatment of 1 ppt SW-adapted fish with E_2 (4 injections of 2 μg g^{-1} body mass on alternate days; Experiment 4) significantly decreased gill K^+-*p*NPPase activity (P=0.0016, one-way ANOVA) and increased plasma osmolality (P=0.003, one-way ANOVA) (Fig. 2) and Na^+ values (P=0.007, one-way ANOVA) (Table 2). After transfer to SW gill K^+-*p*NPPase activity remained unaltered in both control and E_2-treated fish. However, plasma osmolality significantly increased in E_2-treated fish compared to controls (Fig. 2, bottom); no significant differences were observed in plasma Na^+ or Cl^- values (Table 2).

Table 2
Effects of four injections of vehicle or vehicle plus E_2 (2 μg g^{-1} body mass) on alternate days on plasma Na^+ and Cl^- values prior to and following 1 ppt SW to SW transfer

	Before transfer		After transfer	
	Oil	E_2	Oil	E_2
Na^+ (mM)	167±2[a]	182±4[b]	195±4[a,*]	202±4[a,*]
Cl^- (mM)	132±4[a]	139±3[a]	157±3[a,*]	166±2[a,*]

Fish were kept in 1 ppt SW for 6 h after the last injection and then transferred to SW for 18 h.
Values are the means±S.E.M. (n=7–8). Same letters: No differences among groups before or after transfer (P<0.05, one-way ANOVA). Asterisks: significant difference relative to controls before transfer (P<0.05, two-way ANOVA).

Table 3
Effect of a single dose of E_2 on plasma Na^+ and Cl^- levels following SW to 1 ppt SW transfer

	SW	0 μg g^{-1}	1 μg g^{-1}	2 μg g^{-1}	5 μg g^{-1}
Na^+ (mM)	171±4[a]	164±3[a]	169±4[a]	168±4[a]	169±2[a]
Cl^- mM)	159±4[a]	136±4[b]	139±3[b]	140±3[b]	145±4[b]

Fish were kept in SW 6 h following treatment and then transferred to 1 ppt SW for 18 h.
Values are the means±S.E.M. (n=7–8). Same letters indicate no differences among groups (P<0.05, one-way ANOVA).

3.2. SW–1 ppt SW transfer

Transfer from SW to 1 ppt SW did not decrease plasma osmolality (P=0.0067, one-way ANOVA) or gill K^+-*p*NPPase activity (P=0.08, one-way ANOVA). A single injection of E_2 (5 μg g^{-1} body mass, Experiment 3) before transfer from SW to 1 ppt SW decreased gill K^+-*p*NPPase activity, and such effect was statistically significant with the higher doses (P=0.015, one-way ANOVA) (Fig. 3, top). Plasma osmolality after transfer from SW to 1 ppt SW was not influenced by E_2 treatment (Fig. 3, bottom); also plasma values for Na^+ and Cl^- did not change significantly after transfer (Table 3).

After prolonged treatment with E_2 only a tendency for a decrease in gill K^+-*p*NPPase activity (P=0.162, one-way ANOVA) and for an increase in plasma osmolality (P=0.057, one-way ANOVA) was seen in SW-adapted fish (Fig. 4). However, after transfer to 1 ppt SW gill K^+-*p*NPPase activity decreased significantly in E_2-treated fish compared to controls. Values for Na^+ and Cl^- followed a similar pattern as observed for plasma osmolality (Table 4).

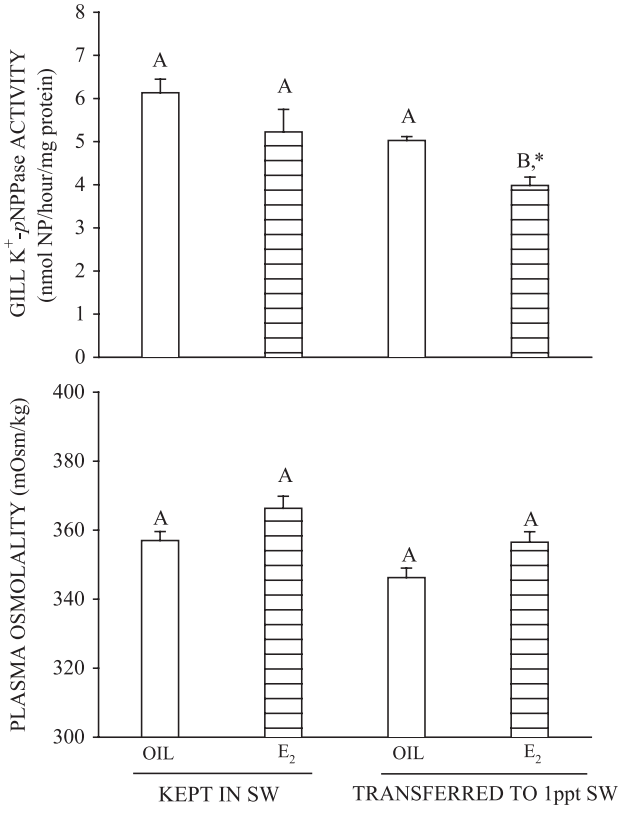

Fig. 4. Effects of four injections of E_2 (2 μg g^{-1} body mass) on alternate days on gill K^+-*p*NPPase activity (top) and plasma osmolality (bottom) after transfer from SW to 1 ppt SW. Fish were kept in SW for 6 h after the last injection before transfer to 1 ppt SW for 18 h. Values are the means±S.E.M. (n=7–8). Same letters indicate no difference among groups before or after transfer (P<0.05, one-way ANOVA). Asterisks indicate significant difference compared to the same fish before transfer (P<0.05, two-way ANOVA).

Table 4
Effect of four injections of vehicle or vehicle plus E_2 (2 μg g^{-1} body mass) on alternate days on plasma Na^+ and Cl^- levels before and after SW to 1 ppt SW transfer

	Before transfer		After transfer	
	Oil	E_2	Oil	E_2
Na^+ (mM)	171±3[a]	180±4[a]	164±3[a]	174±4[a]
Cl^- (mM)	159±4[a]	167±4[a]	135±3[a,*]	140±3[a,*]

Fish were kept in SW 6 h after the last injection and were then transferred to 1 ppt SW for 18 h.
Values are the means±S.E.M. (n=7–8). Same letters: no differences among groups before or after transfer (P<0.05, one-way ANOVA). Asterisks: Significant difference relative to controls before transfer (P<0.05, two-way ANOVA).

4. Discussion

4.1. Observations on 1 ppt SW killifish (fish adapted to a hypoosmotic medium)

Estradiol appears to impair particularly the hypoosmoregulatory capacity of killifish. In two established experimental paradigms, this effect was seen: A single injection with a high dose (5 μg g^{-1} body mass) of estradiol and a prolonged treatment (4×2 μg g^{-1}) compromised the powerful hypoosmoregulatory response normally seen in control (this study) or untreated (Marshall et al., 1999; Mancera and McCormick, 2000; Katoh et al., 2001) fish of this species. Estradiol inhibited the branchial sodium pump and this inhibition coincided and inversely correlated with a strong increase of plasma osmolarity and sodium (and to a lesser extent chloride) levels following transfer of the treated fish from hypoosmotic to hyperosmotic medium.

The two types of experiments performed do not allow discrimination between genomic and non-genomic effects of estradiol. There is increasing evidence for many steroids (Borsky, 2000; Falkenstein et al., 2000; Sunny and Oommen, 2001) that these signal substances not only act via cytosolic transcription factors but also exert effects via membrane bound receptors and second messenger pathways. In the single dose experiment, the time between injection and analysis is 24 h and this time span would allow for genomic effects (see references above). Experiments with membrane-impermeable analogues will elucidate whether non-genomic effects of estradiol contribute to this relatively quick response to estradiol. One could question whether estradiol affects the gills, or more specifically the chloride cells (the predominant site of Na^+,K^+-ATPase expression; Hootman and Philpott, 1979; Pisam and Rambourg, 1991), or other osmoregulatory organs. To the best of our knowledge E_2 receptors have not been demonstrated in chloride cells of

fish. However, both intestine and kidney were shown to express estradiol receptors (Persson et al., 2000; Socorro et al., 2000) and for that very reason it would not be surprising if the other main osmoregulatory organ in fish, namely the gills (or more specifically the chloride cells therein), express an E_2 receptor. Available negative data on salmonids (Persson et al., 2000) and gilthead seabream (Socorro et al., 2000) would argue against this statement, but more species need to be investigated and the resolution improved (i.e., focus on the chloride cells in the epithelium). Clearly, more research in this field seems warranted.

Another option is of course that E_2 influences the gills indirectly through other endocrines. Estradiol is well known to influence PRL cells in vertebrates including fish (for references, see Brinca et al., 2003). Whether an effect on PRL cells should be considered a specific osmoregulatory effect or relates to the regulation of reproduction (Brinca et al., 2003; Cavaco et al., 2003) is of less importance when exogenous estradiol is administered. Such (artificial) treatment may overrule in vivo actions, and just reveal one of the other actions of the pleiotropic PRL. In several teleosts (*G. mirabilis*, *O. mossambicus* and *A. anguilla*) E_2 treatment activates PRL cells (Nagahama et al., 1975; Olivereau and Olivereau, 1979). This appears a direct effect as E_2 treatment of organ-cultured rostral pars distalis of *O. mossambicus* increased spontaneous PRL release (Wigham et al., 1977; Barry and Grau, 1986; Poh et al., 1997). Thus, E_2 treatment may enhance circulating PRL levels. In teleosts, including *F. heteroclitus*, it is well established that PRL reduces gill Na^+,K^+-ATPase activity and increases plasma osmolality and ion concentrations (Pickford et al., 1970a; McCormick, 1995; Manzon, 2002). In teleosts, PRL cells are more active in fish adapted to hypoosmotic than hyperosmotic media (Hirano, 1986; Manzon, 2002). Consequently, the presumably more active PRL cells of 1 ppt SW-adapted killifish could be stimulated by E_2 treatment to release more PRL, whereas the inactive PRL cells of SW-adapted fish would prevent such an effect. Further studies on the presence of E_2 receptors in osmoregulatory organs or PRL levels in SW- and 1 ppt SW-adapted fish after E_2 treatment could clarify the functional pathway of E_2 on the osmoregulatory system. Any stimulation then of the PRL cells following estradiol treatment could explain the results obtained in this study.

Another target for E_2 could be the growth hormone–insulin-like growth factor (GH/IGF) system. Poh et al. (1997) showed that estradiol elevates GH production in *O. mossambicus*. If the latter system is stimulated by E_2 in (male) killifish, higher levels of GH may be predicted. However, it is known that the GH/IGF system improves hypoosmoregulatory capacity in teleosts including *F. heteroclitus* (McCormick, 1995; Mancera and McCormick, 1998). Our results show that treatment with E_2 did not increase the hypoosmoregulatory capacity of the fish and thus do not support this explanation for E_2 action.

Estradiol is without effect on cortisol production in tilapia (Vijayan et al., 2001). Extrapolating this finding to the killifish (and these modern teleostean species are comparable in many aspects of their euryhalinity and osmoregulatory capacity) we dare to exclude an indirect effect of E_2 via cortisol, as cortisol is well know for its stimulatory action in this species (Pickford et al., 1970b; Mancera and McCormick, 1999), and an enhanced cortisol production would be at variance with an inhibition of the enzyme activity seen in the present study.

The two experiments performed together strongly indicate a dose-dependency of the E_2 effect. Whereas a single injection produced only effects at the middle and highest doses but not for all parameters tested, the prolonged treatment with the middle dose resulted in clear effects for all parameters. Interestingly, a prolonged elevation of E_2 as we presume occurs (we have no plasma E_2 data for this experiment for reasons relating to the size of the fish) when the fish are injected over a week's time, results in clearer effects and thus seems to mimic better natural and longer lasting elevations of E_2 as occur when the fish undergo sexual maturation. One could argue then that treating male fish with E_2 is an experimental artefact. This seems however, in our view a rather anthropocentric statement as male fish in general respond well to E_2 treatment, as female fish do, for instance with vitellogenin production (Mommsen and Walsh, 1988). The notion of the existence of many protogynous and protandrous hermaphroditic species may help those not familiar with fish to understand such apparently deviating endocrinology.

The transient rise in plasma osmolality and plasma ions during the adaptive period following transfer of *F. heteroclitus* from hypoosmotic to hyperosmotic medium is well documented (Jacob and Taylor, 1983; Zadunaisky et al., 1995; Marshall et al., 1999) and our results are in perfect line with such observations. Branchial Na^+,K^+-ATPase activity tends to increase after transfer from 1 ppt SW to SW but not in a statistically significant way, an observation reported before for this species (Mancera and McCormick, 2000). However, a single injection of E_2 given to 1 ppt SW-adapted fish evokes a highly significant decrease in branchial Na^+,K^+-ATPase activity and increase in plasma osmolality when the fish are transferred to SW. The endocrinology underlying this rapid effect of E_2 on the osmoregulatory system of the fish is not understood as discussed above. From a "cell biology point of view" it is interesting to mention that the turnover of chloride cells is enhanced by high salinity (Uchida and Kaneko, 1996), and even the Na^+,K^+-ATPase may show higher turnover and shorter half-time when activated or following exposure to stressful or hyperosmotic conditions (Mancera and McCormick, 2000; Tipsmark and Madsen, 2001). Our results would suggest a negative effect of E_2 on the synthesis of new Na^+,K^+-ATPase pumps or a stimulatory effect on

catabolic processes of these pumps. Further studies will be necessary to test these suggestions.

4.2. Observations on SW killifish (fish adapted to a hyperosmotic medium)

Plasma osmolality of SW-adapted killifish was not influenced by transfer to 1 ppt SW. This is at variance with reports on other teleosts subjected to such transfers (Maetz, 1974; Zadunaisky, 1984) but agrees with previous reports for this species (Marshall et al., 1999). The differences in response are likely related to the much stronger euryhalinity of the killifish compared to, e.g., salmonids. Effects of E_2 on SW-adapted killifish are far less clear than those seen in 1 ppt SW killifish. In this way, plasma osmolality of SW-adapted killifish was not influenced by any E_2 treatment and only the highest dose or the long term treatment with E_2 decrease branchial Na^+,K^+-ATPase activity, as determined following transfer to 1 ppt SW. Again we have no clear explanation for this action of E_2 on gill Na^+,K^+-ATPase activity, but a stimulatory effect on catabolic processes of these pumps could be suggested.

Several lines of reasoning may explain this apparent discrepancy in results obtained with SW- compared to 1 ppt SW-adapted killifish. The endocrinology of the fish in SW will be drastically different from that in 1 ppt SW fish. PRL cells generally are less active or even completely inactive in fish in hypersaline environments (Hirano, 1986; Manzon, 2002). It may thus be anticipated that an indirect effect via manipulation of PRL cell activity as described above for 1 ppt SW killifish is less pronounced or absent. On the other hand, the GH/IGF system, so important for survival of fish, including *F. heteroclitus*, in seawater, may be more active in a SW-adapted killifish and through its presumed stimulatory activity (McCormick, 1995; Mancera and McCormick, 1998; see reasoning above) could alleviate inhibitory effects on branchial Na^+,K^+-ATPase activity of E_2 treatment. Importantly, the branchial Na^+,K^+-ATPase activity of SW-adapted killifish is essentially twice as high as that seen in 1 ppt SW-adapted fish. This may provide the fish with a larger buffer capacity of the sodium pump and a greater resistance of the fish to 1 ppt SW transfer. Moreover, the higher sodium pump capacity may better counteract passive ion loss following transfer (and the required active uptake of ions from the water to restore plasma osmolality). The fact that plasma osmolality does not seem at risk following transfer and to be independent of E_2 treatment lends strong support to this suggestion.

Transfer of SW-adapted killifish to 1 ppt SW induced a rather specific decrease in plasma Cl^- levels, an effect that is not reflected by significant changes in plasma osmolality. We explain this observation due to a lack of resolution in the measurement of plasma osmolality. The lower plasma Cl^- levels may relate to a decreased secondary active transport of Cl^- (in fish in a hypoosmotic medium Cl^- enters the gills via channels and is transported into the blood via Na^+,K^+,$2Cl^-$ cotransporter activity, which directly depends on Na^+,K^+-ATPase activity (see Marshall, 2002); the mild decrease of Na^+,K^+-ATPase activity seen after the single high dose and prolonged E_2 treatments would support such interpretation. In addition, transfer of the fish to 1 ppt SW is likely to induce a temporary loss of control over branchial permeability to water and ions, including Cl^-. A secondary transport such as that of Cl^- depending on the electrochemical conditions of the branchial epithelium (that reverse completely between 1 ppt SW and SW conditions) may be more at risk than a primary active transport such as of Na^+. The drastic changes in ambient calcium levels (10 mM in SW, 1–2 mM in 1 ppt SW) as well as transient changes in plasma Ca^{2+} levels may further contribute to alterations in epithelial integrity and permeability to water and ions.

4.3. General considerations

E_2 treatment increases plasma vitellogenin and calcium in teleost fish (Persson et al., 1994; Mosconi et al., 1998; Vijayan et al., 2001; Guerreiro et al., 2002) and again this may be an indirect effect mediated, in this case, by the hypercalcemic parathyroid hormone related protein (PTHrP; Guerreiro et al., 2001). We did not evaluate plasma E_2 or vitellogenin levels but we did observe higher calcium levels in E_2-treated fish (compared to control fish), a fair indicator of effective estradiol treatment (Mommsen and Walsh, 1988). Due to the small size of the fish, we pooled plasma samples of long-term treated fish and could indeed establish elevated plasma calcium levels compared to control (1 ppt SW-adapted fish: 2.04 mM in E_2-treated versus 3.2 mM in oil-treated; SW-adapted fish: 2.32 mM in oil-treated versus 4.0 mM in E_2-treated). According to the data shown for other teleostean fishes (see above), we take this hypercalcemia as a further proof of successful E_2 treatment.

The inhibitory effect of E_2 on branchial Na^+,K^+-ATPase activity is in good agreement with reports on several salmonid species (Miwa and Inui, 1986; Ikuta et al., 1987; Madsen and Korsgaard, 1989, 1991; Madsen et al., 1997), but at variance with results on medaka (Yada and Ito, 1999) and tilapia (Vijayan et al., 2001). Differences among species as well as in the history of the fish and in their ambient medium may be at the basis of such discrepancies. Interestingly, in *O. mossambicus*, E_2 treatment prevents (and thus inhibits) the typical increase in branchial Na^+,K^+-ATPase after transfer from freshwater to 50% SW, a phenomenon explained by a negative effect of estradiol on the metabolic capacity of liver and gills (Vijayan et al., 2001). Further studies will be necessary to clarify the role of E_2 in the interrelationship between energy metabolism and osmoregulation in *F. heteroclitus* and other euryhaline teleosts. The high osmoregulatory capacity of *F. heteroclitus* (Wood and Marshall, 1994) apparently provides this species with a compensatory mechanism for negative reproduction-related signals (such as E_2), such that osmoregulatory

performance is not compromised (nor survival). We here show that this statement holds true independently of the environmental salinity the fish encounters and may explain the reproductive success of this species independently of ambient salinity.

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