Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes

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Abstract

Osmoregulation is critical for survival in all vertebrates, yet the endocrine regulation of this metabolically expensive process is not fully understood. Specifically, the function of leptin in the regulation of energy expenditure in fishes, and among ectotherms, in general, remains unresolved. In this study, we examined the effects of acute salinity transfer (72 h) and the effects of leptin and cortisol on plasma metabolites and hepatic energy reserves in the euryhaline fish, the tilapia (*Oreochromis mossambicus*). Transfer to 2/3 seawater (23 ppt) significantly increased plasma glucose, amino acid, and lactate levels relative to those in the control fish. Plasma glucose levels were positively correlated with amino acid levels ($R^2 = 0.614$), but not with lactate levels. The mRNA expression of liver leptin A (*lepa*), leptin receptor (*lepr*), and hormone-sensitive and lipoprotein lipases (*hsl* and *lpl*) as well as triglyceride content increased during salinity transfer, but plasma free fatty acid and triglyceride levels remained unchanged. Both leptin and cortisol significantly increased plasma glucose levels *in vivo*, but only leptin decreased liver glycogen levels. Leptin decreased the expression of liver *hsl* and *lpl* mRNAs, whereas cortisol significantly increased the expression of these lipases. These findings suggest that hepatic glucose mobilization into the blood following an acute salinity challenge involves both glycogenolysis, induced by leptin, and subsequent gluconeogenesis of free amino acids. This is the first study to report that teleost leptin A has actions that are functionally distinct from those described in mammals acting as a potent hyperglycemic factor during osmotic stress, possibly in synergism with cortisol. These results suggest that the function of leptin may have diverged during the evolution of vertebrates, possibly reflecting differences in metabolic regulation between poikilotherms and homeotherms.

Key Words
- osmoregulation
- leptin
- hyperglycemia
- teleost fishes

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Introduction

Teleost fishes are the largest group of vertebrates and are important models for understanding the endocrine and physiological mechanisms governing hydromineral balance (Evans *et al.* 2005). Euryhaline species have the remarkable ability to acutely withstand wide fluctuations in environmental salinity. During acute seawater (SW) acclimation, these animals overcome an increase in plasma osmolality of as much as 175 mOsm through active excretion of ions, consuming 20–68% of their total metabolic energy demand (Morgan *et al.* 1997, Bœuf & Payan 2001). The primary site of ion exchange is the gill (Evans *et al.* 2005), which has a low capacity for the

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oxidation of fatty acids or ketones (Segner *et al.* 1997, Crockett *et al.* 1999). Gill ionocytes utilize glucose from adjacent glycogen-rich cells (Tseng *et al.* 2007), and collectively the gill may represent only a fraction (3–8%) of the energy demand (Morgan & Iwama 1999), suggesting that other tissues contribute to the bulk of the expenditure (e.g. brain and kidney; Sangiao-Alvarellos *et al.* 2005, Polakof *et al.* 2006, Tseng & Hwang 2008). Previous studies indicate that systemic mobilization of glucose, derived in part from the liver, is critical for meeting the energy requirements of acute salinity adaptation (Bashamohideen & Parvatheswararao 1972, Chang *et al.* 2007).

The full complement of hormones regulating energy mobilization and expenditure in fishes is unclear, but it may differ to some degree from classic paradigms demonstrated in mammals. For example, glucose loading does not induce a subsequent period of hyperinsulinemia (Kelley 1993), and glucagon-like peptide 1 (GLP1), a hypoglycemic hormone in mammals, promotes hyperglycemia in fishes (Mommsen 2000). Similarly, cortisol promotes hyperglycemia through gluconeogenesis in fishes and mammals (Vijayan *et al.* 1996, Khani & Tayek 2001), but it also promotes sodium excretion through the gill during SW adaptation (Evans *et al.* 2005). This steroid is lipolytic in vertebrates (Mommsen *et al.* 1999), yet no changes in plasma free fatty acid levels or lipid oxidation rates reportedly occur in salinity-challenged salmonids (Bystriansky *et al.* 2007).

The function and regulation of leptin in fishes, and among poikilotherms, in general, are also poorly understood. Leptin is an adipostatic cytokine exhibiting marginal hypoglycemic (Borba-Murad *et al.* 2004, Benomar *et al.* 2006) and strong lipolytic (Wang *et al.* 1999, Huang *et al.* 2006) effects in mammals. Plasma leptin levels are correlated with total fat stores in human subjects (Weigle *et al.* 1997) and decline during fasting while promoting satiety when high in rats and mice (Ahima & Flier 2000), effects that remain unclear in non-mammalian vertebrates. In teleost fishes, the liver is considered to be the primary site of leptin production (Kurokawa & Murashita 2009, Won *et al.* 2012), as opposed to adipose tissue in mammals, with current evidence suggesting that the hormone may suppress appetite (Won *et al.* 2012), but also paradoxically increases it during periods of fasting (Zhang *et al.* 2012; for a review, see Won & Borski (2013)). Therefore, leptin may serve discrete roles of mobilizing energy during periods of stress or catabolism among vertebrate groups. Two leptin paralogs (*lepa* and *lepb*) exist in many teleosts, suggesting genome duplication as a potential mechanism of functional divergence (Gorissen *et al.* 2009, Kurokawa & Murashita 2009, Zhang *et al.* 2012).

These studies aimed to evaluate the effects of an acute hyperosmotic challenge on plasma metabolites, hepatic energy reserves, and hepatic expression of leptin A and its receptor (*lepr*) in the euryhaline Mozambique tilapia (*Oreochromis mossambicus*) and the potential roles that leptin and cortisol may play in the regulation of elevated energy expenditure characteristic of this response. Newly developed recombinant tilapia leptin A (rtLepA) has been evaluated along with commercially prepared human leptin (recombinant human leptin (rhLep)) to assess potential differences in metabolic effect. Despite considerable divergence in peptide sequence (~13% identity), the tertiary structures of human and fish leptins appear quite similar (Huising *et al.* 2006, Copeland *et al.* 2011), and mammalian forms have been commonly used to evaluate the function of leptin in teleost fishes (de Pedro *et al.* 2006, Gorissen *et al.* 2012, Won *et al.* 2012; see Won & Borski (2013) for review). This study directly contrasts the effects of recombinant tilapia and human leptins, where many but not all responses appear to be conserved. Collectively, our findings suggest that the expression of tilapia leptin A is enhanced during SW adaptation and may function along with cortisol in the mobilization of glucose during acute periods of salinity stress.

Materials and methods

SW challenge

Adult male tilapia (44±17 g mean body weight (BW)) were allowed to acclimate for 3 weeks in freshwater (FW; salinity 0–0.5 ppt, hardness 74–84 mg/l, alkalinity 126–178 mg/l, and pH 8.0) recirculating tank systems at 24–26 °C with a photoperiod of 12 h light: 12 h darkness. The fish were fed 2% of their BW daily (40% protein/10% fat; Ziegler Brothers, Gardner, PA, USA). All the fish were treated in accordance with the North Carolina State University Institutional Animal Care and Use Committee. Acclimated fish were then transferred either to 23 ppt SW (Crystal Sea Salt Mix, Marine Enterprises, Baltimore, MD, USA) or to FW (sham transfer). The fish were fasted 24 h before the first sampling and for the duration of the challenge (72 h). Fish ($N=72$; $n=8$ fish/group) were sampled at 0, 4, 12, 24, and 72 h after transfer.

Recombinant leptins

The rhLep was purchased from Dr A F Parlow (97±2% purity; National Hormone and Peptide Program, Harbor–UCLA Medical Center, Torrance, CA, USA).The rtLepA was

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produced using the Champion pET151 Directional TOPO Expression Kit (Life Technologies). Briefly, cDNA encoding the mature peptide sequence of Mozambique tilapia *lepa* (GenBank: KC354702) was cloned into an IPTG-inducible vector. The propagated cells were induced and lysed with a native isolation buffer for selective purification of free cytosolic proteins (kit-provided protocol), which were then purified by two rounds of double nickel-affinity chromatography (Life Technologies). The preparation was further purified by filter centrifugation (5000 ***g*** for 15 min) through Amicon Ultra protein purification columns with 30 and 10 kDa molecular weight cutoffs (EMD Millipore, Billerica, MA, USA). The retained protein fraction was then dialyzed in $NaCO_3$ for 4 h and lyophilized overnight. This method produced a single visible band of 16 kDa (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). The relative purity of rtLepA was determined by quantitative SDS–PAGE blot imaging (Odyssey Infrared Imager, LI-COR Biosciences, Lincoln, NE, USA) using Coomassie Fluor Orange Reagent (Invitrogen) and found to be 96.1% relative to that of the commercially prepared rhLep (Supplementary Fig. 1B). The bioactivity of the leptin(s) was evaluated *in vitro* using cultured hepatocytes from hybrid striped bass (*Morone chrysops*×*M. saxatilis*). In cells incubated with 50 nM human leptin, the mRNA expression of growth hormone receptor 1 (*ghr1*) increased significantly after 24 h (Borski *et al.* 2011, Won 2012). In cells incubated with 5 and 50 nM rtLepA, the mRNA expression of *ghr1* increased after 18 h, with the higher concentration leading to an eightfold elevation in gene expression (mean ± S.E.M.; 0 nM = 1.00 ± 0.51; 5 nM = 2.70 ± 0.80; and 50 nM = 8.15 ± 1.01; $P<0.01$; data not shown), indicating that the rtLep produced is bioactive.

Hormone injection studies

Adult male tilapia (95 ± 19 g mean BW) were allowed to acclimate for 3 weeks in recirculating tank systems (salinity 11–13 ppt, hardness 209–263 mg/l, alkalinity 127–152 mg/l, and pH 7.5–8.0) and fed daily (2% BW). The temperature and photoperiod regimens were the same as those described previously. Fishes were anesthetized using buffered MS-222 (Aquatic Eco-Systems, Apopka, FL, USA), weighed, and then administered one of the following i.p. injections: 0.5 or 5.0 μg/g BW of rhLep dissolved in PBS (45 mM NaCl, 3 mM Na_2HPO_4, and 0.6 mM NaH_2PO_4, pH 8.0 and 329 mOsm); 0.5 or 5.0 μg/g BW of rtLepA (in PBS); or 10.0 μg/g BW of cortisol in soybean oil (Sigma–Aldrich). Control fish were injected with either PBS or soybean oil (vehicle). Fish injected with cortisol and leptin were held in tanks that did not share a common water supply and were sampled at 6 and 24 h after injection ($N=112$; $n=8$ fish/group). Before the present experiment, a preliminary range-finding study was carried out to test the effects of three dosages of rtLepA (1, 10, and 100 ng/g BW) and one dosage of rhLep (100 ng/g BW) on tilapia plasma glucose levels. None of these dosages elicited a response (Supplementary Fig. 2, see section on supplementary data given at the end of this article). Therefore, we tested the effects of higher hormone concentrations on metabolic parameters in this study, comparable to those levels previously shown to be effective for other cytokines to induce responses in tilapia (e.g. prolactin and GH; see Shepherd *et al.* (1997)).

Sampling

The fish were anesthetized using buffered MS-222, and blood was collected from the caudal vein using heparinized syringes. Plasma was separated by centrifugation (3000 ***g***) and stored at −20 °C. Tissue samples were collected following decapitation. Gill and liver samples (100 mg) collected for RNA isolation were stored in 1 ml of RNAlater (Life Technologies) and kept overnight at 4 °C before freezing (−80 °C). Liver (50–100 mg) samples collected for glycogen and triglyceride assays were frozen in liquid N_2. Liver samples (50–100 mg) collected for alanine transaminase activity were immersed in 1 ml of ice-cold SEI buffer (150 mM sucrose, 50 mM imidazole, 10 mM EDTA, and pH 7.5) for 30 min before freezing (N_2).

Assays

Plasma osmolality was measured in duplicate with a VAPRO vapor pressure osmometer (WesCor, Logan, UT, USA). Metabolic assays were carried out using kit colorimetric reagents and protocols (Abcam, Cambridge, MA, USA) using an ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA) fitted with 450 and 570 nm optical filters. Sample loading volumes were tested to ensure that they were within the standard curve range ($R^2=0.98$–0.99). Specified background controls were utilized in each assay. Except for the following, assays were carried out in duplicate using 2–5 μl of plasma.

Glycogen Liver samples were weighed before homogenization in 1.25 ml of water and then boiled for 5 min and centrifuged (13 000 ***g*** at 4 °C for 5 min).

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The supernatant (4 μl), diluted 1:25 in water, was used for the assay.

Triglycerides Liver samples were weighed and homogenized in 1 ml of 5% Triton X-100. The homogenate was heated to 85 °C and cooled twice before centrifugation. The supernatant (1–8 μl) was used for the assay.

Alanine transaminase activity Liver samples were thawed on ice and residual SEI buffer was removed before weighing. The tissue samples were sonicated for 15 s in 250 μl of ice-cold assay buffer and the homogenate was centrifuged (13 000 ***g*** at 4 °C for 10 min). The supernatant (2 μl) or a positive control was compared with a non-kinetic standard (1–12 nmol pyruvate) after the color had stabilized. The kinetic readings were taken at 1-min intervals for 60 min, with a linear range observed from 11 to 19 min.

Free fatty acids Long-chain fatty acids (C-8 or greater) were detected ($OD_{570\ nm}$) by enzymatic production and oxidation of acyl-CoA derivatives (free fatty acid quantification kit; Abcam). Neat plasma samples of 30 μl were assayed in duplicate with a negative control (containing no acyl-CoA synthetase) used for background corrections. Values were determined using a palmitic acid standard curve (1–10 nmol).

Gene expression

Total RNA was isolated from gill and liver tissue samples using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), coupled with column affinity purification and DNAse treatment using Direct-zol minipreps (Zymo Research, Irvine, CA, USA). RNA quality was assessed by 18S and 28S rRNA integrity and $OD_{260:280}$ ratios (range 1.9–2.0). Total RNA (1.25 μg) was reverse-transcribed using random hexamers (High Capacity cDNA Synthesis, Life Technologies). The mRNA expression of the following genes was then measured by real-time quantitative PCR: gill – *atpa1a* and *atpa1b*; liver – *hsl*, *lepa*, *lepr*, *lipe*, and *lpl*; and both gill and liver – glucose transporter 1 (*glut1*), *glut4-l*, and *pyg*. All primers, except *atpa1a* and *atpa1b* (see Tipsmark *et al.* (2011)), were designed using ABI Primer Express (v3.0), and they are listed out in Supplementary Table 1, see section on supplementary data given at the end of this article, along with measurements of PCR efficiency calculated using the LinReg analysis of the log (fluorescence) amplification curves (Ramakers *et al.* 2003). The mean efficiency range for all the qPCRs was 95.8–105.3%. Assays were carried out using 12–25 ng of cDNA template and 75 nM primer concentrations with Brilliant II SYBR Green Master Mix (Agilent, Santa Clara, CA, USA). Triplicate runs for all the samples, standards, and negative controls were performed on an ABI 7900HT sequence detection system. Pooled cDNA samples were used for across-plate normalization with negative controls run on each plate. Cycle threshold (*C*t) values were analyzed by absolute quantification using standard curves derived from serially diluted cDNA for each tissue ($R^2 = 0.98–0.99$). Data were then normalized to the expression of β-actin (*bactin1*). The suitability of *bactin1* as a reference gene for osmoregulatory studies has previously been established through studies in the gill (Velan *et al.* 2011) and liver (Deane *et al.* 2002) of teleost fishes and further verified in the present study by normalization to total RNA (Bustin 2000). We found little change in the expression of this housekeeping gene in the present study among the treatment groups. The mean difference in fold change (Δfc) for the treatment effects was 0.42 for the salinity challenge and 0.20 for the hormone injection studies. For the salinity challenge, values are expressed as relative fold change to the mean of the initial (T_0) group (calibrator). For the injection studies, values are expressed as relative fold change to the mean of the 6-h control. Specific amplification of these genes was verified by melting-curve profile and gel electrophoresis.

Statistical analyses

All the analyses were carried out using the JMP Software (v9, SAS Institute, Cary, NC, USA). A two-way factorial ANOVA was employed for the salinity challenge, followed by Tukey's honestly significant difference (HSD) *post hoc* test if the model was significant. Initial (T_0) fish were treated as both a FW control and SW challenge group for statistical purposes. Linear correlations of plasma glucose were performed separately for each treatment group using a bivariate analysis. A one-way ANOVA was employed for the hormone injection studies, followed by *post hoc* testing against control groups using Dunnett's test. The nominal level for significance for all the tests was $P < 0.05$.

Results

SW challenge

FW tilapia were challenged with 2/3 SW (23 ppt) or with FW for 72 h. The plasma osmolality of the SW fish increased by 4 h and reached a maximum value of

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475 mOsm/kg by 24 h ($P<0.001$; Fig. 1A). The osmolality levels subsequently declined by 72 h, but were significantly elevated compared with those of FW fish (325 mOsm/kg; $P<0.001$). The expression of the gill SW-type Na^+, K^+-ATPase α-subunit (*atpa1b*) increased after 12 h and remained significantly elevated ($P<0.001$; Fig. 1B). By contrast, the expression of the gill FW isoform (*atpa1a*) decreased significantly by 12 h ($P<0.001$) in SW fish, but it did not differ from that of FW fish at later time points (Fig. 1C).

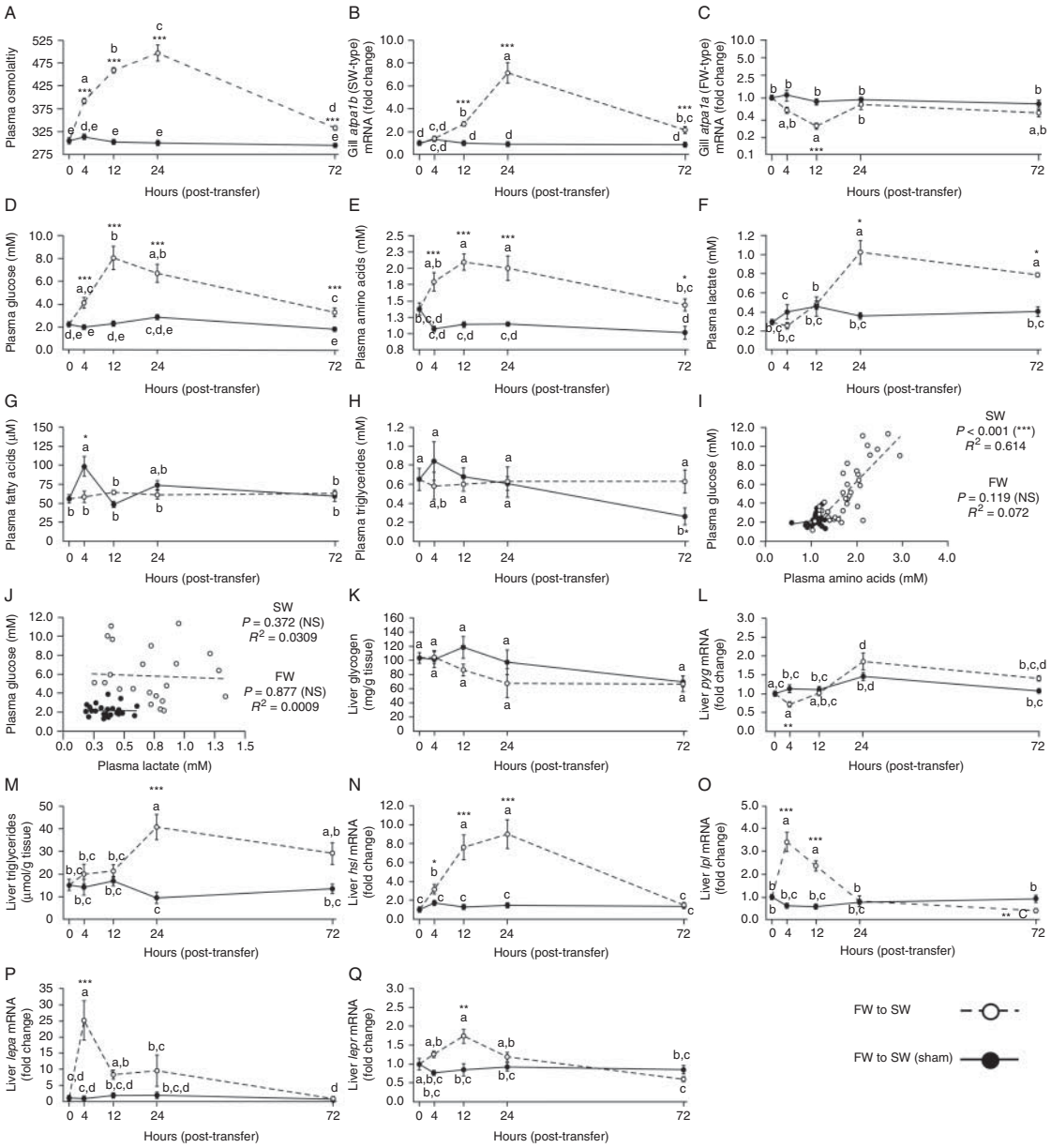

Figure 1

Effects of SW transfer on tilapia (A) plasma osmolality, (B) gill SW-type ATPase (*atpa1b*) mRNA expression, (C) gill freshwater (FW)-type ATPase (*atpa1a*) mRNA expression, plasma (D) glucose content, (E) amino acid content, (F) lactate content, (G) free fatty acid content, and (H) triglyceride content. (I and J) Bivariate correlations between plasma glucose levels and (I) amino acid and (J) lactate levels. Effects of SW transfer on hepatic (K) glycogen content, (L) glycogen phosphorylase (*pyg*) mRNA expression, (M) triglyceride content, (N) hormone-sensitive lipase (*hsl*) mRNA expression, (O) lipoprotein lipase (*lpl*) mRNA expression, (P) leptin A (*lepa*) mRNA expression, and (Q) leptin receptor (*lepr*) mRNA expression. Asterisks indicate time-dependent effects relative to those in the FW fish (***$P<0.001$, **$P<0.01$, and *$P<0.05$; ANOVA); letters indicate effects across time ($P<0.05$). Expression data are shown as fold change relative to T_0 fish. Except for amino acid and lactate levels (I and J), data represent means ± S.E.M. ($n=5$–8 fish/group).

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Plasma metabolites

Plasma glucose levels were significantly higher in the SW fish following transfer ($P<0.05$; Fig. 1D), reaching 8 mM ($P<0.001$) by 12 h and declining to 4 mM by 72 h ($P<0.05$). Plasma amino acid levels were also significantly higher in SW fish after transfer ($P<0.05$; Fig. 1E), reaching maximum values at 12 h (2 mM) and then declining. Mean plasma lactate levels were significantly higher from 24 to 72 h in the SW fish ($P<0.01$; Fig. 1F). Non-esterified (free) fatty acid levels in SW fish were not different from T_0 levels at any time; however, levels were significantly higher in the FW fish at 4 h ($P<0.05$; Fig. 1G). Plasma triglyceride levels in SW fish did not differ from T_0 levels, but in FW fish the levels decreased from 4 to 72 h and were significantly lower than those in the SW fish by 72 h ($P<0.05$; Fig. 1H). Plasma amino acid and lactate levels were correlated with plasma glucose levels (Fig. 1I and J). No significant correlation with plasma glucose was observed for plasma amino acids ($R^2=0.072$) or lactate ($R^2=0.0009$) for fish transferred to FW. A positive correlation was observed between glucose and amino acid levels in SW-challenged fish (slope >0; $R^2=0.614$; $P<0.001$; Fig. 1I). No significant correlation was observed for lactate levels in the SW fishes ($R^2=0.0309$; Fig. 1J).

Liver catabolism

Liver glycogen levels in SW fish were lower from 12 to 24 h, but did not differ significantly (Fig. 1K). The expression of liver glycogen phosphorylase (*pyg*) mRNA in SW fish was significantly lower at 4 h ($P<0.01$), but did not differ from that of FW fish at later time points (Fig. 1L). In SW fish, liver triglyceride levels rose sharply at 24 h ($P<0.001$) and declined by 72 h (Fig. 1M). The expression of liver hormone-sensitive lipase (*hsl*) mRNA was significantly elevated in SW fish from 4 to 24 h ($P<0.05$–0.001) and then declined by 72 h (Fig. 1N). The expression of liver lipoprotein lipase (*lpl*) mRNA in the SW fish was significantly higher at 4 and 12 h ($P<0.001$), but it declined and was significantly lower than that of FW fishes by 72 h ($P<0.01$; Fig. 1O). The expression of liver leptin A (*lepa*) mRNA in the SW fish increased 25-fold after 4 h ($P<0.001$) and then declined from 12 to 24 h, but remained elevated with respect to that of FW fish (Fig. 1P). The expression of *lepr* mRNA increased by 4 h and was significantly higher after 12 h in SW fish ($P<0.01$) and then returned to control levels (Fig. 1Q).

Effects of leptin and cortisol

rtLepA and rhLep were tested with cortisol 6 and 24 h after injection. Both hormones increased plasma glucose levels at 6 h at the high dosage (5.0 μg/g BW; $P<0.05$), but only rhLep exerted significant effects at the low dosage (0.5 μg/g BW; $P<0.01$; Fig. 2A). At 24 h, only rhLep (low dose) increased glucose levels ($P<0.05$; Fig. 2A). The cortisol-injected fish had significantly higher glucose

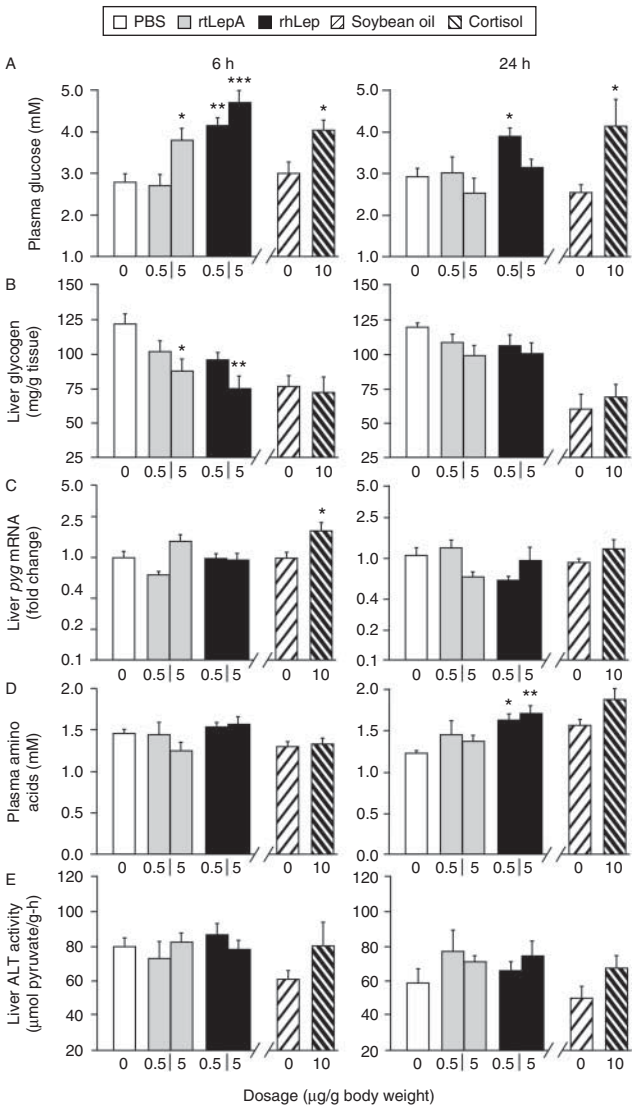

Figure 2
Effect of leptin and cortisol injection (6 and 24 h) on tilapia (A) plasma glucose content, (B) liver glycogen content, (C) liver glycogen phosphorylase mRNA expression, (D) plasma amino acid content, and (E) liver alanine transaminase (ALT) activity. Asterisks indicate significant effects against the control (***$P<0.001$, **$P<0.01$, and *$P<0.05$; ANOVA). Expression data are shown as fold change relative to 6-h controls. Data represent means ± S.E.M. ($n=5$–8 fish/group).

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levels at 6 and 24 h ($P<0.05$; Fig. 2A). Both leptins decreased liver glycogen levels by 6 h ($P<0.05$), but the levels were not different at 24 h (Fig. 2B). No significant differences in liver glycogen levels were observed in the cortisol-injected fish (Fig. 2B). The expression of liver *pyg* mRNA did not differ significantly in leptin-injected fish (6 or 24 h), but the cortisol-injected fish exhibited significantly higher expression of *pyg* mRNA at 6 h ($P<0.05$), but not at 24 h (Fig. 2C). Plasma amino acid levels remained unchanged 6 h after leptin or cortisol injection, but they were significantly higher after rhLep injection by 24 h ($P<0.01$–0.05; Fig. 2D). Liver alanine transaminase activity remained unchanged after either leptin or cortisol injection (Fig. 2E).

Mean liver triglyceride levels were significantly higher ($P<0.05$) in the rhLep-injected fish (high dose) after 6 h relative to those in the control fish (Fig. 3A). No difference in triglyceride levels was observed at 6 or 24 h after cortisol injection (Fig. 3A). The expression of liver *hsl* mRNA declined twofold by 6 h following low-dose rtLepA administration ($P<0.05$; Fig. 3B). The cortisol-injected fishes exhibited significantly higher expression of *hsl* mRNA relative to the control fish at 6 h ($P<0.01$; Fig. 3B). By 24 h, no significant changes were observed in the expression of *hsl* mRNA with either hormone injection (Fig. 3B). The expression of liver *lpl* mRNA was significantly lower at 6 h at both doses of rtLepA ($P<0.01$), and it remained lower in the low-dose rtLepA-injected fish at 24 h ($P<0.05$; Fig. 3C). The cortisol-injected fish exhibited higher expression of *lpl* mRNA at 6 h relative to the control fish ($P<0.05$; Fig. 3C). The expression of liver *lepr* mRNA in the rtLepA-injected fish was lower by 6 h (low dose; $P<0.001$), but not at 24 h (Fig. 3D). Cortisol increased the expression of *lepr* mRNA by 6 h ($P<0.01$), but no difference was observed at 24 h (Fig. 3D). Neither leptin nor cortisol administration significantly affected the expression of liver *lepa* mRNA (Supplementary Fig. 3A, see section on supplementary data given at the end of this article). Both doses of rtLepA significantly decreased the expression of liver *glut1* mRNA relative to that in the control fishes at 6 h ($P<0.05$), but not at 24 h (Fig. 3E). Cortisol increased the expression of *glut1* mRNA by 6 h ($P<0.01$), but not at 24 h (Fig. 3E).

Discussion

The hormonal components of energy mobilization during osmotic stress remain elusive, despite significant contributions to osmoregulatory physiology (Evans *et al.* 2005, Tseng & Hwang 2008). In this study, the SW-challenged fish had significantly higher plasma glucose levels, observed maximally 12 h after transfer and before peak increases in plasma osmolality and gill ATPase mRNA expression (Fig. 1A, B and D). Parallel changes in plasma free fatty acid or triglyceride levels were not observed (Fig. 1G and H). These findings are in agreement with historical reports of enhanced glucose utilization during osmotic stress (Basha-mohideen & Parvatheswararao 1972, Fiess *et al.* 2007). Concomitantly, we observed a 25-fold increase in the expression of liver *lepa* mRNA in the SW fish at 4 h, followed by an elevation in the expression of *lepr* mRNA after

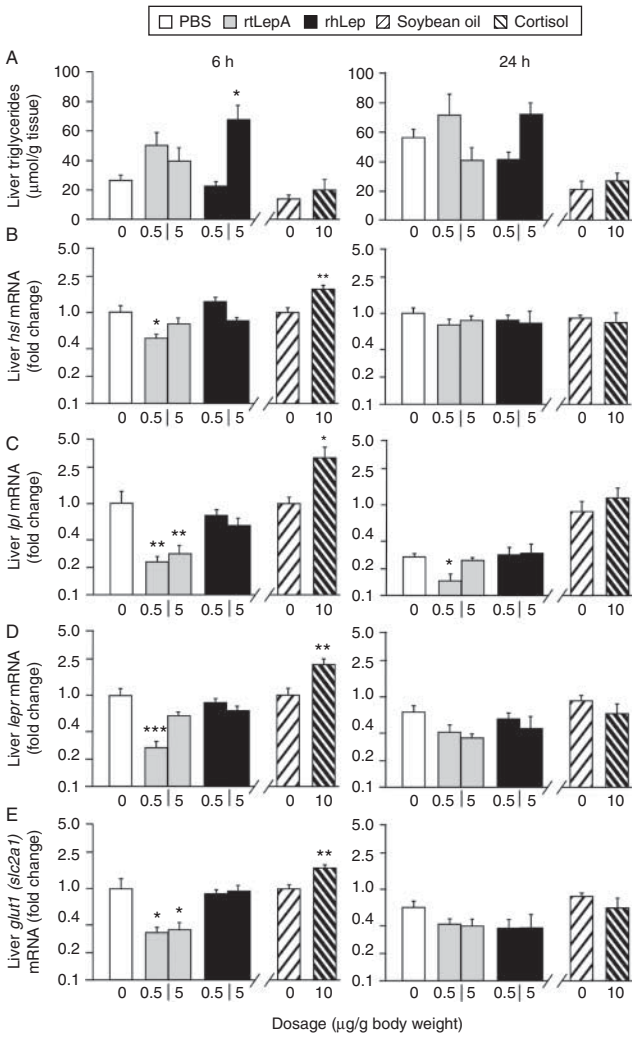

Figure 3
Effect of leptin and cortisol injection (6 and 24 h) on tilapia liver (A) triglyceride content, (B) hormone-sensitive lipase mRNA expression, (C) lipoprotein lipase mRNA expression, (D) leptin receptor mRNA expression, and (E) glucose transporter 1 (*glut1*) mRNA expression. Asterisks indicate significant effects against the control (***$P<0.001$, **$P<0.01$, and *$P<0.05$; ANOVA). Expression data shown as fold change relative to 6-h controls. Data represent means ± S.E.M. ($n=5$–8 fish/group).

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12 h (Fig. 1P and Q). These novel findings of *lepa* sensitivity to hyperosmotic perturbation, along with results demonstrating hyperglycemia (Fig. 2A), suggest that tilapia leptin A functions in the mobilization of glucose to meet the energy demands of SW adaptation and possibly other catabolic stressors, such as fasting and hypoxia (Bernier *et al.* 2012, Gorissen *et al.* 2012).

In previous work, liver glycogen levels have been found to decline in the first 12 h of a SW challenge in tilapia, with sparing occurring thereafter (Chang *et al.* 2007). We found that liver glycogen levels decreased, but not significantly, compared with those of control fish (Fig. 1K). As a decline in glycogen levels was also observed in the control group, short-term fasting may have masked the detection of a significant salinity effect (Fig. 1K). Nevertheless, we observed a significant increase in plasma glucose levels in fish injected with the high dose of rtLepA (Fig. 2A), similar to observations made during the salinity challenge. Elevated plasma glucose levels were accompanied by significant decreases in liver glycogen levels (Fig. 2B), suggesting that this hormone induces hepatic glycogenolysis. A similar action has been proposed for leptin in lizards (*Podarcis sicula*; Paolucci *et al.* 2006), suggesting functional conservation in other poikilotherms. Furthermore, leptin A downregulated the expression of liver *glut1* mRNA (Fig. 3E) and, to a lesser extent, that of *glut4*-like mRNA (Supplementary Fig. 3B), but neither changed in the gill (Supplementary Fig. 3C and D), suggesting that the hormone reduces hepatic glucose uptake. In contrast to what is observed in mammals, our findings identify teleost leptin A as a novel hyperglycemic factor critical for meeting the energy requirements of SW adaptation. Although circulating leptin A levels were not measured due to lack of homologous antisera, previous studies in fishes have shown that increases in the expression of liver *lepa* during fasting correlate with circulating hormone titer (Trombley *et al.* 2012). The liver is the primary site for leptin A production in perciform fishes and hence the source of circulating hormone (Won *et al.* 2012). The rise in the expression of *lepr* during SW acclimation (Fig. 1Q) also suggests that the paracrine effects of leptin signaling are enhanced through greater sensitivity in the liver.

The mechanism(s) by which leptin promotes glycogenolysis or is responsive to salinity is unclear. Leptin reduced liver glycogen levels without appreciable changes in the expression of *pyg* mRNA (Fig. 2C). As this enzyme is rate limiting in glycogen catabolism (Tseng *et al.* 2007), we hypothesize that leptin A may act in a paracrine fashion to exert effect(s) on the activity of this enzyme and/or may operate through the induction of other factors, including GLP1 (Mommsen 2000), catecholamines (Takekoshi *et al.* 2001), and glucagon. A sympathetic response has been postulated as a mediator for leptin in the regulation of 'glucose sensing' in the brain of rainbow trout (*Oncorhynchus mykiss*; Aguilar *et al.* 2010). As neither homologous nor heterologous leptin appears to alter gill *pyg* mRNA, gill ATPase mRNA, or plasma osmolality levels (Supplementary Fig. 3E, F, G and H), the actions of leptin may be limited to the regulation of systemic energy expenditure during osmoregulation. Future studies are required to assess other potential effects of leptin on hydromineral balance, as well as factors responsive to ionic perturbation (e.g. osmotic pressure and osmoregulatory hormones), which may enhance leptin production during SW adaptation.

Leptin is adipostatic in mammals, promoting lipolysis through oxidative metabolism in the liver and adipose tissue and inhibiting hepatic very low density lipoprotein (VLDL) secretion (Wang *et al.* 1999, Ahima & Flier 2000, Huang *et al.* 2006). However, there is no clear evidence that this hormone induces lipolysis in fishes (Li *et al.* 2010, Copeland *et al.* 2011, Liu *et al.* 2012). We found little or no effects on lipid catabolism in this study. Human leptin increased liver triglyceride levels, and the expression of lipase mRNAs (*hsl* and *lpl*) was reduced by as much as 50% in fishes injected with homologous hormone (Fig. 3A, B and C). Furthermore, liver triglyceride levels were increased in SW-challenged fish, while plasma triglyceride and free fatty acid levels remained unchanged when the expression of liver *lepa* mRNA was elevated (Fig. 1G, H, M and P). This is more consistent with a marginal lipogenic response, rather than with a lipolytic one, suggesting that leptin A may have a function that is distinct from that of mammals in regulating carbohydrate and lipid energy reserves. Factors that may have led to this divergence could include partial or complete neofunctionalization of leptin A relative to leptin B, the function of which is yet to be evaluated (Gorissen *et al.* 2009, Kurokawa & Murashita 2009), or the inherent differences in the energy requirements and metabolic regulation between homeotherms and poikilotherms (Paolucci *et al.* 2006). If the effects of leptin B, newly identified in the tilapia genome, are found to be different from those of leptin A, then the derived and ancestral functions of this hormone may be further elucidated, which will be important for understanding the evolution of energy balance in vertebrates.

Commercially prepared rhLep is commonly used for comparative studies in teleosts (Tipsmark *et al.* 2008,

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Gorissen *et al*. 2012, Won *et al*. 2012). In particular, *in vivo* administration of rhLep has been shown to decrease appetite in fishes, a classic effect of leptin in mammals (Ahima & Flier 2000, Won *et al*. 2012). The development of rtLepA provided the opportunity to functionally contrast the effects of both human and homologous forms in our experimental design. Interestingly, we found consistent responses, such as those observed for plasma glucose and liver glycogen content (Fig. 2A and B), for the two hormones, but also observed effects that were quite distinct. rtLep regulated the expression of liver lipases (*hsl, lpl*), *lepr*, and *glut1* mRNAs, while rhLep was altogether ineffective on these parameters (Fig. 3B, C, D and E). It is unlikely that any of the disparities between the responses for tilapia and human leptin reflect differences in hormone purity, and hence effective dosage, as our rtLepA preparation was 96% pure, only 4% lower than that of commercial rhLep (Supplementary Fig. 1A and B). The mechanism(s) by which some responses for tilapia and human leptin are shared, while others appear divergent, is unclear, but may include differences in receptor binding or cell signaling, perhaps further pointing toward potential sub- or neofunctionalization of leptin paralogs (leptin A and B) in fishes. Regardless of this, our results suggest that while heterologous hormone may be useful in some instances, homologous or closely related forms are probably better for discriminating the potential scope of physiological actions of leptin in fishes.

Hepatic gluconeogenesis has been examined only recently during early SW adaptation, despite historical reports of elevated nitrogen excretion rates (Evans *et al*. 2005, Bystriansky *et al*. 2007, Aragão *et al*. 2010). This is probably due to two factors: i) free amino acids were thought to act as solutes to counter osmotic forces (Tseng & Hwang 2008) and ii) decreases in glycogen pointed to glycogenolysis as the source of glucose (Bashamohideen & Parvatheswararao 1972, Chang *et al*. 2007). In the present study, the levels of plasma free amino acids and lactate, substrates for gluconeogenesis, were elevated in the blood of the SW tilapia (Fig. 1E and F), but only amino acid levels were correlated with glucose levels (Fig. 1I and J). Moreover, our findings and the work of others show that declines in glycogen levels stabilize 12–24 h after SW transfer (Fig. 1K; also Chang *et al*. (2007)), despite elevated glucose, amino acid, and lactate levels beyond this period (Fig. 1D, E and F). Sustained exposure to SW has been shown to increase the activity of hepatic transaminases in Gilthead seabream (*Sparus aurata*), enzymes that are mediators of gluconeogenesis (Polakof *et al*. 2006). This finding and the present data suggest that gluconeogenic conversion of amino acids may augment blood glucose levels during later periods (>12–24 h) of salinity acclimation (Fig. 4). The conversion of free amino acids by the liver may also explain, in part, the paradoxical rise in liver triglyceride levels in SW-challenged fish. Liver triglyceride levels doubled after 24 h (Fig. 1M), suggesting increased rates of fatty acid synthesis and lipogenesis. The accumulation of acetyl-CoA (fatty acid precursor), through the catabolism of ketogenic amino acids, may explain this effect (Jürss & Bastrop 1995; Fig. 4), as increases in plasma levels of the ketogenic amino acids leucine and isoleucine have been reported in SW-challenged Arctic char (*Salvelinus alpinus*; Bystriansky *et al*. 2007). Thus, in addition to exerting an osmotic effect, SW-induced aminoacidemia may provide a mechanism for *de novo* synthesis of both carbohydrates and lipids.

We evaluated whether leptin and cortisol might alter metabolites in a manner reflective of gluconeogenesis. Previous studies have shown elevated cortisol levels in the blood of teleosts during salinity adaptation (Evans *et al*. 2005). In Mozambique tilapia challenged with 2/3 SW (20–25 ppt), plasma cortisol levels (Kammerer *et al*. 2010) and tissue sensitivity (glucocorticoid receptor; see Dean *et al*. (2003) and Aruna *et al*. (2012)) were found to be enhanced when plasma osmolality was elevated (0–4 days). In the present study, the cortisol-injected fish had elevated glucose levels at 6 and 24 h (Fig. 2A), yet we found no significant difference in plasma free amino acid levels and liver transaminase activity (Fig. 2D and E). Whether this lack of effects for the common indicators of gluconeogenesis is reflective of a single acute dosage, as opposed to chronic treatment with cortisol (Vijayan *et al*. 1996), is unclear, as previous studies have established a clear role for cortisol in the mediation of gluconeogenesis (Van Der Boon *et al*. 1991, Khani & Tayek 2001). The steroid was effective in increasing the mRNA expression of SW-type ATPase in the gill (Supplementary Fig. 3H), which is consistent with a role in the modulation of ion transport (Evans *et al*. 2005). Human leptin, but not homologous leptin, increased plasma free amino acid levels (Fig. 2D), but neither altered liver transaminase activity (Fig. 2E). Apart from promoting glycogenolysis, it remains inconclusive whether leptin may act in part to induce hyperglycemia through gluconeogenic pathways. Previous studies have shown that cortisol increases leptin synthesis and secretion in human adipocytes (Wabitsch *et al*. 1996), with leptin, in turn, suppressing the hypothalamic–pituitary–interrenal axis in both fish and humans (Szücs *et al*. 2001, Gorissen *et al*. 2012). In the present study, we demonstrated that cortisol upregulates

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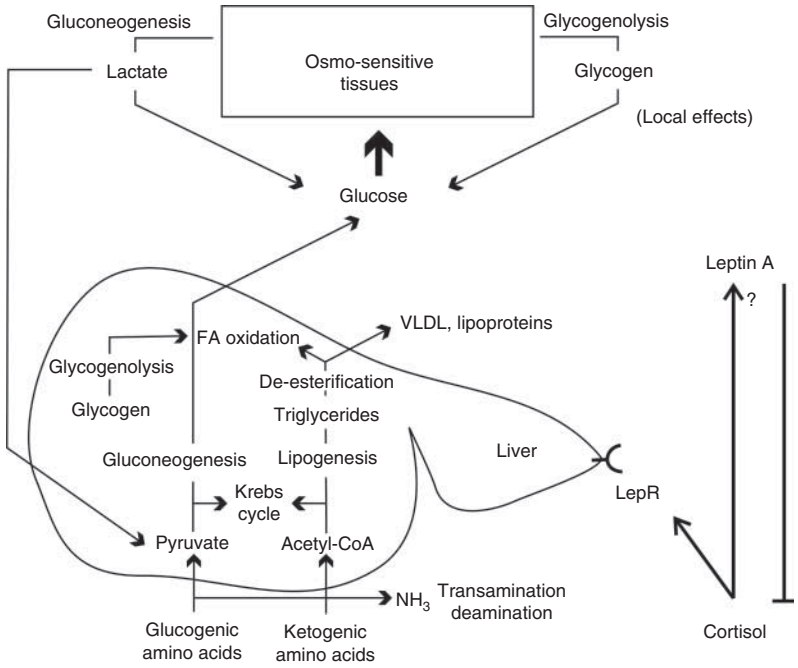

Figure 4
Bioenergetic model of hepatic energy mobilization during salinity adaptation and the regulatory interactions between leptin and cortisol. Local tissues are augmented by hepatic glycogenolysis or gluconeogenesis of glucogenic amino acids or lactate. Ketogenic amino acids are utilized by the liver or converted into triglycerides (lipogenesis). Triglycerides may be de-esterified and oxidized or exported to the blood (as lipoproteins). The regulatory interactions of leptin A and cortisol suggest that these hormones are tightly linked to the regulation of glucose mobilization, with leptin A stimulating glycogenolysis and cortisol probably involved in gluconeogenesis of amino acids. Arrow point, stimulatory effect; bar line, inhibitory effect; and question mark, potential interaction.

the expression of *lepr* in the liver of tilapia (Fig. 3D), further suggesting that these hormones are tightly linked with energy regulation in vertebrates.

In contrast to leptin, cortisol produced a 2.5-fold increase in the mRNA expression of liver lipases (*lpl* and *hsl*; Fig. 3B and C), consistent with a known lipolytic function for this hormone (Mommsen *et al.* 1999), which may coincide with the increases in the expression observed during the SW challenge (Fig. 1N and O). This suggests that cortisol, in addition to inducing hyperglycemia, functions along with other factors (e.g. GH) in the modulation of hepatic lipolysis. The stimulation of *hsl* mRNA expression may reflect greater rates of lipid catabolism needed for gluconeogenesis (Van Der Boon *et al.* 1991). This is despite elevated liver triglyceride levels being observed in SW fish, indicating that the rate of lipid accumulation exceeds that of catabolic breakdown. The enhanced accumulation could be derived, in part, from elevated lipoprotein lipase levels, which may increase fatty acid uptake in the liver (Merkel *et al.* 1998).

Osmoregulation is both a critical and a bioenergetically expensive component of survival in vertebrates, particularly for euryhaline fishes subjected to wide salinity fluctuations. We demonstrate that energy requirements of acute SW adaptation are principally met through the elevation of plasma glucose levels (hyperglycemia) in tilapia. Our findings suggest that two sequential mechanisms of mobilization occur following acute SW transfer, hepatic glycogenolysis, followed by potential gluconeogenesis of free amino acids. This study demonstrates for the first time that *lepa* mRNA is sensitive to osmotic stress and may function as a hyperglycemic factor in teleost fishes, through the induction of hepatic glycogenolysis. Although interactions of leptin with other regulators of glucose metabolism or hydromineral balance remain to be described, evidence suggests that leptin A may act in concert with cortisol to promote hyperglycemia during salinity adaptation and perhaps other catabolic processes.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-13-0292.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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