RESEARCH ARTICLE

Diet influences salinity preference of an estuarine fish, the killifish *Fundulus heteroclitus*

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SUMMARY

Understanding the interplay among the external environment, physiology and adaptive behaviour is crucial for understanding how animals survive in their natural environments. The external environment can have wide ranging effects on the physiology of animals, while behaviour determines which environments are encountered. Here, we identified changes in the behavioural selection of external salinity in *Fundulus heteroclitus*, an estuarine teleost, as a consequence of digesting a meal. Fish that consumed high levels of dietary calcium exhibited a higher preferred salinity compared with unfed fish, an effect that was exaggerated by elevated dietary sodium chloride. The mean swimming speed (calculated as a proxy of activity level) was not affected by consuming a diet of any type. Constraining fish to water of 22 p.p.t. salinity during the digestion of a meal did not alter the amount of calcium that was absorbed across the intestine. However, when denied the capacity to increase their surrounding salinity, the compromised ability to excrete calcium to the water resulted in significantly elevated plasma calcium levels, a potentially hazardous physiological consequence. This study is the first to show that fish behaviourally exploit their surroundings to enhance their ionoregulation during digestion, and to pinpoint the novel role of dietary calcium and sodium in shaping this behaviour. We conclude that in order to resolve physiological disturbances in ion balance created by digestion, fish actively sense and select the environment they inhabit. Ultimately, this may result in transient diet-dependent alteration of the ecological niches occupied by fishes, with broad implications for both physiology and ecology.

Key words: behavior, digestion, ionoregulation, calcium, *Fundulus heteroclitus*, feeding.

INTRODUCTION

Globally, fish are found in a wide range of salinities from freshwater (0 p.p.t.) to saltwater (~32 p.p.t.), and in some cases even hypersaline (>35 p.p.t.) waters. Regardless, fish maintain relatively uniform plasma ion concentrations, resulting in significant environmentally dependent differences in physiology. In essence, freshwater fish must be able to combat water gain and ion loss, while the opposite is true for fish found in saltwater environments. Much is known about the physiological processes involved in ion and water regulation, from branchial, intestinal and renal transport (reviewed by Cerda and Finn, 2010; Grosell et al., 2009; Evans, 2008; Ando et al., 2003; Karnaky, 1998) to hormonal regulation (reviewed by Takei, 2008; Manzon, 2002; McCormick, 2001). There are also many euryhaline species that are able to transition between the two extremes of freshwater and seawater. An extreme case of euryhalinity is seen in fish that inhabit estuaries (e.g. killifish, *Fundulus heteroclitus*) where large daily variations in environmental salinity may occur with the tidal cycles in the estuary (e.g. Elliot and McLusky, 2002; Marshall, 2003; Telesh and Khlebovich, 2010; Potter et al., 2010).

Euryhalinity is enabled by complex changes in the physiology of fish in response to the opposing osmoregulatory and ionoregulatory demands of inhabiting freshwater and seawater, such as alterations in branchial transport processes (e.g. Karnaky, 1998; Wood and Marshall, 1994; Marshall et al., 1999; Marshall, 2002; Katoh et al., 2008; Madsen et al., 2009; Tipsmark et al., 2010). While knowledge of the basic physiology of fishes with

respect to ion and water regulation has progressed greatly from the first historic investigations (Smith, 1930; Keys, 1931; Krogh, 1937), most studies have been based on constraining fish to controlled environments and in many cases subjecting them to single step changes in salinity (e.g. Katoh et al., 2008; Scott et al., 2008; Madsen et al., 2009). However, in nature, these animals are capable of movement throughout their habitats, and they may rapidly encounter a variety of salinities. Hence, fish behaviour may dictate their physiology over a fine temporal scale. Experimental evidence of preferences for temperature and salinity exists for a variety of euryhaline species (e.g. Fritz and Garside, 1974; Deacon and Hecht, 1995; Cardona, 2000; Serrano et al., 2010). Interpretation of these studies has focused on correlating choices with optimal growth (Jobling, 1981) or food conversion (Larsson, 2005). Indeed, common snook (*Centropomus undecimalis*) maintained at apparent optimal salinities invested more energy in processing food than fish maintained at suboptimal salinities (Gracia-Lopez et al., 2006). Additionally, there is evidence that fish can learn [e.g. associative learning (Bratland et al., 2010; Siebeck et al., 2009), social learning (Strand et al., 2010)] (reviewed by Kieffer and Colgan, 1992; Bshary et al., 2002) and remember (e.g. Harvey-Girard et al., 2010; Nordgreen et al., 2010). Taken together, evidence of environmental preferences and the ability to learn suggests that fish can discriminate and select physiologically optimal conditions, and may ultimately be able to learn and remember which habitats allow for this.

An additional factor that can influence ionoregulation and osmoregulation is feeding. A meal presents the animal not only with energy for growth, reproduction, etc., but also with large ion loads and potential disruptions in acid–base balance. The effect of feeding on a variety of physiological parameters in teleosts has been investigated recently in seawater [e.g. acid–base regulation (Bucking et al., 2009; Taylor et al., 2007; Taylor and Grosell, 2006; Taylor and Grosell, 2009; Wood et al., 2010), salt balance (Bucking et al., 2011; Taylor et al., 2007; Taylor and Grosell, 2006)] and freshwater [e.g. acid–base regulation (Bucking and Wood, 2008; Cooper and Wilson, 2008; Taylor et al., 2007; Wood et al., 2010), salt balance (Bucking and Wood, 2006a; Bucking and Wood, 2007; Taylor et al., 2007), water balance (Bucking and Wood, 2006b)]. In general, feeding appears to result in salinity-dependent alterations in ion, water and acid–base transport processes in order to alleviate digestion-induced disruptions in homeostasis.

One goal of the present study was to measure the salinity preference of an estuarine fish that would be exposed to changing salinities in its natural environment. We employed killifish, *Fundulus heteroclitus macrolepidotus* (Walbaum 1792), which are ubiquitous along the Atlantic coast of Canada and the USA and are frequent inhabitants of intertidal marshes, creeks and estuaries. Killifish move with ease between salinities, adapt quickly (Marshall et al., 1999) and can survive direct transfer from freshwater to full-strength seawater (Jacob and Taylor, 1983; Zadunaisky et al., 1995). However, ionoregulation and osmoregulation are energetically expensive, requiring ATP-driven transporters to maintain homeostasis (reviewed by Boeuf and Payan, 2001), diverting energy away from growth and reproduction. Hence, our hypothesis was that the killifish would preferentially select a salinity that would allow them to ionoregulate and osmoregulate while expending the least amount of energy – i.e. an isotonic salinity of 10–12 p.p.t.

A second goal was to examine the effect of feeding and diet composition on salinity choice. Killifish are indiscriminate predators and have been variously classified as omnivores, detritivores and piscivores (e.g. Allen et al., 1994; Prinslow and Valiela, 1974; Able et al., 2007). Being an indiscriminate predator makes the killifish an excellent animal to study in the lab as this species will readily consume a variety of diets. It also makes them important members of the intertidal food web in salt marshes and estuaries (Kneib, 1986). Digestion in killifish does not result in an alkaline tide but rather in an 'acid tide' in freshwater-acclimated killifish (but not in seawater-acclimated killifish), nor does it result in altered acid/base excretion to the water (Wood et al., 2010). However, feeding does increase Cl^- and fluid absorption in the intestine both in seawater

(Marshall et al., 2002) and in freshwater (Wood et al., 2010). Consuming a meal may present killifish with internal ion regulation challenges that depend on the external salinity. For example, consumption of a meal of commercial pellets creates an avenue for water loss and ion gain in freshwater trout (Bucking and Wood, 2006a; Bucking and Wood, 2006b; Bucking and Wood, 2007) and increased uptake of water and certain ions in seawater trout (Bucking et al., 2011). Based on our initial hypothesis that killifish would inhabit an isotonic salinity, we predicted that killifish would be more similar physiologically to seawater fish in terms of transport properties of the gill and gut (Scott et al., 2008). Hence, our second hypothesis was that after a meal, killifish would experience an increased uptake of water and ions as seen in seawater trout, causing them to seek out a higher salinity to alleviate their water (by osmosis) and ion load (by active excretion).

MATERIALS AND METHODS
Experimental diets

All diets used in the following experiments are presented in Table 1. Live blackworms (Aquatic Foods, Fresno, CA, USA) were held in ~5 l plastic containers. The worms were supplied with enough freshwater to cover them, and the water was changed every 48 h. Worms were used for experimental studies within 7 days of arrival in the lab. Blackworms were chosen as the base for most diets as they have a low ionic content and were readily ingested by killifish during preliminary trials. Diet 2 was made by baking blackworms (at 60°C) for 24 h. The dried blackworms were subsequently ground into a fine powder using a mortar and pestle. The powder was then formed into dough by adding ~50% v/w distilled water and extruded through a 20 ml syringe to make a thin rope of compressed feed. The feed was then dried overnight and subsequently broken into pellets by hand. A similar protocol was used to make diet 3, with the exception that ~950 $\mu mol\,g^{-1}$ dry mass of $CaCO_3$ was added to the worm powder before it was formed into dough, while diet 4 was made with an additional ~900 $\mu mol\,g^{-1}$ dry mass NaCl in addition to $CaCO_3$ (see Table 1).

Diet 5 was prepared by grinding commercial fish flakes (TetraMin fish flakes) into a fine powder using a mortar and pestle. Frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA) were defrosted and mixed with the powder (40% w/w) to again form a paste, which was then extruded through a 20 ml syringe into long thin ropes of food paste. The food was dried in an oven (60°C) for 24 h and then crumbled by hand to form food pellets.

With the exception of diet 1, all diets were stored at –20°C before use.

Table 1. Composition of diets and water used throughout experiments

Diet	Na$^+$ ($\mu mol\,g^{-1}$ dry mass)	Cl$^-$ ($\mu mol\,g^{-1}$ dry mass)	Ca^{2+} ($\mu mol\,g^{-1}$ dry mass)	^{45}Ca^{2+} ($\mu Ci\,g^{-1}$; series 2.2 only)	Water content (%)
1 Live blackworms	40	38	1.5		87±1.4
2 Blackworm pellets	38	41	1.8	20	11±1
3 High Ca^{2+} blackworm pellets (added as CaCO$_3$)	36	39	952	20	12±1
4 High NaCl/high Ca^{2+} blackworm pellets (added as NaCl and CaCO$_3$)	910	918	938		9.4±0.5
5 Fish flakes and brine shrimp pellets	911	922	951	20	8.1±0.2

Water	Na$^+$ (mmol l^{-1})	Cl$^-$ (mmol l^{-1})	Ca^{2+} (mmol l^{-1})
Miami seawater (35 p.p.t.)	485	569	11
Miami freshwater (dechlorinated)	1.1	1.2	0.4
Hamilton artificial seawater (22 p.p.t.)	275	303	5.1
Hamilton artificial seawater (35 p.p.t.)	441	537	10

Effect of feeding on salinity preference – series 1

The experiments described here and below were approved by institutional animal care committees at the University of Miami and McMaster University. Wild-caught (New Hampshire, USA) *F. heteroclitus macrolepidotus* (both sexes; 2–6 g) were purchased from Aquatic Research Organisms Ltd (Hampton, NH, USA), and held at the Rosenstiel School of Marine and Atmospheric Sciences (University of Miami, FL, USA) in 50 l aquaria supplied with running seawater (22–24°C, 37.5 p.p.t.; mmol l^{-1}: Na$^+$ 485, Cl$^-$ 569, K$^+$ 10.6, Ca^{2+} 10.7, Mg^{2+} 59.5, SO$_4^{2-}$ 31.6) (Wood and Grosell, 2008). The animals were fed a daily 1.5% ration of commercial pellets (mmol kg^{-1}: Na$^+$ 230, Cl$^-$ 170, K$^+$ 326, Ca^{2+} 628, Mg^{2+} 77) (Wood et al., 2010) during laboratory acclimation and holding.

To determine the salinity preference of the killifish, a shuttle box system (Loligo Systems, Tjele, Denmark) was used, which has been described in detail elsewhere (Serrano et al., 2010). Briefly, the system consisted of two large tanks connected by a small tunnel, which allowed the animal to 'shuttle' between the two compartments. One compartment was connected to a seawater source (composition above; referred to as the seawater compartment) while the other compartment was connected to a dechlorinated freshwater source [Miami City tap water; mmol l^{-1}: Na$^+$ 1.06, Cl$^-$ 1.21, K$^+$ 0.08, Ca^{2+} 0.43, Mg^{2+} 0.13, SO$_4^{2-}$ 0.14 (Wood and Grosell, 2008), referred to as the freshwater compartment]. However, the water flow (i.e. which water source, either seawater or freshwater, was turned on) was not continuous, but was determined by the position of the animal within the system. Pumps connecting the two chambers maintained the salinity differential between the two tanks at 5 p.p.t. at all times. The position of the animal was continuously recorded (uEye camera with infra-red capabilities, IDS Imaging Development Systems, Obersulm, Germany) and the image was converted into *X,Y*-coordinates by LoliTRACK software (Lolitrack Lite 1.1, Loligo Systems) once every second. The *X,Y*-coordinates were relayed to the Labtech Notebook program (Omega Engineering Inc., Stamford, CT, USA), which analysed the coordinates in real time to determine whether the fish was located in the high salinity compartment or the low salinity compartment of the system. When the fish was located within the high salinity compartment, the Labtech Notebook program activated the seawater source to deliver seawater to the compartment (and subsequently increased the salinity of both chambers in the system). Correspondingly, the Labtech Notebook program activated the freshwater source when the fish was located within the low salinity compartment (subsequently decreasing the salinity of the system). Hence, by selectively delivering either seawater or freshwater to the system, the salinity was controlled by the location of the animal with a constant offset 5 p.p.t. between the two compartments.

Throughout each experiment, the salinity of each compartment was also continuously measured using two conductivity probes (TetraCon 325 conductivity probe, WTW, Weilheim, Germany) and meters (WTW conductivity meter, WTW). The salinity of each compartment was also relayed every second to the Labtech Notebook program for later analysis along with the *X,Y*-coordinates (and corresponding time stamp) described above. At the beginning of each trial, the system was filled with 100% seawater in both chambers to match the acclimation salinity of the animals. Once a fish was placed in the system, the tracking software was activated and the fish could control the delivery of water depending on which chamber it chose to occupy. The entire system was housed in an environmental chamber to maintain a constant temperature (22–24°C) as well as to control the light:dark cycle, which was set at 14 h:10 h. During the dark cycle, infrared lights placed beneath the system allowed the fish to be visible to the camera, which enabled the system to be operational over a complete 24 h cycle.

Each trial consisted of monitoring a single fish for 48 h within the system. Before experimentation, feeding was suspended for all animals for 48 h, to encourage feeding when food was presented to the fish within the system as well as to ensure an initial unfed state. A single fish was then indiscriminately chosen and placed in the shuttle box system following weighing. For 24 h the position of the fish within the system as well as the salinity of each chamber were recorded. Following 24 h, the fish were fed to satiation with one of the diets presented in Table 1, or sham fed. Sham feeding consisted of feigning all actions involved in feeding (entering the chamber, moving an arm over the tank as one would during feeding, etc.) but without actually providing any food for the fish. Subsequently, the location of the fish and salinity of the system were recorded for an additional 24 h. Excess food was siphoned from the tanks (tanks with sham-fed fish were also siphoned) upon satiation. Care was taken to randomize which chamber the food was presented in, as well as to begin each trial and feed at a standard time (16:00 h was chosen for both). At the end of the experiment, fish were lightly anaesthetized (MS-222, tricaine methanesulphonate, Syndel Laboratories, Qualicum Beach, BC, Canada; buffered to pH 7.00) and their body length (BL, cm) measured. Overall, 7 fish were used for each dietary treatment. Care was taken to expose only naive fish to the system (i.e. fish were not reused between treatments). All fish chosen for the experiments utilized the system and none were excluded. An attempt was made to ensure an even number of males and females for each trial (a total of 16 male fish and 19 female fish were used).

To determine the salinity preference of individuals, the overall salinity of the system was calculated as the mean salinity recorded between the two chambers at each time point (every second of the 48 h trial). The mean salinity was chosen because the fish must travel back and forth between the two chambers to establish an overall preferred salinity, given the nature of the set-up, and thus we cannot be certain that the chamber the fish was occupying at any particular time point was chosen because the salinity in that chamber was the more preferred salinity or because the salinity in the other was the less preferred salinity. The mode salinity of the system was then calculated over 30 min time periods. As the mode represents the most commonly measured salinity over the 30 min, this reflects the most commonly chosen salinity of the fish (i.e. mean preferred salinity).

To determine the variability in the preferred salinity during each treatment, the mean (±s.e.m.) salinity preference of individuals (*N*=7) was then calculated at each 30 min time point. The overall preferred salinity of fish in each treatment was also calculated as the mean preferred salinity over each 24 h period, as well as for each light and dark cycle, and for selected post-feeding time intervals. Finally, swimming speed (BL s^{-1}) was calculated by measuring the distance moved (in cm) by the fish every second using the *X,Y*-coordinates of the fish recorded by the Labtech Notebook program (Serrano et al., 2010). The swimming speed of each fish at every second was then averaged over a 30 min time period excluding periods of inactivity (i.e. when swimming speed was 0). The mean length of inactive periods during the trials did not vary significantly between treatments (see Results). The first 4 h of each trial was excluded from statistical analysis as a training period.

Physiological effects of external environment during feeding – series 2.1

Fundulus heteroclitus macrolepidotus were obtained as above for the salinity choice experiments, and maintained in a large fibreglass tank

(~500 l; re-circulating 10 p.p.t. artificial seawater; Instant Ocean, Atlanta, GA, USA) at McMaster University (Hamilton, ON, Canada) for several months. The animals were then transferred into two large aquaria for a 1 month acclimation period, and supplied with charcoal-filtered re-circulating artificial seawater (35 p.p.t.; Instant Ocean). The animals were then gradually acclimated to 22 p.p.t. over a 24 h period based on the results seen when the animals actively controlled the environmental salinity (series 1). Fish were subsequently fed diet 2 (Table 1) until satiation, and one aquarium was maintained at 22 p.p.t., while another aquarium was subjected to a salinity increase to 35 p.p.t. over 2 h (again based on observations from series 1). Animals were terminally anaesthetized in MS-222 after 4 h (a total of 6 h following feeding; N=7 for each treatment). This time frame was chosen based on data collected during series 1 that suggested this was the time of greatest change in salinity preference during digestion. A caudal blood sample was then obtained with an ice-cold, heparinized capillary tube (Fisher Scientific Canada, ON, Canada) and centrifuged to obtain a plasma fraction, which was subsequently placed in liquid nitrogen and stored at –80°C for later analysis. The gastrointestinal (GI) tract was quickly removed and the entire contents were collected, placed in liquid nitrogen, and stored at –80°C for later analysis. The experiment was then repeated with fish fed two additional diets (diet 3 and diet 4; Table 1).

The GI tract contents were digested with acid (5 volumes of $1 \, mol \, l^{-1} \, HNO_3$) and heat (60°C) for 48 h. The plasma and GI tract contents were analysed for Na^+ and Ca^{2+} by atomic absorption spectrophotometry (AAS; Varian Australia Model 220FS, Mulgrave, Vic, Australia) using certified standards (Na^+, Radiometer, Copenhagen, Denmark; Ca^{2+}, Fisher Scientific); interference by other ions during measurement was corrected by adding Cs^{2+} and La^{3+} to the samples, respectively.

Physiological effects of external environment during feeding – series 2.2

Animals were acclimated to artificial full-strength seawater for a 1 month period and then subjected to a gradual decrease in salinity (from 35 to 22 p.p.t.) over a 24 h period, as in series 2.1. The animals were subsequently fed to satiation with diet 2, 3 or 5 (Table 1). However, diets 2, 3 and 5 were spiked with $^{45}Ca^{2+}$ (20 μCi $CaCl_2 \, g^{-1}$ dry food mass; PerkinElmer, Waltham, MA, USA). Immediately following feeding, individual fish were removed from the aquaria and placed into individual aerated chambers (500 ml), which were initially filled with 22 p.p.t. artificial seawater. Subsequently, over 2 h the water in each chamber was either gradually replaced until the salinity was 35 p.p.t. or kept at 22 p.p.t. as in series 2.1. Once the final salinity was reached, an initial water sample was taken, the fish were held statically for 4 h, and a final water sample was taken. Animals were then terminally anaesthesized in MS-222 as above, the GI tract was removed and the whole body was reserved for later analysis of $^{45}Ca^{2+}$ content. $^{45}Ca^{2+}$ content was measured in the initial and final water samples (1 ml samples), acid-digested chyme and intestine, diet (digestion protocol as for series 2.1; 200 μl of acid-digested sample) and the body of the whole animal (acid-digested as for the chyme and intestine; 200 μl acid-digested samples). ACS fluor (Aqueous Counting Scintillant, Amersham, Little Chalfont, UK) was added to each water sample, while Ultima Gold scintillation fluor (Packard Bioscience, Meriden, CT, USA) was added to the acid-digested samples, and both were stored in the dark overnight before measurement to reduce chemi-luminescence. The activity of $^{45}Ca^{2+}$ was then measured using a liquid scintillation analyser (Tri-Carb 2900TR; PerkinElmer). Quench curves were determined for each sample and fluor type. The distribution of $^{45}Ca^{2+}$ among the water, the body and the GI tract (including the

chyme) was determined as a percentage of the whole $^{45}Ca^{2+}$ activity for each fish. The absolute amount of dietary Ca^{2+} found in the GI tract, the water and the body was calculated based on the activity level of each compartment (c.p.m. g^{-1} fish mass) and the specific activity of the food (cpm μmol^{-1} Ca^{2+}).

Statistics

Data are expressed as means ± 1 s.e.m. (N). For series 1, changes in salinity preference and swimming speed at each 30 min time point over the 48 h experiment and with treatment (unfed, diet 1, diet 2, diet 3 and diet 5) were examined with a two-way repeated measures ANOVA followed by *post hoc* testing (Holm–Sidak) as appropriate. Changes in mean, overall salinity preferences (i.e. Table 2, diel effects, sex effects and treatment day effects) were examined using paired and unpaired *t*-tests as appropriate, followed by appropriate Bonferroni corrections. For series 2.1, plasma and chyme Na^+ and Ca^{2+} concentrations, along with water, GI tract and body Ca^{2+} concentrations were examined using a two-way ANOVA with salinity (22 *vs* 35 p.p.t.) and diet (unfed, diet 1, diet 2, diet 3, diet 4 and diet 5) as factors followed by Holm–Sidak *post hoc* testing. And, finally, for series 2.2, the $^{45}Ca^{2+}$ percentage distribution was transformed (arc sine transformation) and a two-way ANOVA (with salinity and diet as factors as in series 2.1) was used to examine the data, followed by Holm–Sidak *post hoc* testing. When two-way ANOVA analyses were employed for data analysis the significance of the interaction between the factors is reported in the text. Where the interaction effect between factors was significant, each factor was examined individually. Where the interaction effect between the main factors was not significant, but a main factor itself was significant, multiple comparisons (Holm–Sidak *post hoc* tests) were employed to determine specific significant differences. SigmaStat 3.1 was used to analyse the data.

RESULTS
Series 1

There were no significant differences in the preferred salinity of killifish over the first 24 h period of each 48 h trial (interaction term P=0.998; diet P=0.741; time P=0.067) and therefore the preferred salinity over this period was averaged for all treatments (Fig. 1A). Excluding the first 4 h during which the mean salinity in the system decreased from the full-strength seawater where each trial was begun, the mean salinity over the first 20 h for all treatments was 21.5±0.4 p.p.t. (N=35; Fig. 1A). For the second 24 h period of each trial, the treatments were analysed individually as follows because statistical analysis revealed a significant interaction of diet and time (P<0.001) as well as significant effects of diet itself (P=0.011) and time (P<0.001).

For the unfed treatment, the mean salinity preference over the second 24 h of the 48 h trial was 21.3±0.3 p.p.t. (N=7; Table 2, Fig. 1B), identical to that in the first 24 h (Fig. 1A). As with unfed fish, fish that were fed diet 1 (live blackworms) showed no significant changes in salinity preference over the second 24 h trial period despite transient fluctuations (Fig. 1C). The mean preferred salinity was 21.0±0.3 p.p.t. (N=7; Table 2). There were also no significant differences between fish fed diet 1 and unfed fish at any time point (Fig. 1B,C). In comparison, for fish that consumed diet 2 (dried blackworm pellets) the preferred salinity in the second 24 h was significantly lower (17.3±0.5 p.p.t., N=7) relative to unfed fish or fish fed diet 1 (Table 2). When examining individual time points, there were no significant effects of diet or time compared with unfed fish or fish fed diet 1 (Fig. 1B–D). When fish were fed diet 3 (high Ca^{2+} blackworm pellets) and salinity preference was
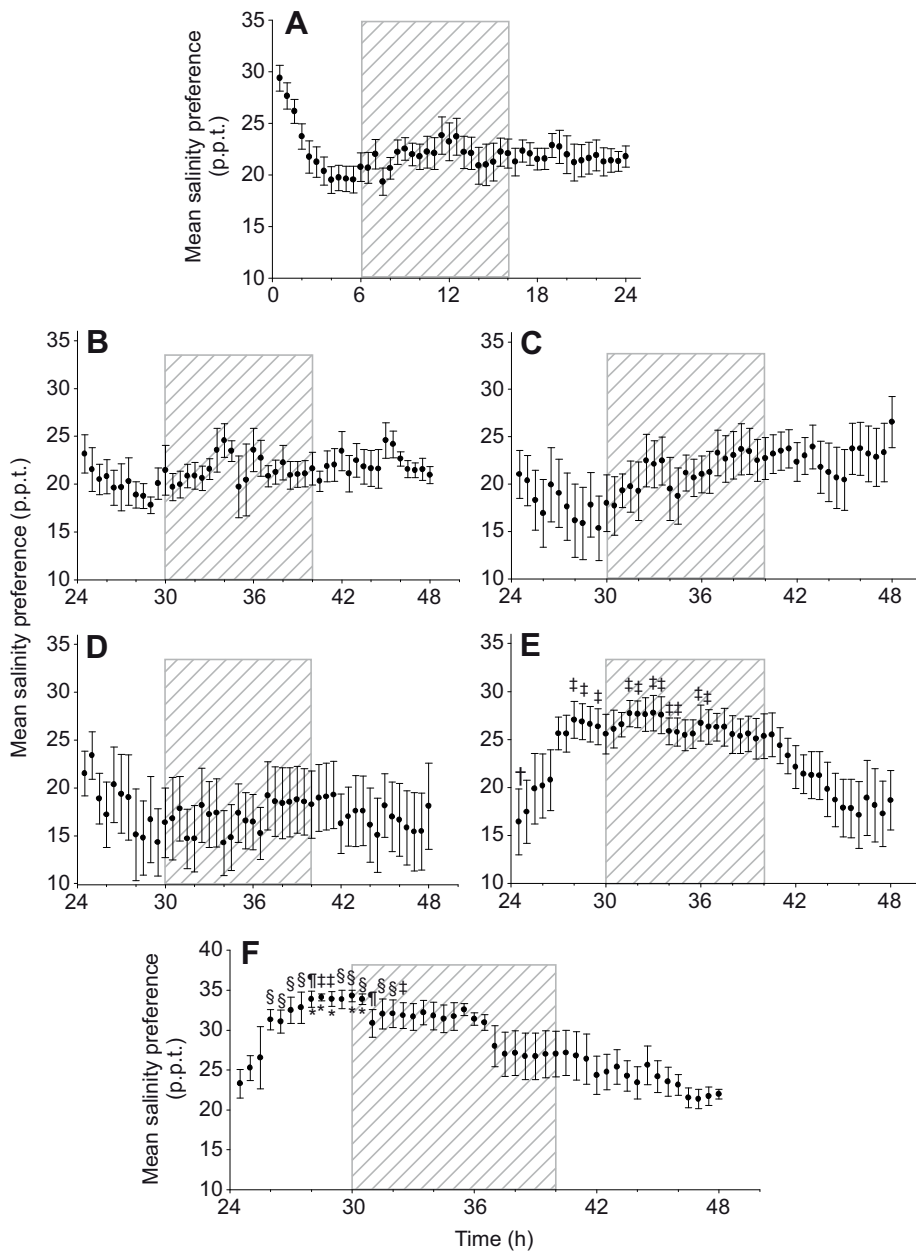
Fig. 1. (A) The mean preferred salinity of killifish over all treatments over the first 24 h of each trial. Feeding (or sham feeding) occurred immediately following this initial 24 h period. (B–F) Mean preferred salinity of killifish that were (B) unfed, (C) fed diet 1, (D) fed diet 2, (E) fed diet 3 or (F) fed diet 5 (see Table 1) following the initial 24 h trial period (see A). The hatched areas represent the dark portion of the light:dark cycle of the chamber. $N=7$ for each diet. *Significant effect ($P<0.05$) of time within each diet treatment. †Significant difference from unfed fish at the same time point. ‡Significant difference from unfed fish and fish fed diet 2 at the same time point. §Significant difference from unfed fish, fish fed diet 1 and fish fed diet 2 at the same time point. ¶Significant difference from unfed fish, fish fed diet 1, fish fed diet 2 and fish fed diet 3 at the same time point.

averaged over the subsequent 24 h, there was no significant effect of treatment on the preferred salinity when compared with unfed fish (overall mean preferred salinity 23.4±0.3 p.p.t., $N=7$; Table 2). However, when examining individual time points there were significant increases in the preferred salinity of fish fed diet 3, when compared with fish that were not fed, or were fed either diet 1 or diet 2 over a brief period following feeding (Fig. 1E). Notably, when examined on this finer time scale (one that more accurately represented the time frame of digestion), the overall mean salinity preference of fish fed diet 3 increased at 0–12 h post-feeding (i.e. 24–36 h) and increased even further at 4–12 h postfeeding (i.e. 28–36 h; Table 2). Fish that consumed diet 5 (fish flakes and brine shrimp pellets that were high in both Ca^{2+} and NaCl) exhibited an even more pronounced effect of treatment when compared with fish that were fed diet 3, and the difference was highly significant relative to unfed fish, or fish fed either diet 1 or diet 2. On average, the preferred salinity of fish fed diet 5 during the second 24 h trial was 28.4±0.4 p.p.t. ($N=7$; Table 2). Again,

when examined on a finer time scale the salinity preference of fish fed diet 5 was much larger, 1.5-fold higher at 0–12 h post-feeding (24–36 h) and 1.6-fold higher at 4–12 h post-feeding (28–36 h) than in unfed fish (Table 2). Specifically, there was an increase in the preferred salinity 2 h after feeding, significantly rising above that of fish fed diet 1 and 2 (Fig. 1F). The preferred salinity continued to increase for another 2 h until full-strength salinity was reached (Fig. 1F). The preferred salinity of fish fed diet 5 then fell to become not significantly different from the other treatments, 9 h following feeding (33 h; Fig. 1F).

Overall, there were no significant diel effects or sex effects on the preferred salinity of animals during each trial (examined using paired *t*-tests comparing the mean salinity for each condition within each treatment) with two exceptions. Fish that were fed diets 3 and 5 showed a significant increase in the mean dark cycle salinity preference during the second 24 h trial period over the light cycle salinity preference (i.e. mean night *vs* day salinity preference during second 24 h of the trial). The increase in salinity preference in the

Table 2. Summary of mean salinity preference (p.p.t.) following feeding or sham feeding at 24 h

Time period	Unfed	Diet 1	Diet 2	Diet 3	Diet 5
24–48 h	21.3±0.3	21.0±0.3	17.3±0.5*	23.4±0.3	28.4±0.4*
24–36 h	20.8±0.4	19.2±0.5	17.2±0.6*	24.9±0.9*	31.6±0.7*
28–36 h	20.9±0.5	19.2±0.6	16.8±0.4*	26.6±0.3*	32.5±0.4*

N=7 for each diet.
*Significant difference from the unfed group over the same time period.

dark was confounded by feeding occurring 6 h prior to the lighting transition.

In contrast with the mean preferred salinity, there were no effects of treatment or time on mean swimming speed (interaction effect P=0.991; diet P=0.336; time P=0.798; Fig. 2), and as such all treatments were combined at each time point. Excluding the first 4 h of experimentation as a training period, the mean swimming speed of killifish, regardless of time or diet, was $1.1\pm0.1\,BL\,s^{-1}$. There were no diel or sex effects as examined with t-tests. On average, there were no significant differences in the stationary time over each 30 min interval between or amongst all treatments (9±3 min, data not shown; N=35).

Series 2.1

Statistical testing revealed that not only was the interaction effect of diet and salinity significant (P=0.009) for plasma Ca^{2+} concentration but also diet and salinity were significant individual factors as well (P=0.001 and P=0.005, respectively). In contrast, when examining plasma Na^+ and chyme Ca^{2+} concentrations, the interaction effect of diet and salinity was not significant (P=0.131 and P=0.695, respectively), nor was the salinity factor itself (P=0.059 and P=0.361, respectively), but the main factor of diet was (P<0.001 for both). For chyme Na^+ concentration, the interaction effects between salinity and diet were not significant (P=0.443) and neither were the effects of diet or salinity when examined individually (P=0.237 and P=0.110, respectively). Hence, for unfed fish, gradual acclimation to 22 p.p.t. from 35 p.p.t. (over

24 h) followed by a rapid increase in salinity (from 22 to 35 p.p.t. in 2 h) had no effect on the plasma concentration of Na^+ (mean for the two salinities, $147.4\pm4.2\,mmol\,l^{-1}$, N=14; Table 3) or Ca^{2+} (mean for both salinities, $2.4\pm0.4\,mmol\,l^{-1}$, N=14; Fig. 3). Consuming diet 1 while maintaining the external salinity at 22 p.p.t. had no significant effects on plasma ion constituents when compared with the same treatment of unfed fish though there was a tendency for higher plasma Ca^{2+} in these fed fish (Ca^{2+} $5.1\pm1.4\,mmol\,l^{-1}$, N=7, Fig. 3). There was also no significant effect of changing the external salinity to 35 p.p.t. on plasma Ca^{2+} concentration ($5.1\pm1.3\,mmol\,l^{-1}$, N=7; Fig. 3). The same held for fish consuming diet 2 when examining Na^+ and Ca^{2+}, with plasma levels being maintained regardless of external salinity, with no differences relative to fish fed diet 1 or unfed fish (mean for the two salinities, Na^+ $148.2\pm4.9\,mmol\,l^{-1}$, N=14; Table 3; Ca^{2+} $3.2\pm0.4\,mmol\,l^{-1}$, N=14; Fig. 3). Plasma Na^+ concentration was unaffected by consuming diet 3 or increasing the external salinity, similar to what was observed following the consumption of diets 1 and 2 (Table 3). However, consumption of diet 3 when fish were constrained to a salinity of 22 p.p.t. resulted in a significant increase in plasma Ca^{2+} to $6.2\pm2.1\,mmol\,l^{-1}$, N=7, substantially above unfed and diet 2 levels. Notably, when the external salinity was increased to 35 p.p.t. this post-feeding plasma Ca^{2+} concentration was no longer statistically different from unfed values ($3.0\pm0.9\,mmol\,l^{-1}$, N=7; Fig. 3). Diet 4 showed the same trends as diet 3, with the post-feeding rise in plasma Ca^{2+} being alleviated when the fish were transferred to 35 p.p.t. (Fig. 3). Diet 5 showed no effect of diet or external salinity on plasma Na^+ as with most other treatments (Table 3). When fish ingested diet 5 there was a very large and significant increase in plasma Ca^{2+} concentration (to $13.4\pm1.0\,mmol\,l^{-1}$, N=7; Fig. 3) but again, the transition to higher salinity helped to attenuate the effect ($8.8\pm0.9\,mmol\,l^{-1}$, N=7; Fig. 3). The concentration of Na^+ in the chyme was maintained irrespective
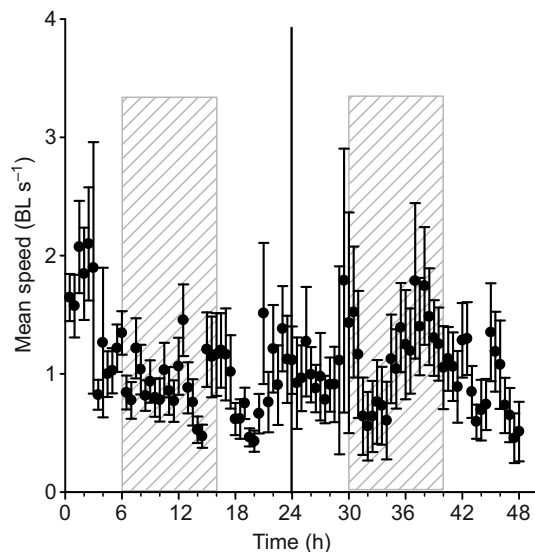

Fig. 2. The mean swimming speed [body lengths (BL) s^{-1}] across all treatments over 30 min time intervals. No significant differences were found when the treatments were examined individually, and therefore treatments were combined (N=35). There was no significant effect of time (P>0.05) on the mean swimming speed of the killifish. Hatched areas represent the dark period and feeding occurred at 24 h as shown by the vertical line.

Table 3. Plasma Na^+ concentration and chyme Na^+ and Ca^{2+} concentration in killifish fed various diets and either constrained to 22 p.p.t. or moved to 35 p.p.t.

| | | Plasma | Chyme | |
| | | Na⁺ | Na⁺ | Ca²⁺ |
Diet	Salinity	$(mmol\,l^{-1})$	$(mmol\,l^{-1})$	$(mmol\,l^{-1})$
Unfed	22 p.p.t.	144±4	–	–
	35 p.p.t.	150±4	–	–
Diet 1	22 p.p.t.	143±6	–	–
	35 p.p.t.	150±3	–	–
Diet 2	22 p.p.t.	144±5	96±6	53±10
	35 p.p.t.	153±6	79±4	58±7
Diet 3	22 p.p.t.	150±2	102±4	510±23*
	35 p.p.t.	146±8	98±3	481±32*
Diet 4	22 p.p.t.	157±4	–	–
	35 p.p.t.	172±5	–	–
Diet 5	22 p.p.t.	149±6	95±4	502±22*
	35·p.p.t.	143±3	106±8	503±31*

N=7 for each salinity and diet.
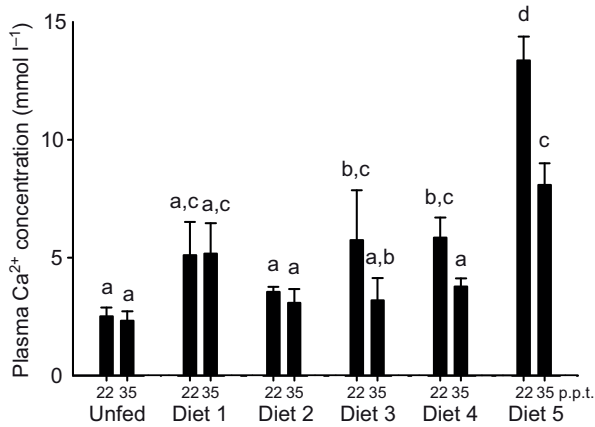*Significant effect of diet (P<0.05).

Fig. 3. Plasma Ca^{2+} concentration of fish fed various diets and constrained to a salinity of 22 p.p.t. or moved to 35 p.p.t. Bars that share letters are not significantly different (P>0.05). N=7 for each treatment.

of diet (mean concentration across all diets and salinities; 95.9±4.1 mmol l^{-1}, N=42; Table 3). Ca^{2+} concentration (µmol g^{-1}) in the chyme of fish fed diet 2 (mean across the two salinities, 55.4±8.2 mmol l^{-1}, N=14) was significantly lower than that seen in the chyme of fish fed diet 3 (mean across the two salinities, 495.0±27.7 mmol l^{-1}, N=14) and diet 5 (mean across the two salinities, 502.4±26.5 mmol l^{-1}, N=14; Table 3).

Series 2.2

Overall, when the proportion of ^{45}Ca^{2+} found in the body and water was examined statistically, there was a significant effect of diet (P=0.009, P=0.015), salinity (P=0.021, P=0.027) and a significant interaction between the two factors (P=0.038, P=0.041). In contrast there were no significant effects found in the gut (interaction P=0.376, diet P=0.064, salinity P=0.978; Fig. 4A). Hence, there was no effect of increasing the external salinity on the distribution of ^{45}Ca^{2+} among the external water, the whole-body compartment and the intestine (including chyme) when fish were fed diet 2 (Fig. 4A). At this time (6 h post-feeding), each compartment accounted for roughly equal proportions of the ingested ^{45}Ca^{2+}, approximately 29–41% each. Fish that consumed diet 3 showed a similar pattern in postprandial ^{45}Ca^{2+} distribution, regardless of external salinity (Fig. 4A). While there was no significant difference between the distributions seen with diet 2 and diet 3, fish that were fed diet 5 and constrained to 22 p.p.t. retained the majority of ^{45}Ca^{2+} within the body compartment, and released only a very small proportion to the external water (2.1±1.4%, N=7). However, when these fish were exposed to an increase in external salinity to 35 p.p.t., there was a significant increase in the proportion released to the water (15.8±6.1%, N=7) and a corresponding significant decrease in the proportion retained in the body compartment compared with fish at 22 p.p.t. (42±12.4%, N=7 at 35 p.p.t. vs 81.6±12%, N=7 at 22 p.p.t.; Fig. 4A). There was then no difference when these proportions were compared with the other two diet treatments (Fig. 4A). There were no significant differences in the proportion of ^{45}Ca^{2+} that was found in the intestine, either within diet 5 between the salinities, or between the other two diets (Fig. 4A).

There was a significant interaction of diet and salinity (P<0.001) as well as significant effects of both diet (P<0.001) and salinity (P<0.001) on the absolute amount of Ca^{2+} found in the water, body and gut (Fig. 4B). Hence, the corresponding total amount of dietary
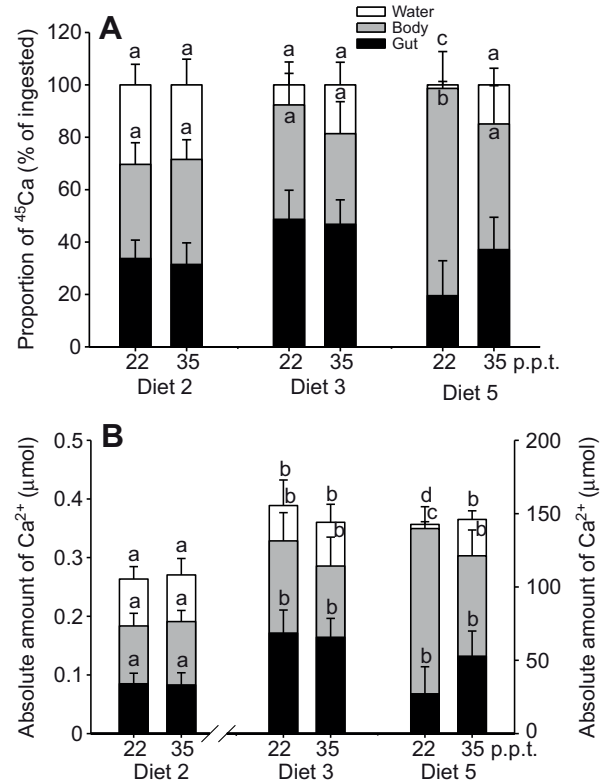

Fig. 4. (A). The proportion of ^{45}Ca^{2+} (% of ingested) in the water, the body and the intestine of killifish fed various diets and either constrained to a salinity of 22 p.p.t. or moved to 35 p.p.t. N=7 for each treatment. Bars that share letters are not significantly different (P>0.05). (B) The absolute amount of dietary Ca^{2+} excreted into the water, absorbed into the body and remaining in the intestine for each killifish fed various diets and either constrained to a salinity of 22 p.p.t. or moved to 35 p.p.t. The left y-axis refers to diet 2; the right y-axis refers to diets 3 and 5. Bars that share letters are not significantly different (P>0.05).

Ca^{2+} [based on the specific activity of the food (Table 1) and the radioactivity found in each compartment (Fig. 4A)] shows the very large body Ca^{2+} burden generated by diets 3 and 5 (note the difference in scale, right-hand axis, for the data for diets 3 and 5). This was particularly pronounced when the salinity was restricted to 22 p.p.t. (Fig. 4B). The calculated load of dietary Ca^{2+} in the body at 22 p.p.t. is 1.7-fold higher than when the animal is moved to 35 p.p.t. (Fig. 4B).

DISCUSSION

The unique system employed in the current study was first used to test the salinity preference of the grey snapper, *Lutjanus griseus* (Serrano et al., 2010). Interestingly, the laboratory salinity preference of *L. griseus* contradicted field observations of *L. griseus* occurrence, stressing the importance of external biotic and abiotic factors (Serrano et al., 2010). In the present study, we used this same system to discover the importance of a combined biotic/abiotic factor, the salt content of a meal, in altering the salinity preference of the killifish.

In our study, the mean preferred salinity of unfed killifish in the laboratory was approximately 22 p.p.t. (Fig. 1A,B) which is similar to a previous study on this subspecies [20 p.p.t. (Fritz and Garside, 1974)] but in marked contrast to our initial hypothesis. A salinity of 22 p.p.t. is above the iso-osmotic point, where most ions would

neither move into nor out of the body. In fact, at 22 p.p.t., the external Na^+, Cl^- and Ca^{2+} levels are approximately 2-fold higher than plasma values. In comparison, an environment of 10–12 p.p.t. has Na^+, Cl^- and Ca^{2+} concentrations that are relatively similar between the water and the blood (e.g. Deane and Woo, 2004; Edwards et al., 2005). Energetically speaking, an iso-osmotic environment would eliminate the need for active regulation of ion and water homeostasis. Above or below this salinity, fish will be forced to ionoregulate and osmoregulate, an energetically expensive endeavour (reviewed by Boeuf and Payan, 2001) as it relies heavily on the Na^+/K^+-ATPase to create ion gradients and to drive transport. Fritz and Garside also showed that prior acclimation to either freshwater or seawater before experimentation did not affect overall salinity preference (Fritz and Garside, 1974). In contrast, prior acclimation of killifish to freshwater appeared to reduce the adaptive responses of transepithelial potentials to varying salinities (Wood and Grosell, 2008). These results suggest that the preferred habitats of euryhaline fish may be distinct from those expected based on individual physiological systems.

Salinity is one of the most important environmental factors to influence the geographic distribution of killifish, another probably being temperature (e.g. Fangue et al., 2006). Although considered euryhaline, their distributions in Atlantic Canada and the Eastern USA are such that killifish occur almost exclusively in estuaries and salt marshes. Additionally, the *Fundulus* genus has more than 15 species, many of which occur in sympatry. Spatial separation of these species is probably not due to physiological barriers in salinity tolerance, as all species can tolerate overlapping ranges of salinity (Weisberg, 1986; Griffith, 1974). However, salinity preference differences [e.g. *F. diaphanus* salinity preference of 8 p.p.t. *vs F. heteroclitus* preference of ~20 p.p.t. (Fritz and Garside, 1974)] may be a factor in partitioning each species within the habitat. Additionally, the subspecies *F. heteroclitus macrolepidotus* used in the current study overlaps geographically with the other subspecies *F. heteroclitus heteroclitus* with whom they interbreed and form hybrids. The *heteroclitus* subspecies tolerates lower salinity less well than the *macrolepidotus* subspecies (Scott et al., 2004). In future, it would be of interest to test whether there are corresponding differences in salinity preference between the two subspecies. Other physiological differences between *heteroclitus* and *macrolepidotus* include temperature preference differences (Fangue et al., 2009a), mitochondrial differences (Fangue et al., 2009b) and differential heat shock responses (Fangue et al., 2006; Healy et al., 2010), suggesting that the two subspecies may have slightly different physiology. Finally, the preference of fish exposed to less frequent salinity changes (e.g. migratory species such as salmonids or eels) or less predictable salinity changes (e.g. tide pool inhabitants such as tide pool sculpins) is unclear. We hypothesize that animals exposed to these types of salinity challenges during their life history would not utilize this strategy of altering their surroundings by local movements to compensate for a physiological imbalance, probably because of a lack of opportunity to do so.

The mean swimming speed (and conversely the lengths of stationary periods) of *F. heteroclitus* was unaffected by feeding regardless of the diet, diel patterns or sex (Fig. 2), suggesting that the salinity preference observed was not influenced by activity levels. In contrast, Edeline and colleagues showed that salinity preferences in *Anguilla anguilla* were linked to locomotor activity, with more active eels preferring a lower salinity (Edeline et al., 2005). They hypothesized that the more active eels were in the process of migrating to freshwater, which suggests that salinity preference can be transient and depend on life history stages. Additionally, Serrano

and colleagues showed that activity levels of unfed *L. griseus* were correlated with external salinity, with spontaneous swimming decreasing at salinity extremes (i.e. both low and high salinities), a phenomenon thought to relate to energy conservation for osmoregulatory needs (Serrano et al., 2010). This particular difference may be partially accounted for by the burst swimming nature of killifish *vs* the continual swimming nature of the grey snapper [current study *vs* that of Serrano and colleagues (Serrano et al., 2010)].

After consuming either live worm prey (diet 1) or worm pellets (diet 2), the salinity preference of killifish was either unchanged or slightly decreased (Table 2). These worms presented the fish GI tract with minimal ion loads. In fact, the ion levels in the live worm diet were much less than those found in the surrounding water (Table 1). This suggests that physiological changes associated with the process of digestion are not responsible for driving the behavioural external salinity selection seen with ion-enriched diets. Addition of Ca^{2+} to the worm pellets (diet 3) caused a marked increase in the salinity preference of killifish (Table 2). This phenomenon was exaggerated when fish consumed not only high levels of Ca^{2+} but also high levels of NaCl (diet 5; Table 2). Interestingly, dietary Ca^{2+} was shown to be assimilated in seawater trout despite high environmental Ca^{2+} levels (Bucking et al., 2011). This assimilation suggests that dietary Ca^{2+} may be important for ion regulation in seawater teleosts despite the fact that absorbing Ca^{2+} may be unfavourable in seawater fish as they are faced with an inwardly directed Ca^{2+} concentration gradient. Additionally, ion regulation imbalances associated with feeding have been suspected of interacting with Ca^{2+}-sensing receptors (CaSRs) in fish through alterations in plasma ion concentrations. Fellner and Parker suggest that increases in plasma Na^+ concentration following feeding reduce the signalling activity of the CaSR found in the rectal gland of elasmobranchs, and consequently increase rectal gland blood flow and salt secretion (Fellner and Parker, 2002; Fellner and Parker, 2004). A controversial role for piscine CaSRs in sensing the external salinity has been theorized. It has been suggested that the CaSRs, located mainly in ionoregulating and osmoregulating tissues such as the gill, kidney and intestine in addition to the brain, are activated by increases in ions associated with increases in external salinity (Nearing et al., 2002). This is either through increases in plasma ion concentrations created by elevated environmental levels or through direct contact with the external environment (Nearing et al., 2002). It is thought that the CaSRs would hence provide a mechanism of notifying organs of changes in external salinity. However, how this theory fits in with previous evidence that Cl^- is the stimulus for salinity sensing and control of drinking rates (Ando and Nagashima, 1996; Hirano, 1974) is unknown.

The reason for the move to higher salinity appears to be plasma Ca^{2+} regulation, as when the fish were confined to 22 p.p.t., the plasma Ca^{2+} levels rose to impressive levels (Fig. 3), apparently caused by a compromised ability to unload Ca^{2+} to the water (Fig. 4A). Generally, Ca^{2+} is handled by three organs: the intestine, the kidney and the gills. Dietary Ca^{2+} appears to be taken up in substantial amounts in both freshwater (Bucking and Wood, 2007) and seawater fish (Bucking et al., 2011). Intestinal uptake of Ca^{2+} is thought to be Na^+ dependent (Flik et al., 1993; Flik et al., 1990) and is likely to involve a Na^+/Ca^{2+} exchanger. The results of the current study suggest that Ca^{2+} uptake from the diet is not influenced by changes in salinity (Table 3, Fig. 4) and probably does not explain the apparent inability to regulate plasma Ca^{2+}. Renal excretion of divalent ions is thought to be important in both freshwater and seawater fish, despite a significant reduction in urine production at

higher salinities. The kidney anatomy of the killifish is consistent with that of other marine stenohaline teleosts; the nephron lacks the distal tublule responsible for reabsorption of Na^+ and Cl^- (Edwards and Schnitter, 1933), suggesting that they may be using this route for Ca^{2+} excretion. In fact, renal calcium reabsorption is significantly reduced in seawater-adapted fish (Schmidt-Nielsen and Renfro, 1975; Bjornsson and Nilsson, 1985; Elger et al., 1987), which ultimately results in higher excretion to the urine.

Finally, Ca^{2+} is actively taken up by the gills of fish for growth and homeostasis (Flik et al., 1985; Perry and Flik, 1988; Flik and Verbost, 1994); however, gill Ca^{2+} transporter activity is not reduced with increasing salinity (reviewed by Flik et al., 1996). Complex electrochemical conditions may offer an explanation, at least in part. The increased plasma Ca^{2+} levels may trigger a move to higher salinity. As this would result in an increase in the transepithelial electrical potential (TEP; blood-side positive) of the gills (Wood and Grosell, 2008), the animal may account for the increase in the equilibrium potential (calculated according to the Nernst equation) of Ca^{2+} caused by the increased plasma concentrations, and maintain a net outward driving force for passive Ca^{2+} loss. Indeed, it would be of interest to record the TEP in these Ca^{2+}-loaded fish. Elevated dietary NaCl levels may also result in elevated plasma Na^+ concentrations, increasing the activity of the branchial Na^+/Ca^{2+} exchanger, which would drive internal plasma Ca^{2+} concentrations even higher, resulting in the need to move to water of even higher salinity to maintain the equilibrium potential of Ca^{2+} below that of the TEP. However, Prodocimo and colleagues found Ca^{2+} influx to increase with increasing salinity in killifish, with no change in Ca^{2+} efflux (Prodocimo et al., 2007), which contradicts our hypotheses, as does our finding of mainly non-significant changes in plasma Na^+ concentration. Paradoxically, Prodocimo and colleagues also found that at higher salinities, killifish had decreased whole-body calcium concentrations (Prodocimo et al., 2007). Additionally, the influx rates measured at full-strength seawater were more than 2-fold greater than those previously measured (Mayer-Gostan et al., 1983). Clearly, this is a complicated physiological response and much more experimentation is needed to verify that an increase in plasma Ca^{2+} is more easily relieved at higher salinities (either through the kidney or the gill) and that this is the reason for the observed salinity preference in the current study.

Overall, this study confirms the salinity preference of *F. heteroclitus* to be about 22 p.p.t., clearly greater than iso-osmotic values. However, this is influenced by the ionic composition of the diet, with elevated Ca^{2+} driving the preferred salinity higher, an effect that is exaggerated by elevated dietary NaCl. The adaptive significance behind the change in salinity preference may be related to ion regulation, with an inability to excrete the dietary Ca^{2+} load at lower salinities. This is not to say that the Ca^{2+}-loaded killifish were unable to survive at lower salinities, but given the opportunity they sought out an environment that augmented their ionoregulation, showing adaptive behavioural regulation. Based on the current study, killifish actively seek out salinities that can rectify internal Ca^{2+} imbalances created by feeding on Ca^{2+}-rich diets. Ultimately, this could extend to a multitude of estuarine fish species. As pointed out in the Introduction, environmental salinity plays a significant role in determining the physiology of fish. However, this study illustrates that the physiology of organisms conversely plays an important role in determining their preferred environment. The implications of this on the overall niche occupation of estuarine fish could be large, affecting the way we interpret the ecology of these fish species.

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