ABSTRACT

Teleost fish experience passive osmotic water influx in fresh water (FW) and water outflux in salt water, which is normally compensated by water flow driven by active ion transport mechanisms. Euryhaline fish may also minimize osmotic energy demand by “behavioral osmoregulation”, seeking a medium isotonic with their body fluids. Our goal was to evaluate the energy requirement for osmoregulation by the euryhaline fish *Fundulus heteroclitus*, to determine whether it is of sufficient magnitude to favor behavioral osmoregulation. We have developed a method of weighing small fish repetitively for long periods without apparent damage, which was used to assess changes in water content following changes in external salinity. We found that cold (4 °C) inhibits osmoregulatory active transport mechanisms in fish acclimated to warmer temperatures, leading to a net passive water flux which is reversed by rewarming the fish. A sudden change of salinity at room temperature triggers a transient change in water content and the initial slope can be used to measure the minimum passive flux at that temperature. With some reasonable assumptions as to the stoichiometry of the ion transport and ATP-generating processes, we can calculate the amount of respiration required for ion transport and compare it to the oxygen uptake measured previously (Kidder et al., 2005) under the same conditions. We conclude that osmoregulation in sea water requires from 6% to 10% of the total energy budget in sea water, with smaller percentages in FW, and that this fraction is probably sufficient to be a significant selective driving force favoring behavioral osmoregulation under some circumstances.

Most teleosts live in water of significantly different osmolarity from that of their plasma, and therefore must perform osmoregulatory work to compensate for the osmotic flux through their gills. Fresh water (FW) fish drink little water, have high glomerular filtration rates (GFR) and strong salt reabsorption in their kidney tubules. They secrete high volumes of dilute urine and reabsorb NaCl through the gills to compensate for urinary salt loss. Salt water (SW) fish absorb salt and water from ingested sea water, have low GFRs and excrete large quantities of NaCl through the gills. In either case, metabolic energy is required for osmoregulation, with euryhaline fish requiring additional energy for the synthesis of new salt-transporting proteins as the fish moves from salt to FW and vice versa. The estuarine fish, *Fundulus heteroclitus* (killifish, mumichog), is capable of surviving indefinitely in fresh (<0.5 ppt) or hypersaline SW (Griffith, ’74). Unlike anadromous or catadromous fish (e.g., salmon or eels) which change environmental salinity only twice in their lives, *F. heteroclitus* may be subjected to osmotic variations at daily intervals (Marshall, 2003). Observations in our laboratory (Kidder, ’97) suggest that, all other factors being equal, this species prefers water approximately isotonic to its blood plasma, where osmotic water flux is
minimal. One might expect the evolution of such behavior if the energy cost of osmoregulation were a significant part of its normal energy budget. In order to determine this energy cost, we need an estimate of the magnitude of the water flux under a variety of temperature and salinity regimes.

There are few data available on this subject (Boeuf and Payan, 2001), and apparently none on *F. heteroclitus*. We report on the water fluxes into and out of *F. heteroclitus* in various conditions. With these water flux data, we calculate the osmotic load and the amount of salt transport, which must be supported by metabolic energy. By accurately measuring oxygen consumption under these same conditions (Kidder et al., 2005), we can calculate the percentage of resting metabolic rate consumed by osmoregulatory work in this species.

In the absence of a specific inhibitor of osmoregulatory salt transport that does not inhibit other systems required by the organism, we elected to cool the fish to 4°C as a way of lowering metabolically driven water movements without markedly changing passive (osmotic) water fluxes. If we reduce the apparent active osmoregulation to zero, the net water flux would consist of the osmotic component alone, and we would then have an accurate measure of passive osmotic flux. We will see that with some reservations and under some conditions, our expectation seems to have been met.

Some of these data have previously appeared in preliminary form (Kidder, 2000).

**METHODS AND MATERIALS**

Short-term changes in the weight of osmotically stressed fish seem primarily due to water movements. Weighing a small fish requires removing it from water, which cannot be done accurately by lifting it in a net. A soft fabric net retains a variable amount of water, resulting in inaccurate weights, while a metallic net damages the fish. We therefore anesthetized the fish with MS-222 and inserted a length of sewing thread through the lower jaw with a sewing needle. After recovery from anesthesia, the fish could be raised by its thread until it was clear of the water, allowed to drain for 1 min, weighed and returned to the water. Fish treated in this manner can survive several weeks of once-per-hour weighing and recover completely after the experiment. Since these fish are often stranded by receding tides and do not panic when removed from water, it is probable that they are well suited to such weighings without undue stress.

To automate the collection of data, and to be able to weigh several fish at a time, an apparatus (Fig. 1) was constructed using four Narco Biosystems (Austen, TX, USA) strain gauges mounted on a board which was raised and lowered by a stepping motor under computer control. We used the Sable Systems (Henderson, NV, USA) "Datacan V" program to drive the motor and to collect and reduce the data to tabular form. The calibration of the strain gauges was periodically checked with a series of weights and found to be linear although different for each sensor.

Several protocols were used. In the first series, fish (1.5–10 g) acclimated to warm (15–20°C) SW (30%) were fitted with threads and were placed in individual 1-l "tall form" beakers containing 500 ml of SW. An aquarium pump was used to bubble air through the solutions to ensure adequate oxygenation, and the beakers were placed in a water bath maintained at 16°C. Once per hour, the fish were withdrawn from the water for about 1 min, weighed and returned to the water. An example of the data collected is shown in Figure 2. At time zero, the bath temperature was lowered to 4°C and the water was changed to cold FW (0.5%). Weighing continued once per hour over the following 6 days. At this time, the bath temperature was raised to 16°C without changing the water, and the weighing continued...
for another 6 days. An example of this sort of experiment is shown in Figure 3.

A second series was conducted in the same manner, using fish acclimated to FW and tested in FW. In this case, the only change at time zero was the temperature. In other experiments, fish acclimated to FW, iso-osmotic (10%) or SW were challenged by cold SW. As a control, fish were cooled in iso-osmotic water to confirm that an osmotic gradient was necessary for a weight change. Finally, a series of experiments was conducted in which fish acclimated to FW or SW at 16°C were placed in the opposite water at 16°C, to determine the time course of development of osmoregulation at “normal” temperature.

Experiments were conducted at two sites. For experiments at Illinois State University (ISU), *F. heteroclitus* were obtained from the Marine Biological Laboratory (Woods Hole, MA, USA), and maintained in the laboratory in aquaria filled with water at 0.5% (FW), 10% (ISO) or 34% (SW), using an artificial salt mixture (Instant Ocean®, Mentor, OH, USA) dissolved in water purified by reverse osmosis. These aquaria were held at room temperature (ca. 25°C). For experiments at the Mt. Desert Island Biological Laboratory (MDIBL) in Salisbury Cove, ME, the fish were trapped in Northeast Creek, a small estuary, and maintained in running natural SW aquaria outside the laboratory building, at temperatures ranging from 10°C to 20°C depending on the weather. Fish were fed commercial flake fish food (TetraMin, Melle, Germany). Since transferring these fish from one salinity to another results in a transient change in their plasma osmolarity which requires about 4 days for full correction (Zadunaisky et al., '95; Kidder, '97), they were acclimated to a single salinity for at least 7 days before use. Experimental salinities were always produced with artificial salt water (ASW) for consistency. All fish were retained after the experiment, fish used at MDIBL were returned to their source, and no fish were reused. All procedures used are consistent with the American Physiological Society’s guidelines for use of laboratory animals and were approved by the relevant Institutional Animal Care and Use Committees.

Examination of the results of individual fish recorded at MDIBL showed an apparent circadian rhythm in their weights. This was traced to diurnal fluctuations in laboratory temperature, which changed the sensor calibration. The maximum variation of the most temperature-sensitive sensor was 180 mg, which could introduce an error of 3% or 30 ml H₂O/kg fish into the results for a 6 g fish. These errors do not contribute any serious problems in interpretation of the data, and were not observed in the experiments conducted at ISU in a temperature-controlled laboratory. They were eliminated in later experiments by holding the sensors at a constant temperature with small electric heaters and a thermostat.

The weight data were normalized to the initial weight of the fish, and plotted as ml kg⁻¹, on the assumption that the weight change is due to water movement. Data are reported as mean ± SE for *N* observations. Linear regression lines were fit using a commercial plotting program (SigmaPlot, Pt. Richmond, CA, USA), determination of the standard error of slopes by the regression analysis, and data means were compared by *t*-test.

![Graph](image-url)
RESULTS

Repetitive weighing may be somewhat stressful to fish, and might have some effects on their physiology. Stress effects seem to be minor compared to the osmotically induced changes. At 16°C, it is clear from the “recovery” phases shown (e.g., Figs. 3, 5, 8, etc.) that these fish are osmoregulating while being weighed, and can apparently achieve full compensation. In the cold, the weight change is dependent on an adverse osmotic gradient, and when this is removed, there is no significant weight change. In two replicates, three fish each acclimated to 10% water were weighed in this water at 4°C for 6 days. In one replicate, a slight water loss occurred (−0.13 ml·kg⁻¹·hr⁻¹, r² = 0.57); in the other, a slight water gain occurred (0.091 ± 0.019 ml·kg⁻¹·hr⁻¹, r² = 0.11). Neither of the slopes were significantly different from zero. Cooling to 4°C does not cause weight changes in the absence of an osmotic gradient.

In a different sort of control, SW-acclimated fish were weighed at 16°C in SW for 24 hr. The slope of the regression line for each fish was determined; the average of these slopes was 0.774 ± 0.412 ml·kg⁻¹·hr⁻¹ (N = 12), and the t statistic for the difference between this slope and zero was 1.877, for which P > 0.05. The act of weighing these fish once per hour in an adverse osmotic gradient does not give rise to artifactual weight changes at this temperature.

Figure 3 shows a typical weight experiment with a single fish acclimated to warm (~20°C) ASW, and initially bathed in 16°C ASW. Following an initial sudden weight loss when the water was changed to 4°C FW, this fish gained 15% of its body weight at two different rates (in two phases) over 6 days with no signs of reversal. When the temperature was raised to 16°C in FW, weight loss (apparently osmoregulation) occurs after a lag period of 18 hr. The final weight is below the starting weight, as might be expected since the fish had not been fed for 12 days. It should be clear that this method measures total net water movement, without distinguishing between water-containing compartments. The only compartments of significant size are the plasma, the peritoneal cavity, the gut and the urinary bladder, with the first two in rapid equilibrium, and neither of the latter two being large enough to contain the total water movements observed. Active water uptake in SW is initially by drinking water into the gut, followed by active uptake from gut to plasma.

During osmoregulation, and in the quasi-steady state of linear water gain, the drinking rate must equal the absorption from the gut in the absence of significant fecal flow. The weight measurement is a measurement of both of these processes which occur in series.

When experiments such as that shown in Figure 3 were repeated, the phenomena were consistently reproducible among fish. Figure 4 shows the mean normalized weight (± SE) expressed as calculated water flux (ml·kg⁻¹). The initial sudden weight loss persists, and the subsequent water uptake can be resolved into two straight lines which have slopes significantly (P < 0.001) different from each other.

When the temperature is returned to 16°C, the fish lose weight, apparently by activating their transport mechanisms and actively extruding water, as seen in Figure 5. These fish were SW acclimated, and had not been exposed to warm FW until the temperature change shown here. Note the lag period between the elevation of temperature and the commencement of active water extrusion.

Figure 6 shows fish acclimated to FW at 16°C before the start of the experiment. When cooled to 4°C, water uptake occurs as before, but at significantly lower rates. However, when the temperature is elevated after swelling has occurred, there is an immediate loss of water with no perceptible lag period, as seen in Figure 7. This is in marked contrast to the 8–12 hr lag seen in the SW-acclimated fish (Fig. 5).

Figure 8 shows that FW-acclimated fish lose weight at 4°C in SW and recover it at 16°C.

![Graph](image-url)

Fig. 4. Summary of the swelling phases of six SW-acclimated fish exposed to cold FW at time zero. Data normalized and converted to water flux as discussed in text. The initial extrusion of water (micturation?) is still evident, but only two swelling slopes can be detected.

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As before, the loss can be resolved into two linear phases of different rates (first phase, $-4.29 \pm 0.3 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$; second phase, $-1.86 \pm 0.06 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$), and there is a short (< 6 hr) lag period before active water uptake begins. When the same experiment was conducted with SW-acclimated fish, there was no significant change in weight.

The previous experiments show the water gain or loss at 4°C. If FW-acclimated fish are transferred to SW at 16°C, we expect a transient weight loss followed by a return to steady state as the fish starts osmoregulation. Figure 9 shows that this indeed occurs, and shows that the rate of water loss, nearly $-21 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$, is much higher than at 4°C. The transition of SW-acclimated fish to FW shows a smaller rate of water gain ($8.69 \pm 2.21 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$) and a somewhat faster return to normal weight.

**DISCUSSION**

Since the fish are not fed during the weighing periods, there are some long-term weight changes apparently due to loss of solid body mass, at a rate of about $0.25 \text{ g} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$, as seen in Figure 3 and other data. The osmotically driven, reversible weight changes occur at much higher rates, and can logically be attributed to water movement in...
and out of the body. This net water movement \( (\Delta J_{\text{net}}) \) includes passive osmotic movements \( (\Delta J_{\text{Osm}}) \) and the active osmoregulatory movements \( (\Delta J_{\text{at}}) \) which oppose osmotic flows. Thus,

\[
\Delta J_{\text{net}} = \Delta J_{\text{Osm}} + \Delta J_{\text{at}}.
\]  

When the fish is in an osmoregulatory steady state, \( \Delta J_{\text{net}} = 0 \) and \( \Delta J_{\text{Osm}} = -\Delta J_{\text{at}} \). Only during transient states will we measure a finite \( \Delta J_{\text{net}} \). For instance, cooling the fish should decrease \( \Delta J_{\text{at}} \) and lead to a finite \( \Delta J_{\text{net}} \), since \( \Delta J_{\text{Osm}} \) is not a strong function of temperature (van’t Hoff, 1887). If we could reduce \( \Delta J_{\text{at}} \) to zero, then \( \Delta J_{\text{net}} \) during these transient conditions would measure \( \Delta J_{\text{Osm}} \). When \( \Delta J_{\text{at}} \) is not zero, \( \Delta J_{\text{net}} \) underestimates the value of \( \Delta J_{\text{Osm}} \), so calculations based on the assumption of no active transport in the cold lead to a minimum value for \( \Delta J_{\text{Osm}} \). Likewise, when a sudden reversal of osmotic flow is imposed, as by changing a well-acclimated fish from FW to SW, we assume that active transport in the new direction cannot be activated immediately, and therefore that the initial slope of the weight change curve represents osmotic water flux. If there is residual osmoregulatory ability during the initial few hours, this will likewise lead to an underestimate of the magnitude of the osmotic flux. All of the values reported are therefore minimum values, subject to this caveat.

Since osmosis is defined by a linear force-flow equation, we can define an effective permeability coefficient, \( P_e \), such that

\[
J_{\text{Osm}} = P_e \Delta \text{OsM} = P_e (\text{OsM}_p - \text{OsM}_w),
\]  

where OsM\(_p\) and OsM\(_w\) are the plasma and water osmolarities, respectively. Since OsM\(_w\) can be either above (SW) or below (FW) the plasma osmolarity, \( J_{\text{Osm}} \) can have either sign. This effective permeability coefficient, \( P_e \), in ml H\(_2\)O \( \cdot \) kg\(^{-1} \cdot \text{hr}^{-1} \cdot \text{OsM}^{-1} \) is a phenomenological coefficient which seems largely due to osmotic water flow through the gills, an unavoidable consequence of the high permeability of gill tissue necessary for gas exchange (e.g., Evans et al., 2005). This effective permeability of any membrane is governed not only by its intrinsic water permeability and area, but by the unstirred layers on either side (Teorell, ’36; Dainty, ’63; Dainty and House, ’66), including those due to mucus which impeded bulk water flow (Shepard, ’97) by creating an unstirred layer immediately adjacent to the membrane, lowering the transmembrane osmotic gradient (Bressler et al., ’76). Both the water flow over the gill (ventilation) and the blood flow through the gill (perfusion) will change the unstirred layers on the two sides of the gill lamellae. For a given fish, \( P_e \) is also a function of (gill) membrane area. Thus \( P_e \) is a complex coefficient, and should vary between fish species, and in the same fish under different conditions, as membrane architecture, perfusion and ventilation change. \( P_e \) might be different between the same fish in FW and SW, as the gill membranes are altered by salinity (e.g., Karnaky et al., ’76). We also expect \( P_e \) to change with gill ventilation, which must always be sufficient to supply the oxygen demand.

Table 1 shows the calculation of \( P_e \) from the initial slopes of the weight change curves, using Eq. 2. It is important to note that while the water osmolarity (OsM\(_w\)) is changed immediately, both the plasma osmolarity (OsM\(_p\)) and the membrane architecture which affects \( P_e \) initially remain those of the acclimation condition. Thus, the \( P_e \) calculated from a FW to SW transition is the steady-state value for a FW fish, and is calculated using the plasma osmolality of an FW fish. To calculate the steady-state osmotic water flux for a FW fish, we must use the FW \( P_e \) with the osmotic gradient experienced by a fish in FW. The calculated steady-state flux values are somewhat lower (6.3–11.9%) than the measured flux for this reason, but are the values which must be opposed by active osmoregulation if the fish is to remain in the steady state.
In experiments with any higher animal, there is inevitably a certain amount of "stress" imposed, which can alter the physiology of the organism. In the present experiments, changes in osmolarity of the external medium are a stress, along with the procedures necessary for weighing the fish, the cold temperatures to which they are exposed, and the general stress of handling the fish. Since cortisol seems to be involved in the response of fish to all sorts of stress, including osmotic stress (Nichols et al., '85), it could be argued that these experiments are being conducted on "unnatural" fish. These experiments were conducted with minimal stress, and since the fish survived to be released, these experiments measure fish in a viable physiological state which could be similar to that encountered in the wild.

The data presented show different instances of osmotic swelling or shrinking when $J_{at}$ has been inhibited by cold temperatures. Under these several conditions, this inhibition is followed by a change in fish weight in the direction predicted from Eq. (2) (increase in FW, decrease in SW). We observed two distinct rates of osmotic flow in each experiment (Figs. 4, 6–8). If $J_{at}$ is constant within any given run, the observation of the second lower slope must indicate that $J_{OsM}$ has decreased after the initial 24 hr. If $J_{at}$ is constant, this can only be due to changes in $P_e$ or $\Delta OsM$. Let us consider the possibility that $\Delta OsM$ has changed. The environmental osmolarity ($OsM_a$) is constant in these experiments, as confirmed by salinity measurements before and after the experiments. The smallest change in $J_{net}$ is a decrease from 4.03 to 3.16 ml·kg fish$^{-1}$·hr$^{-1}$, or to 78% of the initial value, after about 32 hr of swelling (Fig. 4). To change the osmotic driving force by this amount with a constant OsMa requires that OsM$p$ decrease by 22%. While the steady-state F. heteroclitus plasma osmolarity does decrease by 10% between sea water and FW conditions (Kidder, '98), the change is not sufficient to account for the minimum change here considered, and much less for the larger changes under other conditions. Nor can a slow change in plasma osmolarity explain the abrupt change in rate that is observed.

One should also see a decrease in $J_{net}$ if $J_{at}$ were to increase. In light of the different mechanisms required for osmoregulation in the two directions, it seems improbable that an increase in $J_{at}$ would have occurred in all fish at the same time after an osmolarity reversal. We therefore suggest, by elimination, that this rate change is probably due to a change in effective permeability, $P_e$ due to a change in ventilation, perfusion or active gill membrane area (Evans et al., 2005).

Referring to Table 1, we see that the osmotic load in F. heteroclitus acclimated to FW and transferred to SW is 4.85-fold higher (19.5/4.02) at 16°C than at 4°C. The oxygen consumption rate has been found (Kidder et al., 2005) to obey the equation $Q_{O_2} = 10^{(0.52+0.04t)}$ which predicts that $Q_{O_2} = 47.9$ ml·kg$^{-1}$·hr$^{-1}$ at 4°C and 144.5 ml·kg$^{-1}$·hr$^{-1}$ at 16°C, a 3.02-fold increase between these temperatures. Since SW at 4°C contains 7.51 ml O$_2$·Litter$^{-1}$ (Weiss, '70), the water volume needed to supply this O$_2$ is 6.38 liter·kg$^{-1}$·hr$^{-1}$, while at 16°C, the solubility is decreased to 5.75 ml·O$_2$·liter$^{-1}$, so the required ventilation flow is 25.11 liter·kg$^{-1}$·hr$^{-1}$, which is 3.94-fold higher than at 4°C. Thus the increased ventilation required by oxygen consumption at 16°C compared to 4°C may be responsible for the majority of the increase in $P_e$ between these two temperatures.

In the SW steady state, if the water lost by osmosis is replaced by drinking and subsequent transport, it appears that these fish must drink at a rate of 19.5 ml·kg$^{-1}$·hr$^{-1}$. Tsuchida and Takei ('98) report a rate around 2.1 ml·hr$^{-1}$ by direct esophageal cannulation, in eels averaging 181.5 g, which is 11.5 ml·kg$^{-1}$·hr$^{-1}$. Hutchinson and Hawkins ('90) report 12–19 ml·kg$^{-1}$·hr$^{-1}$ for 0-group flounder (0.66 g). Others report values ranging from 0.34 to 8.3 ml·kg$^{-1}$·hr$^{-1}$ for

<table>
<thead>
<tr>
<th>Condition</th>
<th>Measured flux (ml)</th>
<th>Plasma mOsm</th>
<th>Medium mOsm</th>
<th>$\Delta$Osm, trans.</th>
<th>$P_e$ (ml/Osm)</th>
<th>$\Delta$Osm, s-state</th>
<th>Calc. flux (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW to SW, 4°C</td>
<td>−4.29</td>
<td>330</td>
<td>974</td>
<td>−0.644</td>
<td>6.66 (FW)</td>
<td>−0.604</td>
<td>−4.02</td>
</tr>
<tr>
<td>FW to SW, 16°C</td>
<td>−20.8</td>
<td>330</td>
<td>974</td>
<td>−0.644</td>
<td>32.3 (FW)</td>
<td>−0.604</td>
<td>−19.5</td>
</tr>
<tr>
<td>SW to FW, 4°C</td>
<td>4.03</td>
<td>370</td>
<td>34</td>
<td>0.336</td>
<td>12.0 (SW)</td>
<td>0.296</td>
<td>3.55</td>
</tr>
<tr>
<td>SW to FW, 16°C</td>
<td>8.69</td>
<td>370</td>
<td>34</td>
<td>0.336</td>
<td>25.9 (SW)</td>
<td>0.296</td>
<td>7.66</td>
</tr>
</tbody>
</table>

The measured fluxes are the slopes of the lines in Figs. 4, 8 and 9. Plasma values are long-term acclimation data, from Kidder ('98). From these and the known water osmolarity, a $P_e$ is calculated which represents the fish in its acclimated condition; FW for an FW to SW transition, etc. Using the plasma osmolarity and $P_e$ appropriate to the steady state, a steady-state flux can be calculated.

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SW-acclimated fish by various methods (Fuentes and Eddy, '97), while Miyazaki et al. ('98) report rates as high as 28 ml·kg⁻¹·hr⁻¹ for SW-acclimated tilapia fry. Potts and Evans (67) measured drinking rates in F. heteroclitus by gut retention of ¹⁴C inulin, and report a drinking rate of 23.0 ± 2.7 ml·kg⁻¹·hr⁻¹ for SW-acclimated fish. They obtained 8.3 ± 3.9 ml·kg⁻¹·hr⁻¹ in FW fish. Malvin et al. ('80) measured the accumulation of PEG in the gut of the same species, reporting values from 4.4 to 8.8 ml·kg⁻¹·hr⁻¹ in SW-acclimated control fish, and from 1.0 to 4.3 ml·kg⁻¹·hr⁻¹ in FW. They explain the Potts and Evans values as spuriously high due to surface binding and fluorescence interference with the radioactive counting, but do not account for their FW-acclimated values being even higher than Potts and Evans'. Significantly, all of their fish had been handled and injected within 5 min of the start of the 1-hr uptake period, which might seriously alter their results. Our values from a completely different method are therefore toward the high end of a very wide range, agreeing well with the values which Potts and Evans considered to be most reliable.

One major determinant of $P_e$ (and thus of $J_{O_{2M}}$ and drinking rate) might be the ventilation rate, which is determined in large part by the oxygen demand. Mass-specific oxygen uptake ($Q_{O_2}$) depends on fish weight; in F. heteroclitus, the relationship is $\log_{10}(Q_{O_2}) = 2.43 - 0.443\log(g)$ (Kidder et al., 2005). The ~5 g fish used in the present experiments had a $Q_{O_2}$ around 140 ml·kg⁻¹·hr⁻¹ at 16°C. A 180 g fish would have a $Q_{O_2}$ of 27 ml·kg⁻¹·hr⁻¹ if this relationship holds, which would require only 19% of the ventilation of a 5 g fish, per unit fish mass. The mass-specific drinking rate of the large eels might therefore be equivalent to 59 ml·kg⁻¹·hr⁻¹ for this reason alone. Our calculated drinking rates are thus within the range to be expected for SW-acclimated fish of this size.

The importance of osmoregulatory work in the energy budget depends on the fraction of available energy it requires. Potts ('54) calculated on thermodynamic grounds that between 0.5% and 1.2% of metabolic energy was used for osmoregulation in various species, assuming theoretical 100% efficiency of the various processes involved. Kirschner ('93, '95) made the corresponding calculations based on reasonable assumptions about the pathways for generation and utilization of ATP, but without solid data on osmotic fluxes and metabolic rates, and concluded that between 10% and 15% of metabolic energy is used for osmoregulation in flounder and sharks.

Table 2 shows these calculations for F. heteroclitus using the present data. The steady-state water flux from F. heteroclitus into SW at 16°C is 19.5 ml·kg⁻¹·hr⁻¹ (Table 1), and is normally compensated by drinking SW at this rate and excreting the salt. Sea water contains 440 mM Na, so the fish must excrete NaCl through the gills at $19.5 \times 0.44$ mmoles Na ml⁻¹ = 8.58 m-moles Na·kg⁻¹·hr⁻¹. If the gills use a 3 ATP transport system (Na/K ATPase), as seems to be the case for Na efflux (Kirschner, '93), this requires 2.86 mmoles ATP·kg⁻¹·hr⁻¹. Since a maximum of three phosphorylations of ADP to ATP occur per oxygen atom (P/O = 3), generating

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**TABLE 2. Na transport required to maintain steady state, and its energy cost (per kg·hr)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Calculated flux (ml)</th>
<th>Tissue</th>
<th>Na (mmoles)</th>
<th>$O_2$ calc. (ml)</th>
<th>$O_2$ meas. (ml)</th>
<th>Osmoregulatory load (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW, 4°C</td>
<td>4.02</td>
<td>Gill</td>
<td>1.77</td>
<td>2.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intestine</td>
<td>0.60</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>2.95</td>
<td>47.8</td>
<td></td>
<td>6.17</td>
</tr>
<tr>
<td>SW, 16°C</td>
<td>19.5</td>
<td>Gill</td>
<td>8.58</td>
<td>10.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intestine</td>
<td>2.92</td>
<td>3.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>14.32</td>
<td>145</td>
<td></td>
<td>9.79</td>
</tr>
<tr>
<td>FW, 4°C</td>
<td>3.55</td>
<td>Gill</td>
<td>0.036</td>
<td>0.088</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.50</td>
<td>0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>0.61</td>
<td>47.8</td>
<td></td>
<td>1.28</td>
</tr>
<tr>
<td>FW, 16°C</td>
<td>7.66</td>
<td>Gill</td>
<td>0.077</td>
<td>0.191</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>1.07</td>
<td>1.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1.33</td>
<td>145</td>
<td></td>
<td>0.92</td>
</tr>
</tbody>
</table>

Using the water influx values from Table 1, Na active transport in the relevant tissues and its energetic (oxygen) requirement is calculated. For the assumptions used, see text.
this ATP requires, at a minimum, 0.48 mmoles O$_2$.kg$^{-1}$.hr$^{-1}$, equivalent to 10.7 ml.O$_2$.kg$^{-1}$.hr$^{-1}$. In addition, since the ingested water is iso-osmotically absorbed across the intestinal wall by a Na/K-ATPase-driven system (e.g., Rankin et al., '83), 19.5 ml.kg$^{-1}$.hr$^{-1}$ of isotonic (150 mM) NaCl requires an additional 3.64 ml.kg$^{-1}$.hr$^{-1}$ of oxygen consumption, by a similar calculation. The kidneys are of little consequence in this energy calculation, since GFR is reduced in sea water fish to perhaps 0.5 ml.kg$^{-1}$.hr$^{-1}$ (Evans, '93). The sum of these values, 14.32 ml.kg$^{-1}$.hr$^{-1}$, is the oxygen cost of osmotic work under these conditions. Since the Q$_{O_2}$ of Fundulus at 16°C is 145 ml.O$_2$.kg$^{-1}$.hr$^{-1}$ (see accompanying paper, Kidder et al., '05), osmotic work represents 9.79% of the available energy. Table 2 lists the results of this calculation and the similar calculations at 4°C which result in a similar cost of osmotic work, 6.17%. These estimates of the cost of osmoregulation are similar to those calculated by Kirschner ('95).

When a SW fish enters FW at 16°C, water enters the fish by osmosis at 7.66 ml.kg$^{-1}$.hr$^{-1}$, which is the rate at which it must be excreted into the urine to achieve a steady state. Thus the kidney filters plasma (~150 mM NaCl) and reabsorbs all but ~10 mM NaCl, thus pumping 0.14 x 7.66 = 1.07 mmoles Na.·kg$^{-1}$.hr$^{-1}$. The overall Na/ATP ratio in fish kidney seems to be between 3.5 and 4.3 (Kirschner, '95); we will take the lower value to maximize the calculated energy cost. We therefore calculate a requirement for 1.14 ml.O$_2$.kg$^{-1}$.hr$^{-1}$. The sodium lost in the urine must be reaccumulated by gill transport, for which Na influx is 0.01 x 7.66 = 0.077 mmoles Na.·kg$^{-1}$.hr$^{-1}$. This consumes 2 ATP per 3 Na transported (Kirschner, '95), adding 0.051 mmoles ATP.·kg$^{-1}$.hr$^{-1}$, or 0.19 ml.O$_2$.kg$^{-1}$.hr$^{-1}$ for a total of 1.33 ml.O$_2$.kg$^{-1}$.hr$^{-1}$, which is only 0.92% of the measured oxygen uptake at this temperature. As in SW, similar results are obtained at 4°C. Note that these calculations are for osmotically linked Na transport only. Additional Na presumably leaks across the gills and must be removed, but this is not a cost of osmoregulation per se, and we have no data on the magnitude of these leaks. If the P/O ratio were less than 3, as has sometimes been reported (e.g., Brand et al., '93), the fraction of resting metabolism used for osmotic work would be correspondingly higher.

Since both the osmotic gradient and the effective permeability are lower in FW than in SW, and since oxygen uptake is not a function of salinity, FW should be less osmotically stressful than SW, while brackish water would be the least stressful. Whether these percentages of total available energy represent an evolutionary-significant selective force leading to isotonic-seeking behavior remains arguable, given the differences of opinion as to the “biological significance” of any selective pressure. We believe that a work load approaching 10% of available energy should be a powerful pressure for the evolution of “behavioral osmoregulation” in estuarine species, as has been predicted (Fritz and Garside, '74; Kidder, '97). Osmoregulation is clearly not the only reason for habitat choice, and other ecological advantages such as decreased predation, increased food availability or increased reproductive success may be more important in particular circumstances.

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LITERATURE CITED


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