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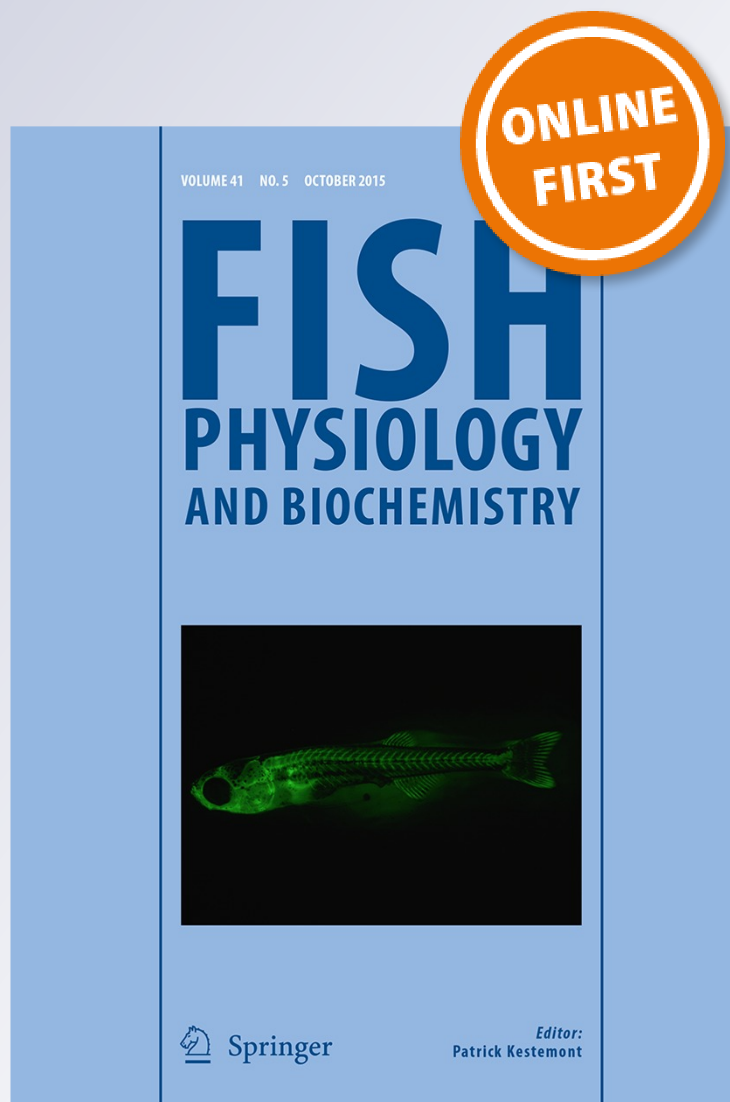

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Abstract Abiotic factors like salinity are relevant to survival of pelagic fishes of the San Francisco Bay Estuary. We tested the effects of 4 parts per thousand (ppt) salinity increases on Delta Smelt (DS) in a laboratory experiment simulating salinity increases that might occur around the low-salinity zone (LSZ) (<6 ppt). Adult DS, fed 2 % body mass per day, starting at 0.5 ppt [freshwater (FW)], were exposed to weekly step-increases of 4 ppt to a maximum of 10 ppt saltwater (SW) over 19 days, and compared to FW controls. DS ($n = 12$/treatment per sampling) were sampled at 24, 72, and 96 h (1, 3, and 4 days) post-salinity increase for analyses of hematocrit, plasma osmolality, muscle water content, gill chloride cell (CC) Na^+/K^+-ATPase (NKA) and apoptosis after being weighed and measured ($n = 3$ tanks per treatment). No apparent increase in length or weight occurred nor did a difference in survival. Following step-increases in SW, hematocrit increased over time. Other fish responses generally showed a pattern; specifically plasma osmolality became elevated at 1 day and diminished over 4 days in SW. Percent muscle water content (%) did not show significant changes. CCs showed increased NKA, cell size and apoptosis over time in SW, indicating that CCs turnover in DS. The cell renewal process takes days, at least over 19 days. In summary, DS are affected by salinities of the LSZ and ≤10 ppt, though they employ physiological strategies to acclimate.

Keywords Delta Smelt · Fluorescent confocal microscopy · Salinity · Na^+/K^+-ATPase · Apoptosis · Chloride cells · Physiology · Low-salinity zone

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Introduction

Delta Smelt (*Hypomesus transpacificus*) (DS), a small endangered pelagic fish endemic to the San Francisco Bay Estuary (SFE), has sharply declined over the past few decades (Sommer et al. 2007). Several factors played a role in the decline of DS, including entrainment in water export pumps (Grimaldo et al. 2009; Kimmerer 2011), loss of habitat from water diversion (Moyle 2002; Moyle et al. 2010), lack of food availability (Moyle et al. 1992; Kimmerer 2002) and changes in physical and chemical factors such as

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salinity, temperature or turbidity, each of which is a key habitat feature of DS (Jassby et al. 1995; Bennett 2005; Sommer et al. 2007; Feyrer et al. 2007; Cloern and Jassby 2012). Over recent decades, the decline in suitable habitat, particularly of the low-salinity zone, has been substantial, highlighting the importance of understanding why and which factors are essential for this fish (Feyrer et al. 2007, 2011). The focus of this work is in regards to salinity, a habitat factor affected both by Delta pumping and export (Kimmerer and Nobriga 2008; Kimmerer 2011) and climate change (Feyrer et al. 2011).

DS are exposed to a range of salinities during their annual life cycle. Larval fish are thought to migrate from FW ($\sim$0.5 parts per thousand (ppt)) into the low-salinity zone (LSZ) (2–6 ppt) in the spring (Bennett 2005; Meng and Matern 2001). Juvenile to sub-adult DS spend the summer and fall in the mixing zone of the estuary, defined as the LSZ (Dege and Brown 2004). Characteristically, this low-salinity habitat has been defined as $\leq$6 but $\geq$2 ppt or X2 (Feyrer et al. 2007; Kimmerer et al. 2013), the 2 % near-bottom isohaline's distance up the SFE from the mouth of the San Francisco Bay at the Golden Gate Bridge (Jassby et al. 1995). Habitat volume of the LSZ increases with spring freshwater run-off, moving X2 toward to the ocean (Kimmerer et al. 2009; Feyrer et al. 2011). Spring and summer snowmelt are projected to decrease with climate change (Knowles and Cayan 2002, 2004), potentially increasing summer salinities in the estuary (Brown et al. 2013) and decreasing habitat volume of the LSZ (Feyrer et al. 2011). DS are mostly found in the lower range of the LSZ. Adult fish have, on rare occasion, been found up to $\sim$18 ppt (Bennett et al. 2002; Bennett 2005). It is thought that their annual migration occurs when adult and/or sub-adult pre-spawning DS, in winter and early spring, move from the lower brackish estuary to the upper mostly FW one, to spawn (Sommer et al. 2011). However, they also move, regularly around the mixing zone of the estuary from FW up to 6 ppt, where they are predominately found $<$6 ppt (Bennett 2005). Reasons for their movements and habitat preference below 6 ppt are unclear. Recent studies have also shown that a primarily FW spawning migration may not be the case and they also spawn in brackish water (Hobbs et al. 2007; Sommer et al. 2011).

Saltwater mixes gradually with the influx of freshwater that in some places forms rather dramatic haloclines (Lougee et al. 2002). Gradients in ever-decreasing low-salinity habitat are also projected to increase by 2–4 ppt with climate change projections as freshwater influx decreases (Knowles and Cayan 2002, 2004). However, steep gradients at these levels also form during the annual wet season in the estuary (Cloern and Nichols 1985). How DS adapt during relocation from 0.5 ppt to salinities above that, over steep gradients resulting from mixing during flow events, is poorly understood.

Here, we examined the influence of changing salinity step-increases within 0.5–10 ppt on adult DS to understand the effects. We hypothesized that the physiology of DS is negatively affected by salinities above 6 ppt, explaining their apparent habitat preference for the LSZ. If this is not the case, and they do not have a physiological limitation to salinities $>$6 ppt, then other factors such as the abundance and availability of their prey items or other cues in salinity zones of the estuary (Kimmerer 2002; Kimmerer et al. 2013) may play a larger role.

In general, euryhaline teleosts osmoregulate to maintain homeostasis in the aquatic environment at $\sim$one-third that of SW. Marine teleosts drink water and lose ions via highly ionic urine, whereas freshwater fish excrete dilute urine to maintain internal osmotic balance (Moyle and Cech 2004; Evans and Claiborne 2006). While the kidney and other mechanisms (Pearson and Stevens 1991; Allen et al. 2009) play a role in maintaining osmoregulatory balance, the gill is the primary organ responsible for ion exchange (Moyle and Cech 2004). Chloride cells (CCs) in the gill exchange Na^{+} and K^{+} primarily via Na^{+}–K^{+} pumps that drive the cell's potential to extrude Cl^{-} (Marshall 2002) through electrochemical conductance (Daborn et al. 2001). Brain and liver also help regulate stress and other protein changes mainly via hormonal control as a result of changes in internal osmolarity (McCormick 1995; Yang et al. 2009), often measured by plasma osmolality and ions (Hwang et al. 1989; Sardella et al. 2008b; Allen et al. 2009; Kammerer et al. 2010). Muscle water content also has been shown to play a role in regulating hyperosmotic stress in Bay-Delta fish (Sardella et al. 2008b), shown to change by resulting in muscle water loss, if an estuarine fish is not near salinities it could tolerate in its natural environment (Kang et al. 2008). Increased hematocrit may be signaled by changes in plasma volume (Pearson and Stevens 1991). We use the measurements of body size,

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plasma osmolality, hematocrit and percent muscle water, shown to be effective measurements of osmoregulatory status in estuarine fish (Sardella et al. 2008b; DiMaggio et al. 2010) to illuminate acclimatory mechanisms DS may employ. Chloride cells have different isoforms in freshwater and in seawater (Moyle and Cech 2004; McCormick et al. 2009), sometimes described as alpha and beta CCs (Marshall 2002). When a teleost fish moves to higher salinity, CCs have been shown to die and regenerate into a new population of chloride cells with more Na^{+}–K^{+}-ATPase pumps (NKA) (Wendelaar Bonga and Vander Meij 1989; Hiroi et al. 2005) and other ion channels (Marshall et al. 1999), the latter which may be determined by how close to the salinity of a fish's natural environment it is (Kang et al. 2008). Our study seeks to identify apoptosis as an adaptative response DS use as a result of salinity exposure. Apoptosis is indicative of cell turnover and death, where caspase 3/7 is a pro-apoptotic protein that activates DNAases responsible for nuclear DNA fragmentation during this process (Kültz 2005; Lüthi and Martin 2007; Paulsen et al. 2008). In SW, a twofold–threefold increase in apoptotic caspase 3/7 or DNA fragmentation relative to FW can be representative of gill cell turnover (Kammerer and Kültz 2009). Likewise, concentration of NKA per CC or size of CCs increases with salinity in teleosts (McCormick 1995), something NKA staining reveals (Sardella et al. 2008a; Allen et al. 2009; Kammerer et al. 2009; Kammerer and Kültz 2009). While these results have been shown in killifish (Lima and Kültz 2004) and also other SF estuary species (Allen et al. 2009), little evidence has been shown of such effects in DS. Thus, changes in apoptotic caspase 3/7, DNA fragmentation, or increased NKA or CC size relative to FW may indicate salinity stress and help describe physiological changes in DS during habitat migration. In the current study, we use measures of apoptotic caspase 3/7 and CC fluorescence to see if they can be used to track the level of salinity increase.

By exposing fish to increasing levels of salinity, this study aims to define responses of DS to longer-term exposures to salinity, for which very little information is available as most work has examined acute tolerances up to high salinities nearer SW, ~18 ppt (Swanson et al. 2000; Hasenbein et al. 2013) after which mortality increases (Komoroske et al. 2014), and where they are rarely found (Bennett 2005). The responses to longer-term salinity exposure could provide baseline information for monitoring how DS respond to increasing salinities that they may inhabit in the wild (Brown et al. 2013). We tested the hypotheses that physiological effects such as changes in body size, hematocrit, plasma osmolarity, muscle water content, gill CC NKA and apoptosis, and gill caspase 3/7 occur when DS are exposed to salinities in particular above 6 ppt relative to those below 6 ppt, when increased at a level of 4 ppt each week.

Methods

Fish handling

Adult DS (3.16 ± 0.36 g, 78.42 ± 2.74 mm) were obtained from the Fish Conservation and Culture Laboratory (FCCL), in Byron, CA, affiliated with the University of California, Davis. Fish were randomly allocated to six carboys and transported to the Center for Aquatic Biology and Aquaculture (CABA) at UC Davis following methods developed by Swanson et al. (1996). They were then acclimated to covered 250-L black polyethylene tanks (210 ± 20 L working volume per tank) in two recirculation systems with a temperature range of 12 ± 1 °C, a density range of 50 ± 2 fish/tank and natural light (≤1–3 lux below tank lids) (Lindberg et al. 2013). Each system was constructed with three fish-holding tanks (250 L), a settling tank (378 L) and a sump tank (≥390 L) with filter. The sump tank was fitted with an internal biofilter consisting of aerated, 1″ bioballs (#CBB1-S & CBB1-F, Pentair Aquatic Ecosystems, Apopka, FL). An upright filter with 20 micrometer (μm) particle size filtration capability (M-series module filter Lifeguard Aquatics, Cerritos, CA), a UV sterilization unit (model QL-40, Lifeguard Aquatics, Cerritos, CA), a 39′ Iwaki magnetic pump (model #MD100RLT, Pentair Aquatic Ecosystems, Apopka, FL), and a 1.5-hp chiller (Premium Line AC/HP Split System Condensers fit with Aqualogic Digital Temperature Controllers) were also part of the systems' construction. The water quality was maintained at >95 % dissolved oxygen, pH 8.19 ± 0.032, 0 ppm total ammonia nitrogen, <0.8 ppm nitrite and <8 ppm nitrate, and fed 2 % biomass per day distributed into 15 feedings per day by automatic feeder (Baskerville-Bridges et al. 2005; Lindberg et al. 2013). Feed was

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withheld for 24 h prior to transport and salinity increases. Feeds contained very low to normal sodium levels (≤1 %) (Salman and Eddy 1988, 1990).

Salinity exposures

After fish were acclimated to the tanks, flow velocities were standardized to ≤10 cm/s so as to not exceed DS gait transition and exhaustion velocities (Swanson et al. 1998), and salinity treatments commenced. Treatments were saltwater exposure, and FW served as a control in separate recirculating systems with three tanks (replicates) per treatment. Beginning with well water (≤0.5 ± 0.1 ppt), salinities were raised by step-increases of 4 ppt in <2 h, using Instant Ocean salt (Instant Ocean, Blacksburg, PA) at the beginning of the week. The experiment lasted for 19 days. Freshwater controls remained at ≤0.5 ± 0.1 ppt (Table 1). Salinity was measured during salinity increases and monitored daily using YSI Pro30 (YSI, Inc., Yellow Springs, OH). Water quality (% dissolved oxygen, pH, total ammonia nitrogen, nitrite, nitrate) was tested three times/week. Approximately 5–20 % water exchanges were performed for both recirculating system during daily maintenance by siphoning 3 times a week and flushing out the excess food and feces twice a week. Temperature was monitored using Traceable Fisher temperature probes (#06-664-23, Fisher Scientific, Inc., Waltham, MA), daily, as was fish mortality.

Fish sampling for physiological and hematological analysis

Fish (n = 12/treatment per sampling, n = 4 per tank) were euthanized by overdose with MS-222, weighed (±0.1 g) and measured (mm) at 24, 72, and 96 h (1, 3, and 4 days hereafter) post-salinity increase in FW and SW treatments according to UC Davis Institutional Animal Care and Use Committee Protocol #17332. Quickly following the anesthesia, blood was extracted using heparinized microhematocrit capillary tubes (Fisherbrand #22-362-566, Fisher Scientific, Inc., Waltham, MA) via the caudal vein. Blood samples were then spun in a microhematocrit centrifuge (Clay Adams MHCT II, Becton–Dickinson, Franklin Lakes, NJ) at 11,700×g for 3 min to remove plasma and separate hematocrit. Hematocrit (%) was measured in duplicate according to Kammerer et al. (2010) after being removed from capillary tubes and pooled by tank and treatment (n = 3/treatment). Plasma osmolality was measured (Kammerer et al. 2010; Whitehead et al. 2012) in <2 months of sampling (Seifarth et al. 2004) after the first thaw (Paltiel et al. 2008).

To prepare samples for histological and protein analysis, gills were perfused to clear gills of red blood cells (a source of possible Na^+/K^+-ATPase activity or background staining), according to Kammerer and Kültz (2009) and Kammerer et al. 2009 and the right 4 gill arches preserved in 10 % buffered formalin, according to Allen et al. (2009). The left 4 gill arches were snap-frozen in liquid N2 for protein analysis. Approximately 75 mg of right dorsal muscle tissue of the caudal peduncle was removed and snap-frozen in liquid N_2 (Sardella et al. 2008b) to determine percent water content. Muscle tissues were weighed, in pre-weighed aluminum weigh pans (#08-732-101 Fisher Scientific, Inc., Waltham, MA), and then again after drying for 24–48 h in a 65 °C oven (Kang et al. 2008; Sardella et al. 2008b; Kammerer and Heppell 2012).

Fluorescent gill immunohistochemistry and confocal analysis

To measure apoptosis, a triDeoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit (APO-BRDU-IHC Phoenix Flow Systems #AH1001, San Diego, CA) was optimized for triple staining of gill chloride cells using immunohistochemistry (n = 8 gill arch/treatment) similar to

Table 1 Average salinities (in parts per thousand (ppt) per treatment type: freshwater or saltwater (≤10 ppt), over weekly salinity adjustment intervals

Treatment	Day 0–4	Day 4–11	Day 11–18	Day 18–19
Freshwater	0.59 ± 0.04	0.55 ± 0.0	0.50 ± 0.00	0.50 ± 0.00
Saltwater	0.65 ± 0.06	3.92 ± 0.10	7.91 ± 0.04	9.78 ± 0.03

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Kammerer and Kültz (2009). To deparaffinize tissue, slides were soaked in Histo-Clear (xylene alternative, cat. #HS-200 National Diagnostics, Atlanta, GA) for 5 min (2×) and rehydrated as described (Allen et al. 2009). Slides were then dipped in 1× PBS before incubation with Proteinase-K (1:100 v/v) (#AHRXB14, Phoenix Flow Systems, San Diego, CA). A positive control for apoptosis was generated by incubating a FW DS gill section of the same fish in 100 µL DNAase solution (5 % Sigma R6273, 10 % 10× reaction buffer, 85 % 1× PBS) for 20 min at 25 °C prior to deparaffinization. A slide from the same FW fish was used as a control for apoptotic staining, serving also as a standard for NKA staining. Nick-end labeling was accomplished with DNA labeling solution as described (#AHRXB14, Phoenix Flow Systems, San Diego, CA) (Ott et al. 2007) for 30 min. Blocking to decrease background staining was accomplished as described in Allen et al. (2009) though with blocking buffer (#AHBB23, Phoenix Flow Systems, San Diego, CA). Slides were then incubated with primary Na^+/K^+ ATPase (NKA) antibody as described by Allen et al. (2009) though dilution was 50-fold. The NKA antibody, developed by Douglas M. Fambrough, was obtained from the Developmental Studies Hybridoma Bank instituted under the auspices of the National Institute for Child Health and Human Development (NICHD) and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. Following primary NKA antibody incubation, staining with Pacific Blue-labeled secondary-NKA antibody was accomplished as described in Kammerer and Kültz (2009), though for 30 min diluted 1:50 on slides. Secondary Anti-BrdU antibody and propidium iodide incubation was also performed similar to Kammerer and Kültz (2009) using Alexa488 (#AX488, Phoenix Flow Systems, San Diego, CA), though also for 30 min and dilution 1:100 in blocking buffer. Slides were rinsed with PBS three times for 5 min after each fluorescent staining step and mounted with coverslips using ProLong Gold Anti-Fade mountant (#36930 Life Technologies, Grand Island, NY).

To analyze triple-stained slides, immunostained gills were viewed using Metamorph for Advanced software (v 7.8.1.0) and overlaid into a compiled image (Pasupuleti et al. 2013). Color images were captured using FITC (green), DAPI (blue) and Texas Red (red) filters of an Olympus inverted metered research scope (1 × 81) at 30, 10 and 10 mHz at a speed of 1000, 569.19 and 196.23 ms, respectively. Gill images [using 16 largest cells (Lima and Kültz 2004; Karnaky et al. 1976)/image] were analyzed with Fiji (ImageJ) for green and blue fluorescent intensity (RFU/chloride cell) and cell size (pxls2/chloride cell), while red staining was used to identify individual cell nuclei. FW controls remained consistent and served as a standard for TUNEL and NKA staining efficacy. For representation in the journal, color images (.tif) were sharpened and adjusted to 300 dpi using Photoshop CS2.

Microplate-based caspase 3/7 activity apoptosis assay

To analyze gills for caspase 3/7 apoptotic analyses, gills were homogenized in 0.15 ml buffer pH 7.5 according to Kammerer and Kültz (2009) with a few exceptions. Briefly, gill tissue (23.7 ± 0.002 g) was homogenized using a tissue tearor (Biospec Products, Inc., Bartlesville, OK). Supernatants removed after centrifugation were diluted fivefold in homogenization buffer, and caspase 3/7 activity was determined using the caspase-glo 3/7 Assay (#G8090, Promega, Inc. Madison, WI). Plates were read for luminescence after 30 min incubation at 25 °C, using a Tecan Infinite M200. To standardize between plates, a recombinant, Human caspase 3 *E. coli* sample (#235417-5000U Upstate/Millipore, Billerica, MA), diluted 1:20 in buffer, was run with each plate to determine relative luminescent units (RLU) ratio (sample:standard).

Statistics

The effects of salinity treatment and time on lengths, weights, plasma osmolality, blood hematocrit (%), muscle water content (%), Na^+/K^+-ATPase fluorescent intensity, chloride cell (CC) size and two measures of apoptosis: (TUNEL-labeled CCs and gill caspase 3/7 luminescence) were assessed by two-way ANOVA by tank ($n = 3$). Because the experimental unit was each tank to which salinity treatments were applied, a repeated measures model was used for ANOVA after the assumptions for normality (Shapiro–Wilks) and equality of variance were tested. Differences in average mortality between FW controls and the SW treatment were assessed using one-way

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ANOVA. Post hoc Tukey multiple comparison tests were used when means were significantly different. All statistical tests were performed in Sigmaplot (v12.0, Systat Software, San Jose, CA) with significance set at $p < 0.05$. Graphical representations of data and tables were created using Microsoft Excel 2007. Data are presented as the mean $\pm$ standard error of the mean (mean $\pm$ SEM), unless otherwise defined. Statistical significance between the interaction of salinity and time are depicted by an asterisk (*) in results graphics.

Results

Increased salinity did not affect growth or survival of DS. No significant increase in length ($p = 0.377$) or weight ($p = 0.310$) occurred during the experiment. Fish mortality was not different between treatments through time ($F = 0.0357$, $d.f. = 1$, $p = 0.859$): an average of 2.3 fish died in FW, while an average of 2.0 fish died in the SW treatment over the course of the experiment. Most mortality occurred at the start of the experiment, just after the acclimation period and transfer.

Salinities were maintained near target levels of 0, 4, 8 and 10 ppt. In FW and pre-treatment controls for day 0 up to day 4 in the SW treatment, salinity levels ranged from 0.0 to 0.6 ppt, averaging 0.59 ± 0.04 ppt (mean $\pm$ SD). After each of the three salinity increases, treatment salinity averaged 3.92 ± 0.10 ppt from days 4 to 11, 7.91 ± 0.04 ppt from days 11 to 18 and 9.80 ± 0.03 ppt from days 18 through 19 (Table 1).

Hematological parameters significantly changed with salinity. Blood hematocrit changed significantly with salinity, increasing over time relative to FW (Fig. 1a). Values were 15–25% higher in the SW treatment relative to FW controls. The interaction of salinity and time was nearly significant ($p = 0.1$) (Fig. 1a). Plasma osmolality also significantly increased with increases in salinity from FW levels 1–3 days post-salinity increase at days 4, 6, 11, and 13, and 19 ($p < 0.05$) (Fig. 1b). Plasma osmolality in SW returned to FW control levels 4 days post-salinity increase ($p > 0.05$) at days 7 and 14 though FW levels also increased at those times (days 7 and 14). Increases in the SW treatment were 9.5 % greater at 4 and 8 ppt relative to FW controls, and as much as 7.5 % greater at 10 ppt. The second and third significant increases did not achieve as high of levels of plasma osmolality as salinities of 8 and 10 ppt relative to the initial increase from FW to 4 ppt ($p < 0.005$) (Fig. 1b). Percent muscle water content did not change significantly though the pattern suggests potential muscle water loss at the first 4 ppt step-increase (Fig. 2). Increases in gill chloride cell fluorescence and size were consistent with changes observed in gill apoptosis, in response to increases in salinity. Chloride cell

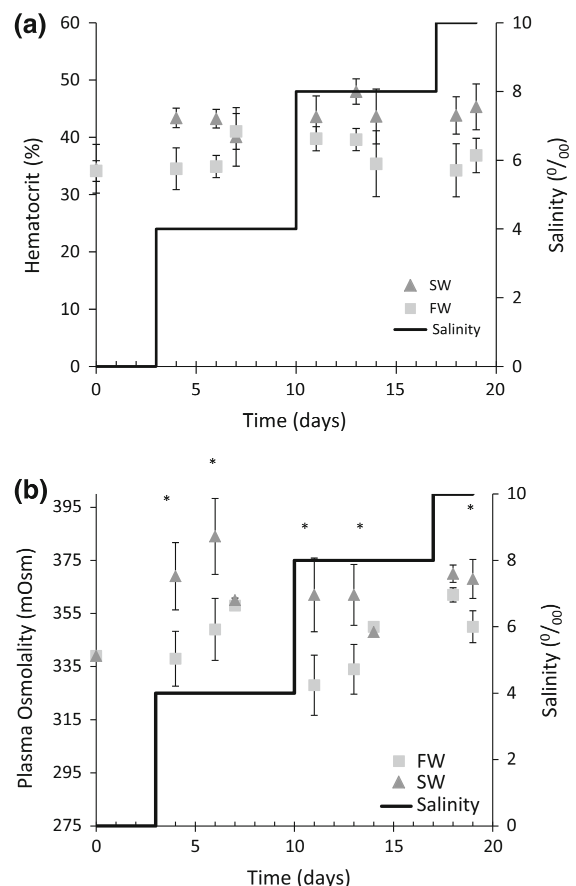

Fig. 1 Hematological responses of Delta Smelt: **a** hematocrit (%) and **b** plasma osmolality in response to salinity, up to 10 ppt, over 19 days. Plasma osmolality and hematocrit are represented by mean values ($n = 3$ tanks, 4 fish per tank). Hematocrit was significantly different between salinity treatments $F = 37.058$, $d.f. = 1$, $p = 0.004$) and over time ($F = 2.434$, $d.f. = 8$, $p = 0.036$), with interactive differences being suggestive ($F = 1.875$, $d.f. = 8$, $p = 0.1$). Plasma osmolarity was nearly significantly different between treatments ($F = 13.724$, $d.f. = 1$, $p = 0.067$), significantly different over time ($F = 52.23$, $d.f. = 8$, $p < 0.001$) and with the interaction of salinity treatment and time ($F = 13.31$, $d.f. = 8$, $p < 0.001$). An *asterisk* significant differences ($p < 0.05$) in pair-wise comparisons using Tukey's multiple comparisons test

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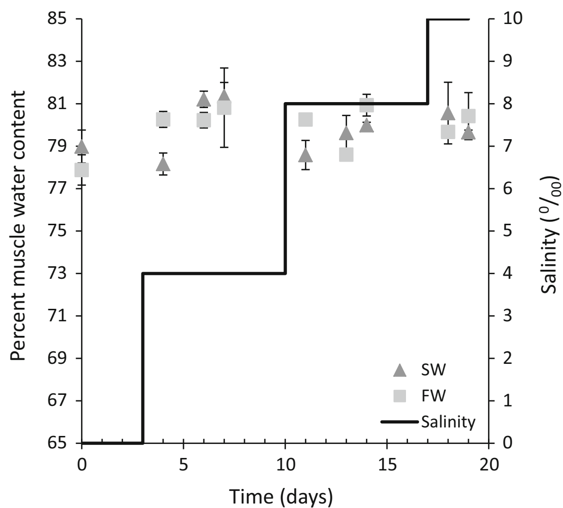

Fig. 2 Delta Smelt percent muscle water content in response to saltwater (SW) (≤10 ppt) and freshwater (FW) (≤0.5 ppt). Percent muscle water content is represented by the difference in tissue weight (75 mg) before and after drying at 65 °C. No significant differences were apparent between treatments ($F = 0.006$, $d.f. = 1$, $p = 0.981$), over time ($F = 2.054$, $d.f. = 8$, $p = 0.071$) or with the interaction of salinity and time ($F = 1.462$, $d.f. = 8$, $p = 0.21$)

(CC) Na^{+}/K^{+}-ATPase (NKA) increased in the SW treatment by day 6 with the 4 ppt increase and remained high in comparison with the FW treatment; it was different between FW and SW over time (Fig. 3). CCs from SW were larger than those from FW ($p < 0.003$) (Fig. 3d). CC apoptosis, as measured by TUNEL assay (Fig. 3a, b), also increased: at days 6, 13 and 19 in the SW treatment up to 3 days post-salinity increase ($p < 0.05$), though was not different by days 7 and 14 (Fig. 4a). Differences were significant with the interaction of salinity and time. Gill apoptosis, measured by caspase 3/7 protein assay, showed a significantly increased RLU ratio by 1 day post-salinity increase at days 4, 11, and 18, with the interaction of both salinity and time ($p < 0.05$). Differences were no longer significant when tested with post hoc Tukey multiple comparison tests by days 6, 13 and 19 ($p > 0.70$) (Fig. 4b).

Discussion

We examined the effects of salinity, increasing from 0.5 to 10 ppt, on adult DS over 19 days. Our study aimed to explain DS's osmoregulatory responses in water with salinities increasing from FW to those of the LSZ and beyond it. Increased salinity did not harm fish in terms of their survival, indicating they were able to acclimate as little mortality occurred over the course of 19 days. Additionally, fish did not show differences in growth between treatments. However, the time course was short compared to similar experiments measuring growth (Kiiskinen et al. 2002), though it was consistent with those performed with similar estuarine fish (Frenkel and Goren 2000). Conditions of current study are also consistent with work with similar species (Ogoshi et al. 2012). To examine growth effects, future experiments with longer time courses may be warranted (e.g., Frenkel and Goren 2000; Kammerer and Heppell 2013). Field abundance distributions for DS are usually centered around the LSZ and they are seldom found above 14 ppt (Moyle et al. 1992; Bennett 2005), suggesting that there are physiological salinity limits for DS above a certain threshold. Nevertheless, the low mortality we observed indicates that the salinities used in this experiment were well within the tolerance range for adult DS. Other work with DS salinity preference has contributed to understanding that DS may be able to acclimate very quickly to increased salinities (Komoroske et al. 2014) and is consistent with similar estuarine species (Kang et al. 2008; Ogoshi et al. 2012). Further attempts to quantify energetic cost may be useful via respirometry (Morgan and Iwama 1991; Morgan et al. 1997; Kammerer et al. 2010).

Marine teleosts maintain their internal milieu at 1/3 that of seawater, or ~280–340 milliOsmoles (mOsm) (Thrall et al. 2012), where seawater is 35 ppt (or 1000 mOsm) (Morgan et al. 1997; Kammerer et al. 2010). More notably, elevated hematocrit also occurred in the salinity treatment over time (Fig. 1a). However, the pattern observed followed that exhibited for plasma osmolality (Fig. 1b), indicative of acclimation to the salinity increase. Hematocrit often increases as a result of decreased plasma volume (Pearson and Stevens 1991). Our observations are consistent with previous work with killifish that showed increases in hematocrit with hyperosmotic seawater transfer (Marshall et al. 2005). Seminole killifish showed little difference in hematocrit with gradual acclimation (Dimaggio et al. 2010), however.

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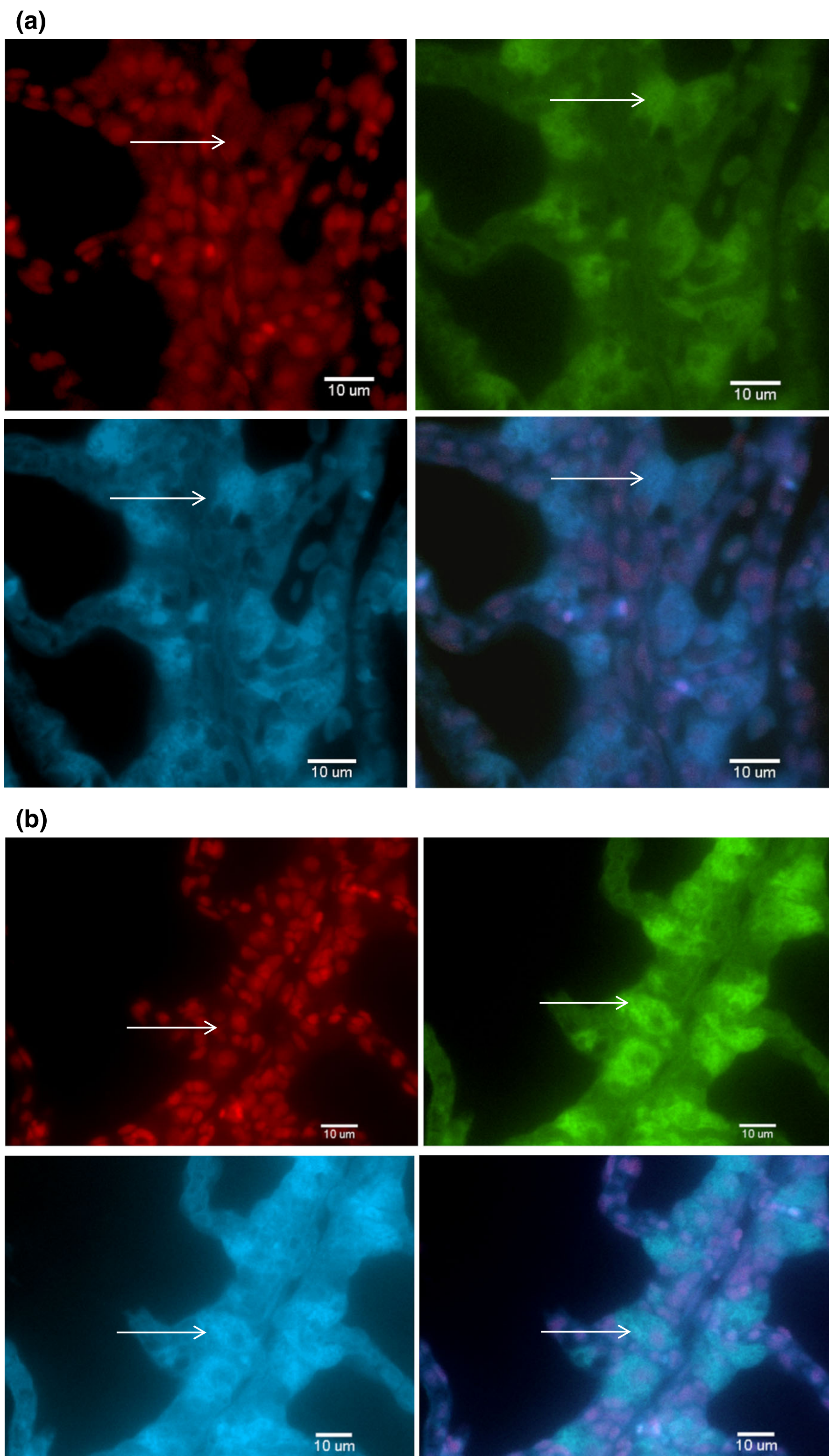

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◀**Fig. 3** Delta Smelt gill chloride cells (CCs) of at the base of gill lamellae have less green and blue fluorescence intensity in **a** freshwater (FW) versus **b** saltwater (SW) at 3 days post-4 ppt step-increase. *Arrows* point to representative CCs. Scale bars represent 10 µn. Graphs depict CC, **c** Na^+/K^+-ATPase (NKA) fluorescent intensity and **d** cell sizes with salinity (≤10 ppt) relative to FW. Images were captured via Metamorph software and confocal microscopy (×40), while differences were quantified with ImageJ analyses measured in $n = 3$ tanks (16 cells per gill arch ($n = 3$–4) in 4 fish per tank per timepoint). CC NKA increased in SW ($F = 54.396$, *d.f.* = 1, $p = 0.002$), and over time ($F = 5.153$, *d.f.* = 8, $p = 0.001$) though not with salinity × time interaction ($F = 1.265$, *d.f.* = 8, $p = 0.296$). CC size was also significantly different in SW relative to FW ($F = 44.036$, *d.f.* = 1, $p = 0.003$), though not over time ($F = 1.77$, *d.f.* = 8, $p = 0.119$), or with the interaction of salinity treatment and time ($F = 1.107$, *d.f.* = 8, $p = 0.385$)

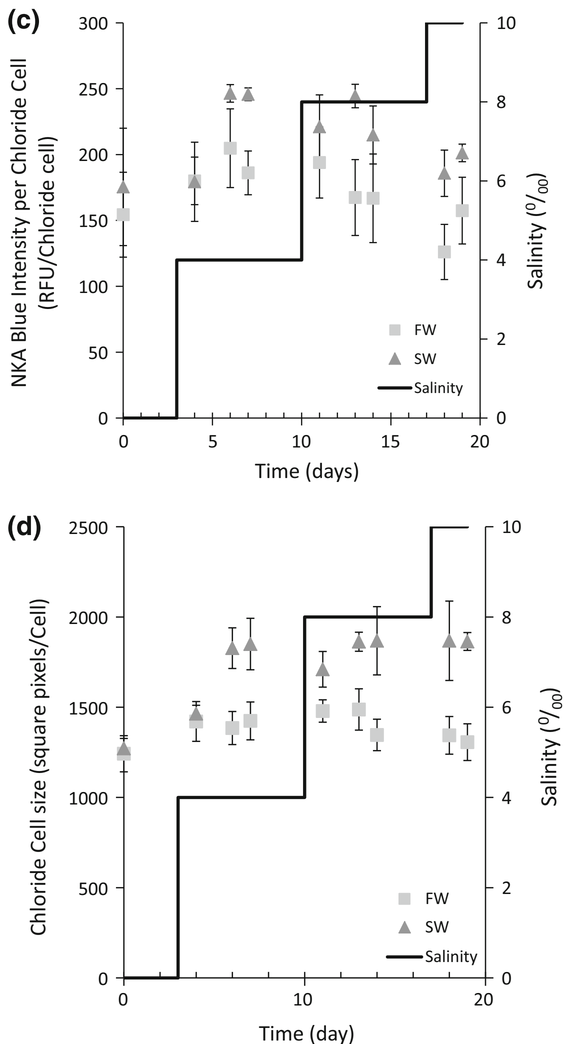

Fig. 3 continued

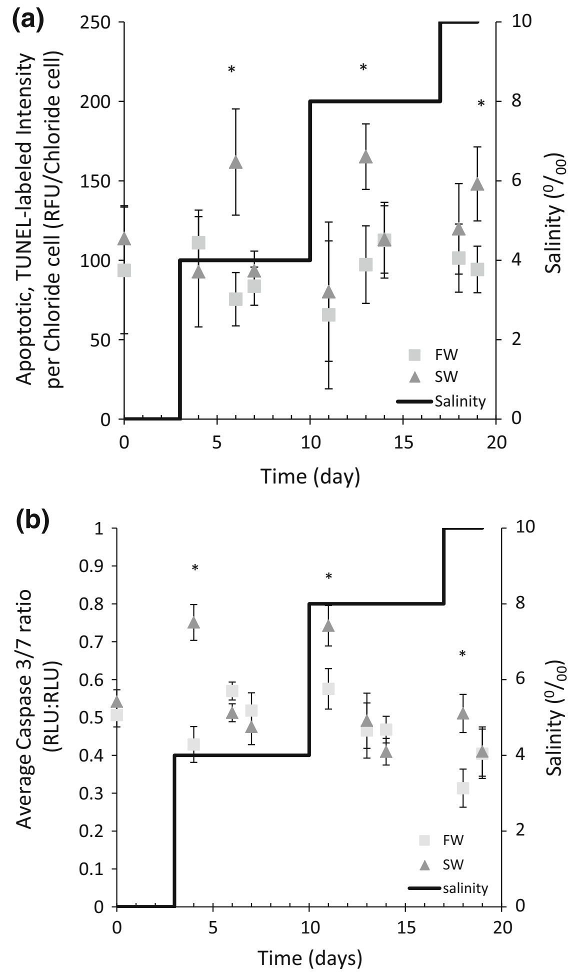

Fig. 4 Gill apoptosis increases in Delta Smelt gill chloride cells and gills. Apoptosis measured by **a** TUNEL assay increased in chloride cells [16 cells per gill arch ($n = 3$–4) in 4 fish per $n = 3$ tanks] up to 3 days post-salinity increase (see also Fig. 3 **a**, **b**) and **b** caspase 3/7 activity in gill homogenates, measured by luminescence plate reader assays, increased in SW (≤10 ppt), after salinity increases ≤4 ppt. TUNEL assay was nearly significant between treatments ($F = 5.651$, *d.f.* = 1, $p = .076$), over time ($F = 1.954$, *d.f.* = 8, $p = 0.086$) and showed significant differences with the interaction of salinity and time (($F = 0.048$, *d.f.* = 8, $p = 0.048$). Caspase 3/7 activity did not show apparent significant differences between treatments ($F = 3.229$, *d.f.* = 1, $p = 0.147$) or over time ($F = 1.488$, *d.f.* = 8, $p = 0.20$), but differences were significant with the salinity × time interaction ($F = 3.316$, *d.f.* = 1, $p = 0.007$). An *asterisk* significant differences ($p < 0.05$) in pair-wise comparisons using Tukey's multiple comparisons test

Our data show adult DS exhibit increases in hematocrit with as little as 4 ppt increases in salinity.

Hematological changes in plasma osmolality were similar to those of hematocrit. Previous metrics for DS

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plasma osmolality are not available, presumably in part, due to the difficulty of collecting enough blood for analyses from this small fish. Notably, plasma osmolality increased from that of the FW controls with increases in salinity at 1–3 days after the salinity step-increase and returned to those of FW by 4 days post-salinity increase (Fig. 1b). Increases in plasma osmolality in FW-acclimated fish exposed to salinities higher than those of their internal environment may be indicative of hyperosmotic stress (Moyle and Cech 2004); presumably the fish is not yet acclimated to higher salinities. However, a number of studies indicate this response may also be evidence that the FW fish is adjusting to a new baseline (Nordlie 2009). Increases in plasma osmolarity have been observed in medaka (Kang et al. 2008) and killifish (Dimaggio et al. 2010). Regardless, return to plasma osmolarities of fish at control levels may indicate acclimation (Sardella et al. 2004a,b ; Nordlie 2009). Interestingly, the response of DS to the initial increase to 4 ppt was greater than that from 4 to 8 ppt (Fig. 1b). Correlating with our data, Hasenbein et al. (2013) observed differences in osmoregulatory mechanisms, something that may lead to chloride cell changes (McCormick 1995). From our results, it appears that the first increase to and maintenance at 4 ppt salinity may help DS acclimate to the subsequent increases, as spikes in plasma osmolality were lower than that of the first (Fig. 1b). In combination with patterns observed for hematocrit (Fig. 1), it suggests that acclimation to 4 ppt salinities are achieved within a few days, and likewise for a subsequent increase to 8 ppt.

Changes in percent muscle water content were consistent with those observed for plasma osmolality and hematocrit. Fish did not show notable changes in percent muscle water content, though it fluctuated (Fig. 2). Our results are consistent with those found in medaka, where an estuarine species did not show changes in this metric when it was near salinities it normally inhabits (Kang et al. 2008).

Our data suggest that changing brackish salinities like those of the LSZ are not necessarily a stressor for DS, as they seem to respond similarly at salinities above 6 ppt than that below. Effects appear to be cumulative where adjustments to 4 ppt levels of salinity, whether within or beyond levels comparable to the LSZ, are well tolerated by DS. Our acclimatory data provide evidence for the apparent habitat preference, based on abundance and distribution (Bennett 2005) of DS $\geq$ 6 ppt to be due to factors other than those of salinity tolerance.

While other organs may contribute (Allen et al. 2009), the gill, with chloride cells, is thought to be the organ that provides the primary exchange site for ions and acclimation to salinity in teleosts (Marshall 2002; McCormick 1995). Given the increases in CC NKA fluorescent intensity and cell size, after salinity increases from FW (Fig. 3), our data suggests that gills may also be responsible for salinity acclimation in DS. Similar results were observed in DS exhibiting increased NKA transcriptomes in increasing salinities (Hasenbein et al. 2013), and greater NKA fluorescence per cell and CC size in other similar estuarine fish studies (Karnaky et al. 1976; Lima and Kültz 2004; Sardella et al. 2008a; Kammerer and Kültz 2009). It is apparent that similar mechanisms occur in DS as they make the transition to saltier water in the LSZ of the estuary above 4 ppt. It is not surprising that apoptosis may provide means for gill and CC restructuring (Kammerer and Kültz 2009; Kammerer et al. 2009). Our data shows that caspase 3/7 significantly increases followed by significant increases in TUNEL-labeled CCs after step-increases in salinity (Fig. 4a, b). Caspase 3/7 is upstream of terminal DNA nick-ends in the cell signaling pathway leading to apoptosis (Kültz 2005; Lüthi and Martin 2007), a fact that sheds light on the timecourse of the observed effects. In combination, the two apoptotic assays suggest that effect in the SW treatment was not significant by 4-day post-salinity step-increase (Fig. 4), indicative of acclimation much like hematological data. Similar work supports our study in a model estuarine fish (Wendelaar Bonga and Vander Meij 1989; Kammerer and Kültz 2009). Apoptotic signaling during salinity acclimation has also been found in killifish (Whitehead et al. 2012). Combined with evidence of cell proliferation (Kammerer et al. 2009) and changes in NKA and cell size (Karnaky et al. 1976; Kammerer and Kültz 2009) that were also observed, in part, in the current study, data is indicative of gill chloride cell turnover. This supposition is consistent with evidence of larger isoforms of CCs with greater NKA activity (Pisam et al. 1988) as well as distinct NKA isoforms in SW CCs (McCormick et al. 2009) of salmonid gills, known as alpha CCs (Moyle and Cech 2004). Further evidence for two types of chloride cell, characterized by PNA+ staining for larger, seawater-type cells and PNA− for smaller FW or accessory gill cells, exists

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(Goss et al. 2001; Galvez et al. 2002), though it is possible that cells differentiate from FW ones, instead of replacing SW types (Kammerer and Kültz 2009). While pavement cells may also express NKA (Lima and Kültz 2004), our analysis focuses on the largest cells, thus ruling out the possibility that FW cells were also accessory ones (Karnaky et al. 1976). Apoptosis may be a very effective measure of salinity especially in lieu of whole-body effects (Sardella et al. 2004b).

Our study suggests that DS utilize larger, more NKA-rich CCs, renewed from those of FW, to regulate ion transfer when faced with increases in salinities of the LSZ and above, to levels up to 10 ppt. In light of field distribution data (Moyle et al. 1992; Bennett 2005), our data also suggest that a suite of physiological mechanisms including changes in blood hematocrit, plasma osmolarity and gill cell turnover enable DS to acclimate to salinity conditions in LSZ salinities, but also those above them.

Conclusions

These works provide evidence of the mechanism DS use to acclimate to changes in salinity. The DS is 'indicator species' of estuary/habitat decline (Meffe and Carroll 1997; Feyrer et al. 2011), somewhat defined by recent investigations to evaluate the collapse of the fishery, part of the pelagic organism decline (POD) of the region (Sommer et al. 2007). Our data show that adult fish acclimate easily to LSZ salinities and above at this level of increase, it points to the other factors, like food and chlorophyll abundances (Kimmerer 2002; Lehman et al. 2010; Slater and Baxter 2014), turbidity (Ferrari et al. 2014), or temperature (Nobriga et al. 2008), playing a greater role in affecting the survival of DS. Predation and competition may also be strong factors (Vermeij 1987; Loboschefsky et al. 2012), though further work is needed to make stronger inferences.

From the present study, our data provides a laboratory baseline of Delta Smelt's physiological response to low salinities and evidence of their adaptation to it. The methods employed may be useful if monitoring the effects of salinity in the estuary becomes necessary. Many of these measurements can be performed easily, with the exception of perhaps fluorescent gill cell staining (Kammerer and Kültz 2009); some of these methods can be performed in labs without access to specialized equipment. Research performed in this paper may also provide useful information for further studies involving adult Delta Smelt spawning and, in particular, physiological responses of larval or juvenile DS that also move through low-salinity gradients in the estuary (Dege and Brown 2004; Wang 2007).

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