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Developing respirometry methodology for the Critically Endangered delta smelt

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ABSTRACT: Delta smelt *Hypomesus transpacificus* is a Critically Endangered fish native to the San Francisco Estuary. Respirometry methods quantify O_2 consumption (MO_2) to estimate metabolic activity, providing insights on organismal energetic budgets critical for devising conservation strategies. Due to its widespread adoption and utility, respirometry protocols have been established to foster quality control. However, delta smelt respirometry attempts over the past 12 yr have been largely unsuccessful, and high mortality rates during tests were only circumvented with very short measurement duration. This prevented the measurement of routine metabolic rate (RMR) and critical O_2 tension (P_{crit}) and obstructed delta smelt physiological research and conservation. Here, we detailed the anecdotal, behavioral, and empirical evidence that culminated in successful delta smelt RMR and P_{crit} measurements at 10, 12, 15, 17 and 19°C. We discovered the delta smelt is physostomous and requires frequent air bladder refills to maintain buoyancy and found the inclusion of an air pocket within their respirometry chamber greatly improved measurements. Daytime respirometry measurements yielded greater survival compared to overnight measurements; time of day did not cause significant RMR differences. Moreover, fasting did not significantly influence MO_2. Delta smelt RMR values at 10 and 12°C were significantly lower than fish acclimated to 15, 17, and 19°C. Delta smelt P_{crit} showed a similar pattern, but only the 19°C treatment showed significantly higher P_{crit} level. Altogether, this methodology allows for acquisition of critical knowledge needed for delta smelt conservation research, reducing conservation aquaculture operating costs, and improving survival during transport and field supplementation.

KEY WORDS: Conservation · Physiology · Physostome · Critical oxygen tension · Routine metabolic rate

1. INTRODUCTION

The delta smelt *Hypomesus transpacificus* was once one of the most abundant fish species endemic to the San Francisco Estuary (SFE) in California, USA, but its population has declined precipitously in recent decades — with recent annual surveys regularly reporting few to no detections (Tempel et al. 2021). The delta smelt is listed as an endangered species on the United States Federal Endangered Species Act (US Office of the Federal Register 1993, 2010, 2020) and the California Endangered Species Act (California Fish and Game Commission 2009) and as a Critically Endangered species on the IUCN Red List of Threatened Species (NatureServe 2014). Delta smelt are functionally extinct in the wild (Scoville 2019), but a genetically managed refuge population exists at the Fish Conservation and Culture Laboratory (FCCL) in Byron (California) to prevent the extinction of the species, and their eventual supplementation into, the SFE — with a production target set at 500 000 fish by 2030 (USFWS 2019). Their population decline has

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been largely attributed to changes in land use, water diversion, and the introduction of contaminants and non-native species across the SFE (Sommer et al. 2007, Hobbs et al. 2017, Tempel et al. 2021, Yanagitsuru et al. 2022, Baerwald et al. 2023). Moreover, the delta smelt has been drawn into the spotlight as their conservation can conflict with water politics, agricultural practices, and other socioeconomic issues in the USA (Moyle et al. 2018, Scoville 2019).

Conservation research has been increasingly incorporating physiological tools, in particular respirometry, to quantify metabolic traits and estimate bioenergetics and ecological performance (Wikelski & Cooke 2006, Cooke 2014, Cooke et al. 2014, Tomlinson et al. 2018, Fangue et al. 2022). Briefly, respirometry is used to measure O_2 consumption rate (MO_2) to determine metabolic traits including standard metabolic rate (SMR), routine metabolic rate (RMR), and critical O_2 tension (P_{crit}). SMR is the minimum rate of energy expenditure necessary to sustain life for a fish that is in a post-absorptive, calm, inactive state after proper thermal acclimation. RMR is higher than SMR and is defined as a resting non-feeding organism with spontaneous or voluntary activity (e.g. growth, reproduction). P_{crit} refers to when the partial pressure of oxygen (pO_2) falls below SMR or RMR, at which point an animal's basic metabolic needs cannot be maintained. Respirometry has allowed pursuit of a broad array of research questions pairing metabolic traits with fish behavior (Hansen et al. 2020), ecophysiology (Zillig et al. 2021, McInturf et al. 2022), aquaculture (Brauner & Richards 2020, Montgomery et al. 2022), and physiological and climate change (Nadler et al. 2016, 2021, Snyder et al. 2016, Norin & Metcalfe 2019, Kwan et al. 2021, Lonthair et al. 2024).

Standardized respirometry protocols have been developed to foster quality control and increase consistency (reviewed in Chabot et al. 2016a,b,c, Killen et al. 2021), but these techniques have been largely developed using model species and may not be universally applicable. For example, there is a wealth of research on salmonids (e.g. Eliason et al. 2011, Zillig et al. 2023a,b) which lend themselves well to the established respirometry protocols and equipment. In contrast, there is considerably less respirometry research data on pelagic fishes. Of the 115 published respirometry studies aggregated by Killen et al. (2016b), only 18 studies (15.7%) examined pelagic fishes. The lack of respirometry data on pelagic fishes (e.g. Osmeridae, Clupeidae, Scombridae) can be attributed, at least in part, to their aversion to isolation and confined spaces, which are inherent to experimental respirometry systems. These complications likely increase respiration rates and ultimately lead to SMR and RMR overestimation (Bernreuther et al. 2013). Therefore, there is a need to develop species-specific respirometry techniques (particularly in pelagic fishes) that do not conform to traditional protocols to enable broader research across fish taxa, especially those of conservation concern like the delta smelt.

Over the past 12 yr, the use of traditional respirometry methodologies and protocols on delta smelt have been largely unsuccessful. The earliest documented attempt at delta smelt respirometry was conducted in June 2012, and the use of annular respirometers for 14 h overnight resulted in 100% mortality (Table 1). Similarly, the use of glass mason jars for 'a few hours' in August 2012 resulted in 100% mortality (Table 1). At the time, delta smelt mortality was attributed to a high orienting flow rate, so smaller recirculating pumps and custom-made cylindrical chambers fitted with barriers were used to slow water flow and generate laminar flow conditions. Testing with these chambers in October 2012 slightly decreased mortality to 91.3% (Table 1). Later in November 2012, researchers discovered that time of day significantly impacted respirometry success. This was most evident when delta smelt were tested at 15°C: overnight respirometry that started in the afternoon resulted in 68–85% mortality, whereas respirometry that started in the late morning (10:00 h) to noon resulted in 16.7–33.3% mortality (Table 1). Despite these improvements, daytime respirometry of smelt acclimated to 19°C resulted in 54.2% mortality. Moreover, it was noticed in 2012 that delta smelt frequently exhibited a nosing behavior towards the surface of the chamber, which prompted the design from a circular chamber to a rectangular chamber. In an attempt to circumvent fish mortality, respirometry measurements were shortened to about 4 h, which reduced mortality to 0–5.6% (Table 1). All of these adjustments led to the publication of juvenile delta smelt MO_2 data (Hammock et al. 2017), though the relatively short 4 h (7 respirometry cycles) duration was likely incompatible with SMR or RMR determination (Chabot et al. 2016c). In an attempt to overcome this significant obstacle, current delta smelt bioenergetic models are based on a surrogate species, the rainbow smelt *Osmerus mordax* (Lantry & Stewart 1993, Rose et al. 2013).

The objective of this study is to present the recent findings that led to significant improvement in delta smelt respirometry. This includes more accurate delta smelt MO_2, RMR and P_{crit} measurements, with survival increasing to 95%. Moreover, we found the

Table 1. Summary of delta smelt respirometry studies. dph: days post hatching; Y: yes; N: no; NA: not applicable; RMR: routine metabolic rate; P_{crit}: critical O_2 tension

Citation	Chamber name	Date	Fish length (mm)	Fish weight (g)	Fish age (dph)	Chamber volume (ml)	Visible conspecific, ceiling	Lighting	Surface access	Duration (h)	Mortality (%)	Starting time
Eder et al. (2013)	Annular respirometer	Jun 2012	NA	NA	NA	10000	Y, N	No light; covered in plastic tarp	N	14	100	Afternoon
Eder et al. (2013)	Glass mason jars	Aug 2012	NA	NA	NA	2000	Y, N	No light; covered in plastic tarp	N	'a few hours'	100	Afternoon
Eder et al. (2013)	Cylinder chamber	Oct 2012	NA	NA	NA	273–975	Y, N	No light; covered in plastic tarp	N	NA	91.3	Afternoon
Eder et al. (2013)	Cylinder chamber with flat plate	Nov 2012	NA	NA	NA	273–975	Y, N	No light; covered in plastic tarp	N	NA	68–85	Afternoon
Eder et al. (2013)	Cylinder chamber with flat plate	Dec 2012	43–62	0.6–2.1	NA	273–975	Y, N	No light; covered in plastic tarp	N	6	11.5–54.2	Mid-morning to noon
D. E. Cocherell unpubl. data	Rectangular chamber	Aug–Nov 2013	Juvenile 50–54; Adult 76–77	Juvenile 1.0–1.2; Adult 3.2–3.7	Juvenile 250; Adult 1050	Juvenile: 250; Adult 970	Y, N	Dim white fluorescent light; covered in plastic tarp	N	4	5.6	Mid-morning to noon
Hammock et al. (2017)	Rectangular chamber	Nov 2013	NA	0.9–3.3	ca. 221	1250	Y, Y	Dim white fluorescent light; covered in plastic tarp	N	4	0	Mid-morning to noon
This study	Rectangular chamber with surface acces	Oct 2023	54–84	1.0–5.2	259–302	RMR: 500; P_{crit}: 700	Y, Y	Overhead red light	Y	17–21.5	40	Afternoon
This study	Rectangular chamber with surface access	Oct and Nov 2023	54–84	1.0–5.2	259–302	RMR: 500; P_{crit}: 700	Y, Y	Overhead red light	Y	10–12	5	Before dawn

incorporation of red lighting and an air pocket (to allow for surface access) within the respirometry chamber allowed for extended and more accurate delta smelt MO_2 measurements. In addition, we tested the influence of time of day (daytime vs. overnight) and fasting on juvenile delta smelt MO_2 across a range of ecologically relevant acclimation temperatures: 10, 12, 15, 17, and 19°C.

2. MATERIALS AND METHODS

The newly collected experimental data reported in this study were conducted between October and November 2023 (Table 1) in accordance with protocol no. 23316 in compliance with the Institutional Animal Care and Use Committee at the Center for Aquatic Biology and Aquaculture (CABA) at University of California Davis. Research activities were authorized by US Fish and Wildlife Service Section 10(a)(1)(A) permit (Permit: TE-027742) under the Endangered Species Act of 1973 and California Department of Fish and Wildlife Memorandum of Understanding (2081a-2022-0011-R3).

2.1. Delta smelt rearing and acclimation

Delta smelt were cultured at the FCCL until 221 d old following hatchery methods (Lindberg et al. 2013), transported by truck in black carboys, and placed into rearing tanks (volume = 200 l; n = 50 $tank^{-1}$) at the CABA. Temperatures were adjusted at a rate of 1°C every 2 d until acclimation temperatures were achieved (ranging from 10 to 19°C). Delta smelt were fed 4% body weight daily (BioPro2 #1 Crum, Bio-Oregon, LongView) via belt feeders. Delta smelt rearing tanks had gray walls and were covered with black (painted) lids made of plastic or styrofoam. Each cover had a small opening that allowed some ambient light and for food from a belt feeder to fall into the tank during the day. Delta smelt were acclimated to their respective target temperature for at least 2 wk prior to experimentation. Respirometry data was generated with delta smelt between 259 and 302 d old (length = 69.7 ± 0.5 mm; weight = 2.6 ± 0.1 g; mean ± SEM).

2.2. Surface restriction behavior

To assess surface restriction behavior, delta smelt were gently water-to-water transferred before dawn and with the assistance of a red-light headlamp into an acclimation tank (20 × 9.5 × 18 cm) to allow the fish to acclimate to the testing area. The testing area was an insulated water bath illuminated overhead with red light and laterally surrounded by black plastic tarp to remove unintentional stimuli. The temperature was set to the respective acclimation temperature (±0.5°C) using a water chiller (DBA-150; Arctica) and heatbar (TH-0800S; Finnex). After 15 min in the acclimation tank, delta smelt were released into 1 of 2 testing arenas. The 2 plastic arenas were completely clear and of the same size (30.5 × 22.5 × 16 cm; L × W × H), and they were stacked on top of each other so that the top arena had surface access whereas the bottom arena did not. Two submersible water pumps constantly delivered aerated water into both arenas. Arenas were rotated 180° horizontally after every test to account for any unintentional stimuli. Recordings were continuously captured by a waterproof CCTV camera (LC-F8036; 101AV Inc.). Fish (N = 8) in the bottom arena were kept 4.5 d without surface access, then moved to the top arena for 2.5 d to monitor their recovery. Fish in the top arena remained there for the entire duration (7 d). Delta smelt were not fed during the behavioral testing. In total, 3 tests were conducted: one each at 10, 12, and 19°C.

2.3. Optimizing time of day and fasting for respirometry

The delta smelt respirometry protocol (detailed below) was optimized by assessing the influence of time of day and fasting on RMR and P_{crit}. First, 10 and 19°C acclimated delta smelt respirometry that began before sunrise (predawn respirometry; starting between 4:00 and 6:00 h; 10 to 12 h in duration) was compared with those that began in the afternoon (overnight respirometry; starting between 15:00 and 18:00 h; 17 to 21.5 h in duration). Second, 10 and 12°C acclimated delta smelt that were fasted for 48 h prior to testing were compared with those that were fed within 12 h of their respirometry test.

2.4. Delta smelt respirometry methodology

Unless stated otherwise, all delta smelt RMR measurements were conducted using the following methods. RMR measurements used bimodal intermittent respirometry, whereas P_{crit} measurements used classic intermittent respirometry. Delta smelt were fasted

for 12 h prior to respirometry, which began before dawn between 4:00 and 6:00 h and was completed between 17:00 and 19:00 h (10–12 h in total).

All respirometry was conducted in a dark room illuminated with overhead red lights (9–31 lm; 1006 839 413; Ecosmart by Home Depot), and light intensity was measured with a light meter (Extech Light Meter 401025; Industrial Electronics). Temperature within the water bath was maintained within ±0.3°C of the acclimation temperature using a heater/chiller (TK-500; Teco). pO_2 of both air and water was measured using optical fiber dipping probes (DP-PSt3; PreSens) inserted into water- and air-tight rubber stoppers and recorded with Autoresp™ software (Loligo), with each measurement cycle having an interval of 90 s of flush, 90 s of wait, and 600 s of measurement (total of 13 min cycle^{-1} with ca. 29 measurement cycles fish^{-1}). O_2 levels within the chambers were not allowed to decline below 95% saturation during the RMR measurements to prevent stress.

Respirometry chambers (14 × 6 × 8 cm; L × W × H) were built with clear acrylic to allow visual access to conspecifics and a clear lid allowed overhead red light to penetrate (Fig. 1). Two sets of 4 chambers were arranged in a row, and all 8 chambers were placed in the same water bath. Individual respirometry chambers were fitted with a rubber gasket to prevent leaks, and 2 perforated acrylic barriers prevented delta smelt from swimming into water inflow or outflow tubing (tubing volume ca. 28 ml; Fig. 1). Each respirometry chamber was equipped with a recirculating pump that passed water over an optical fiber O_2 probe (water O_2 probe; Fig. 1) and returned it to the respirometry chamber. The cover of the respirometry chamber had a port for a second fiber optic O_2 probe to measure within the air pocket (air pocket O_2 probe; Fig. 1). Respirometry chambers were placed at the surface of the water bath, with the top portion of the chamber above the water level to produce an air-pocket. Chambers were carefully balanced and parallel with the water surface. As a result, the water table (height of the water within the water bath) kept the water within the respirometry chamber filled to ca. 5.7 cm, which is equivalent to a water volume of ca. 507 ml and an air volume of ca. 193 ml during RMR measurement. The flush pump (Eheim) was fitted with a motorized ball valve (USS-MSV00007) to prevent backflow and allow for computer control of flush and measurement periods. Air was gently and continuously bubbled within the water bath, and 2 water

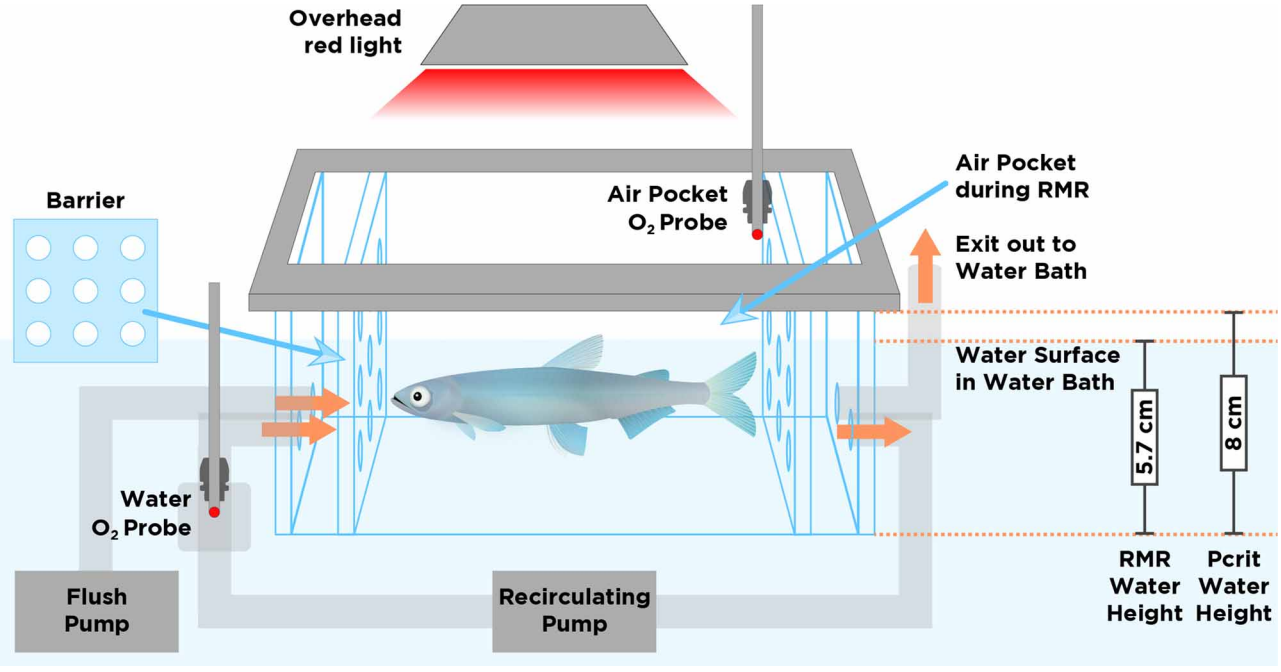


Fig. 1. Schematic of the delta smelt bimodal intermittent respirometer. Delta smelt respirometry was conducted in a rectangular acrylic chamber equipped with 2 optical fiber probes that simultaneously measured O_2 in water and air pocket. Two rigid barriers allowed water flow but prevented the fish from swimming into the tubing. During routine metabolic rate (RMR) measurements, an air pocket provided delta smelt with surface access. To prevent air intake, the recirculating pump and flush pump intakes were placed near the bottom of the chamber. Air pocket was removed during critical O_2 tension (P_{crit}) analysis by water flushing and the temporary removal of the air O_2 probe. See Section 2.4 for additional details

permeable screens were used to limit surface movement that could potentially disturb nearby fish.

Illuminated with a red-light headlamp, delta smelt were transferred from their rearing tanks before dawn to the experimental room in a covered bucket (ca. 3 min walk). Delta smelt were gently coaxed into a plastic tri-corner beaker then gingerly poured into their respective respirometry chambers. The water level in the respirometry chamber was equalized with the surface level, after which the chamber lid was secured. RMR measurements were generated from twenty-nine 13 min measurement cycles (totaling ca. 6 h 30 min fish^{-1}).

One concern with bimodal respirometers is that the air pocket could diffuse O_2 into the water, which would artificially reduce MO_2 during respirometry measurement cycles, as O_2 consumed by the fish is replaced by O_2 from the air pocket. To assuage these concerns, we conducted MO_2 measurements to assess the diffusion between air (97–98%) and water (92–93%) at 19°C over a 55 min period, with the latter representing the greatest amount of O_2 consumed in a 13 min respirometry cycle (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/esr01477_supp.pdf). This no-fish control revealed that dissolved oxygen (DO) decreased at a slope that represents ca. 0.56% of delta smelt RMR at 19°C. However, when DO was further reduced during P_{crit} measurements, O_2 diffusion from the air pocket into the water became evident: positive MO_2 slopes were observed when nitrogen bubbling brought DO levels below 80%. This suggests that the impact of air diffusion into the water during RMR measurement is negligible (when water DO is typically cycled between 97 and 100%) but must be removed during stepwise O_2 drawdown during P_{crit} measurement.

To initiate P_{crit} determination while minimizing disturbance to the fish, the O_2 probe on the lid of the respirometry chamber was removed to create an opening, after which the flush pump would displace the air pocket with water. Air bubbles sometimes became trapped on the lid, and these were removed with a gentle tilting of the chamber and/or the plugging of the outflow. Once all air bubbles were removed, the O_2 probe was re-secured to the lid of the respirometry chamber. During P_{crit} measurements, air bubbling in the water bath was replaced with nitrogen bubbling pumped through a ceramic air stone (1DMBDC300; Pentair). To keep DO drawdown consistently around 5% per cycle, nitrogen pressure was adjusted to 23 psi (158.6 kPa) during 100 to 40% DO saturation, 25 psi (172.4 kPa) during 40 to 20% DO saturation, and 28 psi (193.0 kPa) during 20 to 0% DO saturation (or until all fish lost equilibrium). The use of nitrogen bubbling for O_2 displacement is intended to allow for precise and consistent control over DO drawdown. However, readers are referred to Ultsch & Regan (2019) for a commentary on other P_{crit} methodologies (e.g. simultaneous O_2 drawdown and CO_2 accumulation) and the environmental and physiological implications that could prevent direct comparison across studies.

Background oxygen consumption was measured prior to the introduction of fish into the respirometry chambers and after removal of fish from the chambers. Following fish RMR and P_{crit} measurements, the water bath was re-oxygenated and an air pocket restored within the respirometry chamber. At the highest temperature treatment (19°C), background respiration was calculated to be 0.53% of the fish's MO_2. The respirometry chambers were then cleaned with 10% bleach and rinsed prior to the next respirometry experiment.

2.5. Respirometry data analysis

Metabolic rates and P_{crit} values were quantified by analyzing the AutoResp™ O_2 saturation ($\%O_2Sat$) output. A running average (window length = 29) was applied to smooth the data prior to conversion to O_2 concentration (Chabot et al. 2021). Smoothed O_2 saturation data were converted to O_2 concentration ($[O_2]$ mg O_2 l^{-1}) using Eq. (1):

$$[O_2] = \frac{\%O_2Sat}{100} \times \alpha(O_2) \times BP \quad (1)$$

where $\%O_2Sat$ is the O_2 saturation percentage measured via AutoResp™, $\alpha(O_2)$ is the temperature-corrected O_2 solubility coefficient (mg O_2 l^{-1} mm Hg^{-1}), and BP is the barometric pressure (mm Hg). Per-second O_2 concentration measurements were regressed over time, and the resulting coefficient of this regression (R; mg O_2 l^{-1} s^{-1}) was transformed into metabolic rate (MO_2; mg O_2 kg$^{-0.95}$ min^{-1}) using Eq. (2):

$$MO_2 = R \times V \times M^{-0.95} \times r \quad (2)$$

where V is the volume of the closed respirometer (500 or 700 ml depending on whether a measurement cycle was quantifying RMR or P_{crit}, respectively), M is fish mass (kg$^{-0.95}$), and r is the conversion factor (60 s min^{-1}) which adjusts the rate to min^{-1} from s^{-1}. An allometric scaling exponent of 0.95 was used to account for differences in fish sizes (Lucas et al. 2014).

After the above conversion from O_2 saturation percentage to O_2 concentration, RMR measurement

periods (n = 29 fish^{-1}) were visually inspected. Measurement periods with an $R^2 \leq 0.70$ were individually reviewed, and those indicative of transient temperature fluctuations were discarded (n = 52 of 2320; 2.2%). Final RMR was calculated using the *calcSMR* function (lowest quantile of metabolic rates) from the *fishMO2* package (Chabot 2020) incorporating the remaining RMR measurements (n = 24–29 fish^{-1}). See the Supplement for a summary of the delta smelt MO_2 (Fig. S2) and individual MO_2 (Fig. S3) plots.

The remaining measurement periods attributable to the P_{crit} measurement (n = 12–24 fish^{-1}) were likewise converted from O_2 saturation percentage to metabolic rates as described above. The P_{crit} was calculated using the *calcO2crit* function from the *FishMO2* package. This function uses the metabolic data collected during the P_{crit} measurement as well as the individual fish's RMR (calculated previously with *calcSMR*) to determine the O_2 concentration which restricts the metabolic capacity of the fish. See the Supplement for individual delta smelt P_{crit} (Fig. S4) and treatment-specific $MO_2 \times O_2$ (Fig. S5) plots.

2.6. Statistical analysis

Statistical tests were performed using R (version 4.0.3; R Development Core Team 2013) with packages *nlme* (Pinheiro et al. 2014) and *emmeans* (Lenth 2021). Statistical differences were analyzed using linear mixed-effects models fitted by restricted maximum likelihood (REML) with temperature and (if relevant) another variable of interest (fed or fasted, predawn or overnight) as fixed factors and individual and tank as random factors. Values are reported as mean ± SEM, and an alpha level of 0.05 was used.

3. RESULTS AND DISCUSSION

3.1. Lighting and delta smelt handling

Respirometry measurements of species active during the day (e.g. salmon) are typically conducted in nearly complete darkness in an effort to calm the fish and to prevent sudden changes in lighting from influencing respirometry measurements (Speers-Roesch et al. 2018). However, the delta smelt is a shoaling fish species, and the inability to see conspecifics is likely to induce stress. Indeed, past studies in other shoaling species have reported that visual cues of conspecifics can influence MO_2 (Hall & Clark 2016, Killen et al. 2016b, Nadler et al. 2016). Therefore, initial prototypes of delta smelt respirometry chambers were designed with clear walls and placed in close proximity to allow vision of conspecifics. However, past delta smelt respirometry experiments greatly limited ambient lighting from entering the experimental area with black plastic tarps (Table 1). Moreover, it has been shown that delta smelt collisions with tank features (e.g. fish exclusion screens) are more frequent in dark conditions, which can cause mortality (Swanson et al. 2005). Thus, the lack of lighting during past delta smelt respirometry measurements may have similarly impeded vision of conspecifics, increased collisions within the chamber, and ultimately contributed to greater MO_2 and increased mortality (Table 1).

The rearing protocols of the delta smelt refuge population at the FCCL suggest that this Critically Endangered fish is very light-sensitive. Past studies have documented that indoor lighting promotes hatching and feeding at the early larval stage (<40 d post hatching, dph), but by the late larval stage (40–80 dph) delta smelt prefer lower light/higher turbidity conditions (Tigan et al. 2020). This continues until maturity (about 300 dph), when they are transferred to outdoor tanks with ambient light dampened by shade cloth tank lids to promote sexual maturation and spawning (Baskerville-Bridges et al. 2005). Current best practices in delta smelt transport and rearing require carboys and tanks to be painted black to reduce overhead lighting (Baerwald et al. 2023, Hudson et al. in press, D. A. Gille et al. unpubl.).

Despite the extensive respirometry guidelines compiled by Killen et al. (2021), light levels are rarely reported as respirometry experiments are (or assumed to be) generally conducted in the dark or under heavily shaded conditions to block indoor fluorescent lighting. Of the full light spectrum, red light is known to dissipate rapidly as it permeates through the water column. Though it is thought to be less disruptive to fishes, there is some evidence even deep-sea fishes can still perceive red light (Widder et al. 2005).

In this study, delta smelt under red lighting (9–31 lm) were observed to remain calm and not react to overhead shadows, yet were able to see conspecifics and align themselves accordingly (see Video S1 at www.int-res.com/journals/esr/articles/esr01477/) as well as form and maintain shoals (G. T. Kwan pers. obs.). The sole illumination with red lighting can also be thought of as the omission of other wavelengths, which could simulate turbidity for the delta smelt. In the wild, delta smelt abundance is closely associated with an optimal water turbidity (Hamilton & Murphy 2022, Smith & Nobriga 2023) and associated with increased feeding efficiency (Hasenbein et al. 2013)

and reduced predation risk (Ferrari et al. 2014, Mahardja et al. 2016, Schreier et al. 2016, reviewed in Yanagitsuru et al. 2022).

The use of red lighting allowed us to observe fish behavior during respirometry trials with minimal disturbance and to conduct the experiment more safely and efficiently. Although their shoaling behavior suggests that the delta smelt can see under the red lighting, future research should assess their spectral sensitivity to determine their visual capacities. Moreover, past studies have shown that colored lighting can alter feeding and growth rate (Ruchin 2004, Volpato et al. 2013), cortisol levels (Owen et al. 2010), and liver and gonad conditions (Yuan et al. 2017). In one study on Nile tilapia *Oreochromis niloticus*, red light induced greater feeding behavior though it intriguingly did not significantly increase growth rate (Volpato et al. 2013). Therefore, additional studies are needed to quantify potential differences in metabolic rate across different light wavelengths, lighting intensities, and turbidity levels to ultimately verify whether red lighting can be a useful substitute for turbidity. If red lighting indeed decreases delta smelt metabolic rate and stress levels, then conservation hatcheries could employ these lighting strategies to reduce operational costs associated with the use of turbidity and increase safety and efficiency of animal husbandry routines.

3.2. Surface access is necessary for buoyancy and survival

The prolonged lack of surface access during previous transport and field release attempts may explain the high delta smelt mortality. Initially, open top plastic buckets (ca. 19 l) were fitted with seine netting to prevent fish from escaping during short-distance transport, but delta smelt were often caught in the mesh — leading to struggling and mortality. At the time, this behavior was interpreted as escape attempts, so delta smelt transport protocol was altered to using flat plastic lids to remove the risk of entanglement (which also removed air pockets). Later iterations required transport over longer distances, and the standard operating procedures of delta smelt transport within insulated carboys (ca. 83 l) continued to eliminate the air pocket to prevent injury and mortality caused by splashing (D. E. Cocherell pers. obs.).

Delta smelt appear to be able to tolerate about 4 h of carboy transport without surface access with minimal mortality (<1%). In contrast, transport of roughly 5 h has resulted in ca. 6.25% mortality (ca. 200 of 3200 fish). Later, as conservation focus shifted towards the release of hatchery raised delta smelt to supplement wild populations, researchers tested field cages to allow the fish to acclimatize to the wild prior to their release (Baerwald et al. 2023, D. A. Gille et al. unpubl.). Since delta smelt were thought to be poor swimmers (Swanson et al. 1998, 2000), early prototypes sought to reduce wave action by submerging the field cages. Similar to past carboy transport reports, initial pilot testing with submerged underwater field cages led to over 75% mortality — many of which were wedged into the mesh at the top of the cage (D. E. Cocherell pers. obs.). Later iterations that buoyed the field cages to the surface greatly reduced mortality to <1% (USFWS 2022, 2023).

Respirometry chambers represent another scenario where delta smelt cannot access the surface. In the past, dim or absent lighting prevented researchers from observing delta smelt behavior throughout respirometry runs. In this study, the use of red lighting allowed for extensive observation of delta smelt behavior throughout respirometry runs. In particular, bubbles were observed leaving the mouth of delta smelt during respirometry within cylindrical respirometry chambers (volume = ca. 500 cm^2) (G. T. Kwan pers. obs.). Upon release, delta smelt would doggedly and frequently swim to the surface to air gulp despite normoxic conditions in both the respirometry chamber and their holding tank (G. T. Kwan pers. obs.). Of the fish that died during respirometry, bubbles were observed within the chamber. Post-mortem autopsy revealed that delta smelt are physostomous (air bladders are connected to their esophagus), and comparison to healthy fish in holding tanks showed that submerged fish exhibited a reduced air bladder volume (Fig. 2). These observations imply surface access restriction led to air bladder deflation.

In retrospect, delta smelt behavior during tank and carboy transport, submerged field cage deployment, and the various iterations of respirometry chambers is likely due to restriction from surface access. Perhaps in an attempt to refill their air bladder, delta smelt seeking to reach the surface within respirometry chambers likely led to higher swimming activity, greater energy expenditure, and eventually exhaustion and mortality despite normoxic conditions. Since this discovery, subsequent observations during field release further confirmed our findings: on multiple occasions, delta smelt released into field cages were witnessed to repeatedly air gulp following carboy transport (D. E. Cocherell pers. obs.).

Curiously, other fishes commonly used in respirometry are also physostomes. For instance, Chinook salmon *Oncorhynchus tshawytscha* have survived

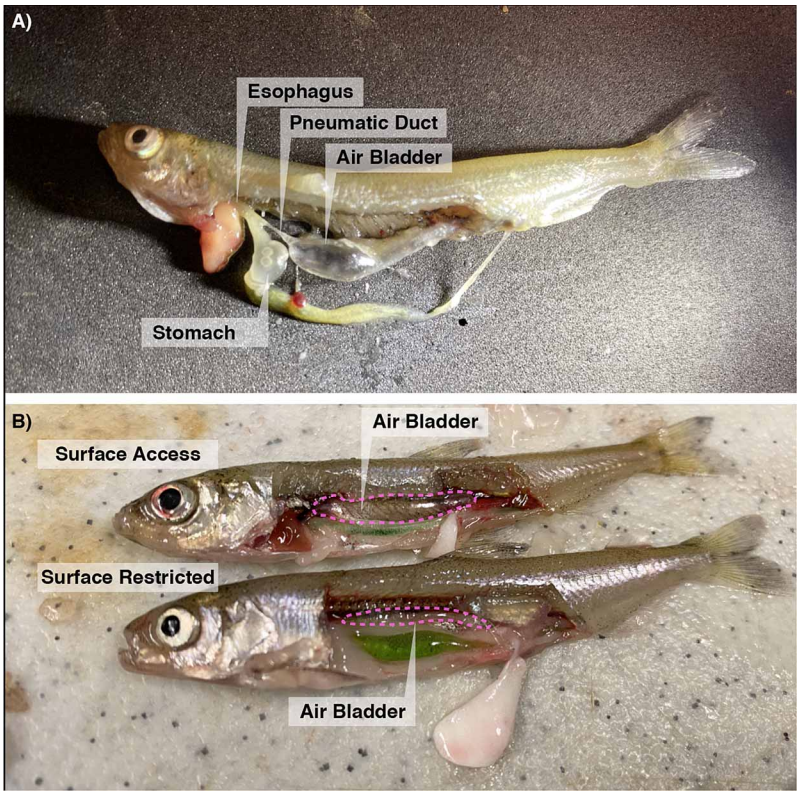


Fig. 2. Delta smelt is physostomous. (A) The esophagus of the delta smelt connects to the stomach and to the air bladder through the pneumatic duct. (B) Air bladder deflation after 8 h long respirometry without surface access. Pink dotted lines: perimeter of the air bladder

enclosure within respirometry chambers for up to 5 d (Lo et al. 2022). Even so, salmonids eventually require surface access: Atlantic salmon *Salmo salar* are known to experience buoyancy problems after 3 wk of submergence (Dempster et al. 2009, Korsøen et al. 2009). Why delta smelt lose air bladder volume more rapidly compared to other physostomes is not known, but this characteristic is consistent with their adaptation to their historical habitat and emphasizes their vulnerability under heavily altered current-day conditions. Historically, optimal delta smelt habitats were relatively shallow and turbid fresh and brackish waters, whereas current-day conditions are channelized systems with significantly reduced shallow, near-surface habitats (Moyle et al. 2016). Given that the tendency of gases escaping from within the air bladder is proportional to water depth (Strand et al. 2005), delta smelt inhabiting today's channelized river conditions may be losing their buoyancy at a greater rate if they reside deeper in the water column during the day and may thereby become more vulnerable to predation as they more frequently access the surface to refill their air bladder. Altogether, the delta smelt's close association with the surface further strengthens the necessity to restore historical floodplain habitats around the SFE to help their populations recover.

When housed in groups of 2 to 4 fish in a larger tank (volume = ca. 3400 cm^2), most (75%; 6 of 8 fish) delta smelt can survive 4.5 d without surface access (Fig. S6). However, delta smelt buoyancy was noticeably more negative within hours of surface restrictions (G. T. Kwan pers. obs.), and fish swam at an angle to avoid sinking (Fig. S2). However, this predicament was reversible: delta smelt recovered their buoyancy when given surface access over 2.5 d (Fig. S2I,J). Altogether, this suggests that earlier delta smelt respirometry measurements without surface access likely resulted in elevated RMR levels as the fish must expend greater energy to maintain their buoyancy or in their attempt to reach the surface. Future research is needed to more fully quantify delta smelt buoyancy and sinking rates in response to surface restriction.

3.3. Incorporating surface access into respirometry

This study is the first to recognize that delta smelt require surface access to maintain their air bladder volume, and its incorporation into our respirometry chamber design allowed delta smelt to survive significantly longer durations compared to past iterations (Table 1). In general, respirometry setups take great pains to remove air bubbles from the chamber, as O_2 diffusion from the air into the water could skew measurements. In order to reconcile this with the delta smelt's need for surface access, we drew upon past bimodal respirometry chamber designs used for air-breathing fishes (Lefevre et al. 2016). Air pockets were created within delta smelt respirometry chambers by positioning individual chambers slightly above the water line (Fig. 1). This allows the water and air levels within the chamber to equilibrate with the water height of the external water bath. Under red lighting, delta smelt were observed to utilize the provided air pocket within the respirometry chamber (G. T. Kwan pers. obs.).

3.4. Influence of time of day

The inclusion of an air pocket successfully reduced overnight respirometry mortality from 100 to 40% (Table 1) and generated the longest delta smelt respirometry measurements to date. To attempt to further reduce mortality levels, we decided to begin respirometry measurements during the day as previous attempts documented considerably higher survival during daylight hours (Table 1). Respirometry measurements beginning before dawn (predawn) further reduced delta smelt mortality from 40 to 5%, though this increase in survival could also be related to the shorter duration within the respirometry chamber. As such, it was necessary to determine whether shorter predawn respirometry measurements would yield comparable MO_2 and P_{crit} measurements as the longer overnight respirometry measurements.

In this study, we found predawn and overnight techniques generated similar MO_2 measurements in both 10 and 19°C delta smelt ($p = 0.9832$; Fig. 3A). As expected, we detected a significant temperature effect ($p = 0.0005$) with fish tested at 19°C having higher MO_2, and their interaction was not significant ($p = 0.9882$; Fig. 3A). In contrast, the P_{crit} of 19°C fish estimated with the overnight method was significantly higher than the 19°C fish estimated with the predawn method ($p < 0.0001$; Fig. 3B). P_{crit} was only affected by temperature ($p < 0.0001$) but not their interaction ($p = 0.1579$; Fig. 3B). We suspect that the unusually high P_{crit} were derived from moribund fish, so shorter respirometry durations would likely yield more accurate measurements at less optimal temperatures. However, RMR estimations did not encounter this issue, as this value is calculated from the mean of the lowest normal distribution, which reflects the period after the delta smelt had settled down. Altogether, the shorter 10 to 12 h long respirometry measurements incurred lower mortality, generated delta smelt RMR comparable to that of the overnight measurements, and provided a more reasonable P_{crit} estimate.

Circadian rhythm patterns are known to influence respirometry measurements (Livingston 1971, Kim et al. 1997, Svendsen et al. 2014), and past studies have indicated delta smelt prefer to feed (Hobbs et al. 2006), spawn (Tsai et al. 2022), and shoal with

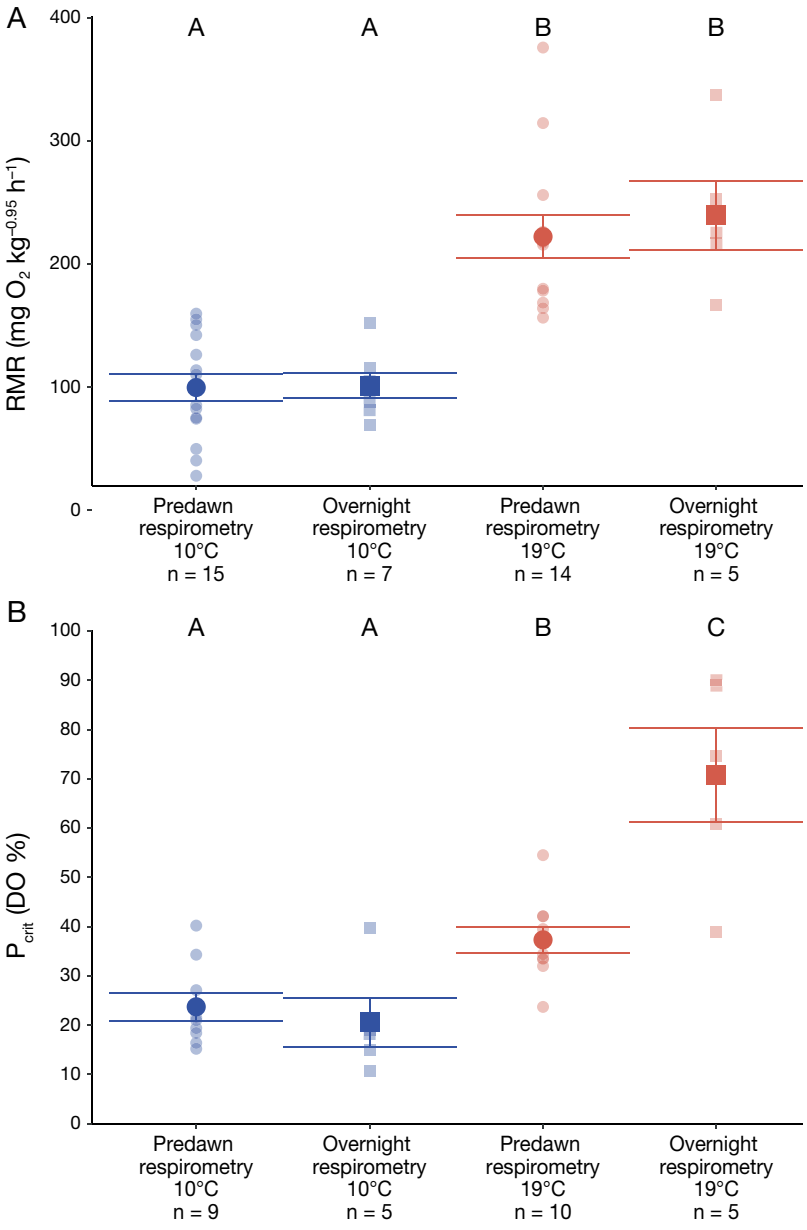


Fig. 3. Predawn and overnight routine metabolic rate (RMR) and critical O_2 tension (P_{crit}) values at 10 and 19°C. The starting time and duration in the respirometry chamber did not significantly influence (A) RMR of 10°C- and 19°C-acclimated delta smelt. (B) P_{crit} in 10°C-acclimated delta smelt was not affected by starting time and duration, whereas P_{crit} was significantly higher in 19°C-acclimated fish that underwent overnight respirometry measurements. Data is shown as mean ± SEM. Different letters denote significant differences among treatments ($\alpha = 0.05$; linear mixed-effects model). DO: dissolved oxygen

conspecifics (Chase et al. 2023) during the daytime. In the wild, delta smelt have also been observed to intentionally change their position within the water column according to the tides (Feyrer et al. 2013). Altogether, the elevated mortality implies delta smelt do not prefer being near the surface during nighttime and may have exhausted themselves as they struggled to swim deeper into the water column. As such, the greater success with daytime delta smelt respirometry despite similar chamber designs (Table 1) is not entirely surprising. Future studies should examine the interplay between time of day and water column position and perhaps design and test a respirometry chamber that incorporates greater vertical space.

3.5. Influence of fasting

Standard respirometry practices advise fasting fish prior to RMR and P_{crit} measurements since feeding is known to increase metabolic rate, thereby inducing greater variance due to differences in food ingested and energetic costs associated with digestion (Chabot et al. 2016a). However, fasting fish prior to respirometry may not always be an option, especially when working with sensitive endangered species and at numbers limited by federal/state permits. As such, we examined the impacts of feeding and 2 d fasting on RMR and P_{crit} levels in 10- and 12°C-acclimated delta smelt. Acclimation temperature significantly affected RMR ($p = 0.0446$) but not P_{crit} ($p = 0.8616$) (Fig. 4). In contrast, neither fasting (RMR: $p = 0.3247$; P_{crit}: $p = 0.1811$) nor their interactions (RMR: $p = 0.3948$; P_{crit}: $p = 0.1983$) affected RMR and P_{crit} (Fig. 4). While we acknowledge our low sample size, these findings are surprising, as feeding typically increases metabolic rates (Goodrich et al. 2022, Lo et al. 2022). In a previous food consumption study, both juvenile and adult delta smelt reared at 10 to 18°C completed digestion within 20 h of ab libitum feeding (Eder et al. 2013). Although we did not verify the stomach contents of our fish, these data suggest that it was unlikely that digestion interfered with the respirometry measurements. Instead, there was a counterintuitive and non-significant trend for increased RMR after the 2 d fasting, which may reflect increased foraging activity given these hatchery-reared fish have been habituated to daily feeding throughout their lives. Perhaps the differences between fed and fasted fish would be more apparent after a longer fasting periods and at higher temperatures and should be confirmed in future studies.

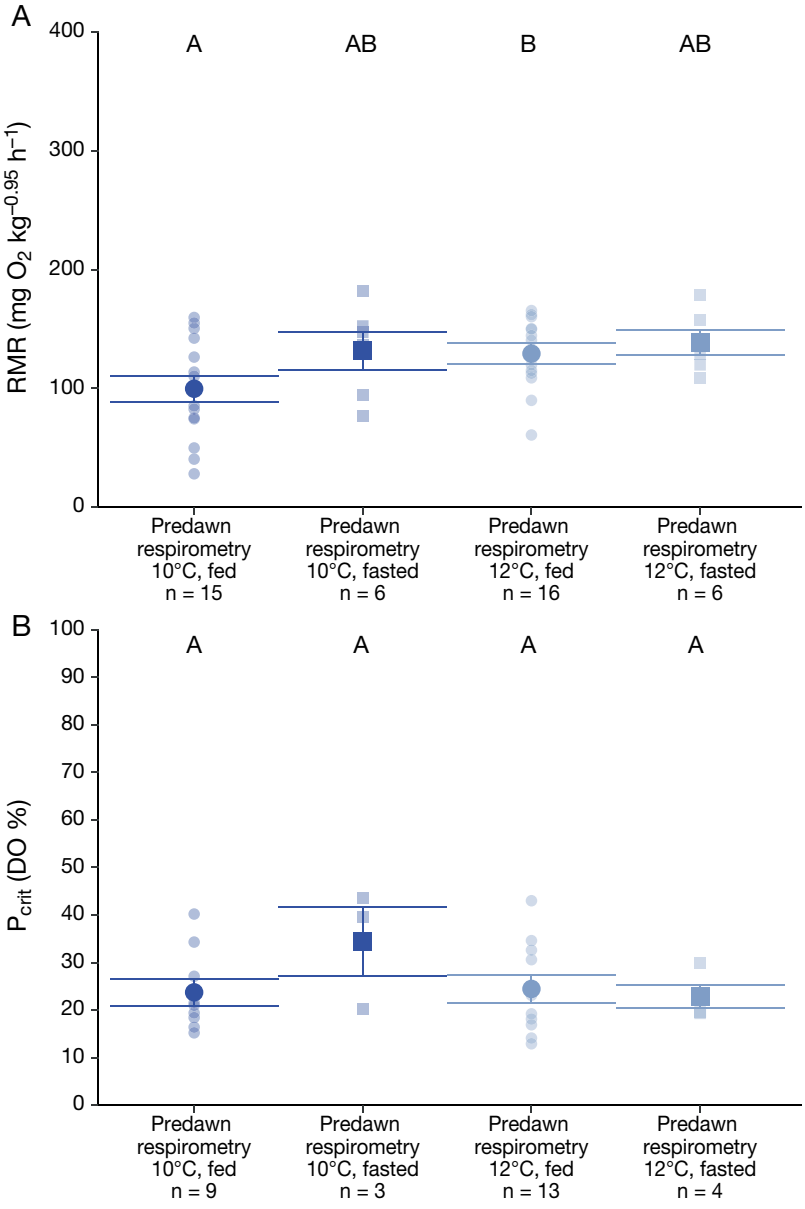


Fig. 4. Fed and fasting routine metabolic rate (RMR) and critical O_2 tension (P_{crit}) values at 10 and 12°C. Fasting did not significantly influence (A) RMR nor (B) P_{crit} of 10- and 12°C-acclimated delta smelt. Data is shown as mean ± SEM. Different letters denote significant differences among treatments ($\alpha = 0.05$; linear mixed-effects model). DO: dissolved oxygen

3.6. Comparison to past respirometry data

Altogether, this study documents our extensive efforts over the last 12 yr and highlights the recent breakthroughs in accurately estimating physiologically relevant RMR and P_{crit}, marking a significant milestone in the determination of delta smelt physiological performance and environmental tolerance. Comparison of our newly collected data to past datasets (Table 1) revealed that the inclusion of red lighting and surface access considerably decreased MO_2 values (Fig. S6). We believe our MO_2 measurements are representative of delta smelt RMR, and longer respirometry measurements will be necessary to identify their SMR.

Comparison to earlier unpublished and published results demonstrate the advancements made in delta smelt respirometry. Here, we compared respirometry data between 15 and 17°C fish with 2013 juvenile and adult delta smelt respirometry data collected over a 4 h measurement period (four 51 min measurement cycles) under low lighting and with no surface access (D. E. Cocherell unpubl. data; Table 1) and with data from Hammock et al. (2017) that measured 16°C fish under low lighting, with no surface access, and for a 4 h duration (five 30 min measurement cycles). Our use of red lighting and air surface access resulted in considerably lower RMRs in both 15°C- and 17°C-acclimated fish compared to Hammock et al. (2017) (Fig. 5). Therefore, the MO_2 measurements presented in Hammock et al. (2017) are likely overestimations as the lack of air surface access may have resulted in increased swimming activity, and the relatively few measurement cycles likely did not overlap with the delta smelt's resting state.

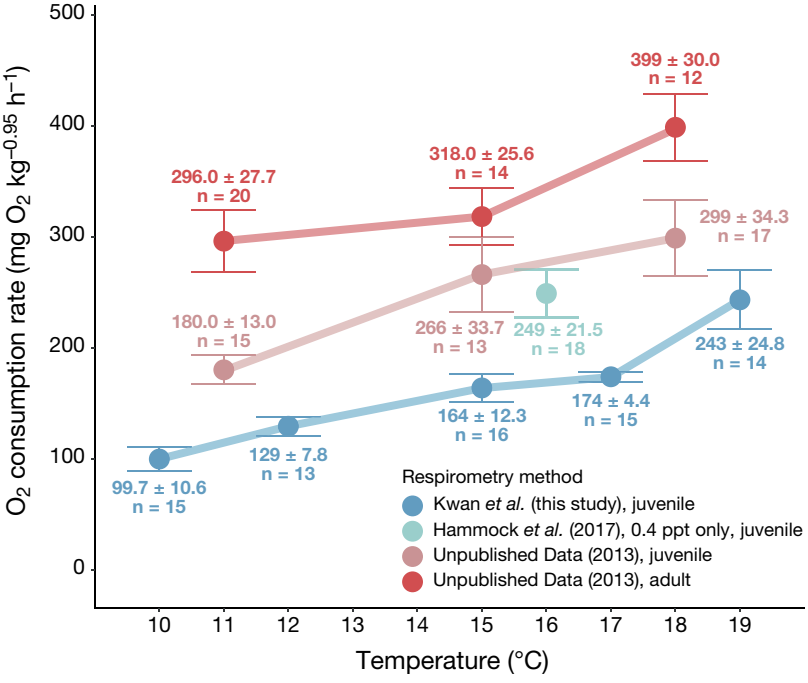


Fig. 5. Comparison of delta smelt respirometry measurements of O_2 consumption rate across methodologies, including unpublished data collected in Aug–Nov 2013 (Table 1), data from Hammock et al. (2017; 0.4 ppt salinity only) and those described in this study. Data is shown as mean ± SEM

3.7. Influence of temperature

Temperature was a significant predictor of delta smelt RMR ($p < 0.0001$) and P_{crit} ($p = 0.0001$). Subsequent pairwise analysis showed delta smelt RMR at 10°C was not significantly lower than those acclimated at 12°C ($p = 0.1616$) but was significantly lower than those at warmer temperatures (15°C: $p = 0.0020$; 17°C: $p = 0.0005$; 19°C: $p < 0.001$ Fig. 6A). Delta smelt P_{crit} results share a similar pattern of increasing hypoxia susceptibility with warmer temperatures, but statistical significance was detected at only the 19°C treatment (Fig. 6B). These experimental results are consistent with the current delta smelt field and husbandry practices: field collections are conducted during cooler conditions (below 12°C) to reduce mortality, and in conservation aquaculture fish spawning occurs between 12 and 15°C and rearing between 15 and 17°C (Baskerville-Bridges et al. 2005).

4. DISCUSSION AND CONCLUSIONS

Beyond the methodological advances, the RMR and P_{crit} measurements presented here provide critical ecological insight into delta smelt physiology. For example, the observed increases in RMR and P_{crit} suggest a narrowing window of metabolic and hypoxia tolerance at higher temperatures, highlighting potential vulnerability to climate-driven warming and declining water quality in the SFE. Future research on delta smelt RMR and P_{crit} across temperatures and turbidities is needed to confirm their preferences, which can then inform optimal supplementation release locations to bolster recovery of this critically endan-

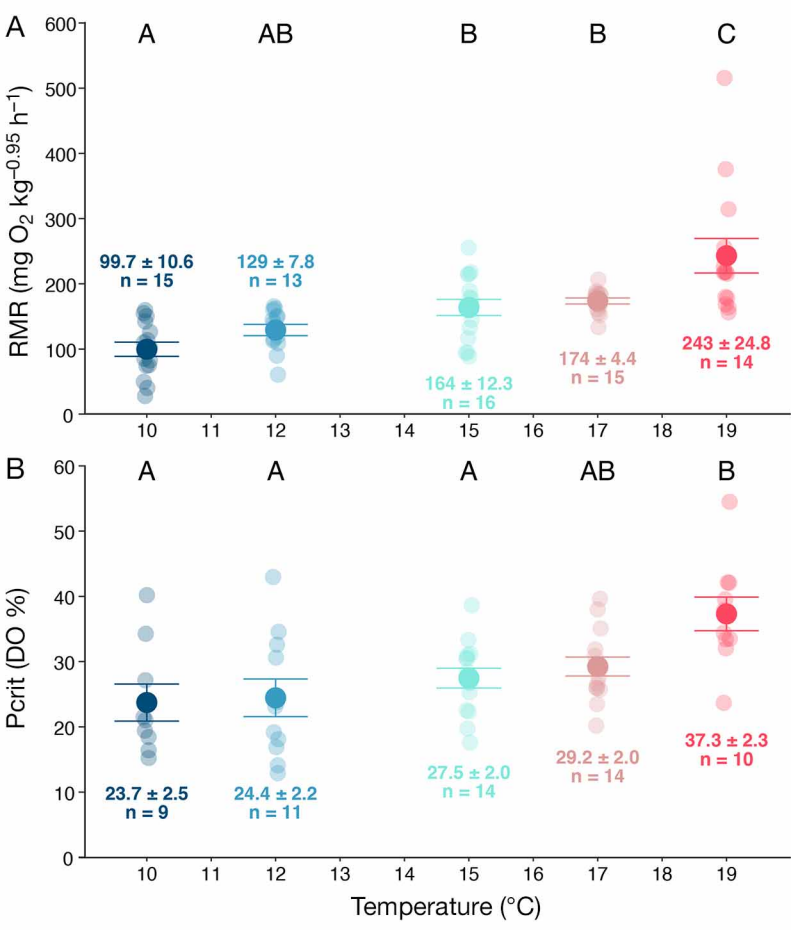


Fig. 6. Delta smelt routine metabolic rate (RMR) and critical O_2 tension (P_{crit}) values across temperature. Delta smelt (A) RMR and (B) P_{crit} increased with temperature acclimation across 10 to 19°C. Data is shown as mean ± SEM. Different letters denote significant differences among treatments ($\alpha = 0.05$; linear mixed-effects model). DO: dissolved oxygen

gered fish. Higher turbidity has been shown to promote feeding success, growth rate, survival (Hasenbein et al. 2013, Tigan et al. 2020), and reduced cortisol levels in larval fish (Hasenbein et al. 2016) and in juvenile and adult delta smelt (Lewis et al. 2021, Pasparakis et al. 2023). Conversely, reduced turbidity across the SFE has been correlated with higher cortisol levels (Pasparakis et al. 2023) and increased predation risk (Moyle et al. 2016). The delta smelt's need to frequently access the surface for buoyancy maintenance could increase their vulnerability to predation during reduced turbidity conditions.

The methods successfully developed here for delta smelt open the door for future studies critical to delta smelt reintroduction and species recovery. For instance, conservation managers can now begin to utilize respirometry to estimate energy consumption across abiotic and biotic factors to ultimately generate a delta smelt-specific bioenergetic model. Moreover, our respirometry methodology now allows for the quantification of sublethal stressors including tagging and post-surgical recovery critical for tracking delta smelt behavior in the wild. The determination of the rate at which delta smelt loses (and recovers) their buoyancy could bolster their survival during long-term transport, which is relevant for meeting fish release goals for ongoing population supplementation into the SFE. Furthermore, deducing delta smelt's perception of light, and whether omission of certain wavelengths could act similarly to turbidity, may serve as a mechanism to facilitate improved animal husbandry and lower FCCL operating costs. Recent estimates suggest delta smelt cost about US $84 per individual fish (Yanagitsuru et al. 2022), which is magnitudes higher than that the cost of producing individual salmon ($0.23 to $0.96 fish^{-1}) (Cavallo et al. 2009, Yanagitsuru et al. 2022). As suggested in this study, it is possible that reduced delta smelt anxiety under red lighting (compared to complete darkness or ambient lighting) could save time, improve fish welfare, and reduce operation costs. These improvements are critical as the current US Fish and Wildlife Biological opinion proposes increasing delta smelt production targets to 500 000 fish by 2030 (USFWS 2019), which is a daunting challenge compared to the 55 733 (USFWS 2022) and 43 725 (USFWS 2023) fish released in the winter of 2021 and 2022, respectively.

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