



Journal of Fish Biology (2010) **77**, 137–152

doi:10.1111/j.1095-8649.2010.02663.x, available online at www.interscience.wiley.com

Characterizing the escape response of juvenile summer flounder *Paralichthys dentatus* to diel-cycling hypoxia

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(Received 27 March 2009, Accepted 8 March 2010)

Swimming speed, angular correlation and expected displacement were measured in juvenile summer flounder *Paralichthys dentatus* acclimated to either oxygen saturation (c. 7.8 mg O₂ l⁻¹; saturation-acclimated fish) or diel-cycling hypoxia (cycling between 11.0 and 2.0 mg O₂ l⁻¹) for 10 days and subsequently exposed to more severe diel-cycling hypoxia (cycling between 7.0 and 0.4 mg O₂ l⁻¹). Saturation-acclimated *P. dentatus* exhibited an active response to declining dissolved oxygen (DO) by increasing swimming speed, angular correlation and expected displacement to peak levels at 1.4 mg O₂ l⁻¹ that were 3.5, 5.5 and 4.2 fold, respectively, greater than those at DO saturation. Diel-cycling hypoxia-acclimated *P. dentatus* also exhibited an active response to declining DO, although it was relatively less pronounced. Diel-cycling hypoxia-acclimated *P. dentatus* swimming speed, however, still doubled as DO decreased from 7.0 to 2.8 mg O₂ l⁻¹. Diel-cycling hypoxia-acclimated *P. dentatus* did not recover as well from low DO exposure as did saturation-acclimated fish. This was reflected in their relatively more random swimming (low angular correlation between successive moves) and poor maintenance of rank order between individuals during the recovery phase. Even saturation-acclimated *P. dentatus* did not resume swimming at speeds observed at saturation until DO was 4.2 mg O₂ l⁻¹. *Paralichthys dentatus* were very sensitive to decreasing DO, even at DO levels that were not lethal or growth limiting. This sensitivity and their poor recovery may preclude juvenile *P. dentatus* from using highly productive nursery habitats affected by diel-cycling hypoxia.

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Key words: acclimation; behavioural avoidance; dissolved oxygen; estuary dependent; juvenile flatfish.

INTRODUCTION

Hypoxia has become an increasingly common feature of estuaries worldwide (Diaz & Rosenberg, 2008). Hypoxia can be classified temporally as: seasonal (weeks to months), multi-day and diel-cycling (hours) (Diaz & Rosenberg, 2008; U.S. EPA, 2000). Diel-cycling hypoxia has been observed for decades in estuaries (Nixon & Oviatt, 1973) and tropical freshwater systems (Junk, 1970). Recently, however, severe diel-cycling hypoxia has been reported in Waquoit Bay, MA, U.S.A. (D'Avanzo & Kremer, 1994), Elkhorn Slough, CA, U.S.A. (Beck & Bruland, 2000;

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Beck *et al.*, 2001), Delaware Bay salt marsh pools (Smith & Able, 2003) and tributaries to the Delaware coastal bays (Tyler *et al.*, 2009). Diel-cycling hypoxia occurs in shallow water in response to the interaction of photosynthesis and respiration over the 24 h day–night cycle (D’Avanzo & Kremer, 1994; Beck & Bruland, 2000). The amplitude of this natural dissolved oxygen (DO) cycle becomes exacerbated in eutrophic estuaries (Beck & Bruland, 2000; Tyler *et al.*, 2009) that are subject to anthropogenic nutrient loading from land runoff and groundwater inputs (Nixon, 1995; Nixon *et al.*, 1996).

The effect of hypoxia on juvenile fishes that rely on estuaries for nursery habitat is difficult to quantify. On one hand, several laboratory studies have demonstrated the direct detrimental effects of hypoxia on the growth (McNatt & Rice, 2004; Stierhoff *et al.*, 2006) and mortality (Miller *et al.*, 2002; Shimps *et al.*, 2005) of juvenile estuary-dependent fishes. On the other hand, laboratory (Wannamaker & Rice, 2000) and field (Bell & Eggleston, 2005; Tyler & Targett, 2007) studies have demonstrated that fishes can successfully avoid areas of low DO. Field avoidance, however, may not be completely effective, as Stierhoff *et al.* (2009) observed a strong negative correlation between diel-cycling hypoxia and *in situ* growth rates of weakfish *Cynoscion regalis* (Bloch & Schneider) and summer flounder *Paralichthys dentatus* (L.) derived from RNA:DNA ratios. Characterizing the behaviour of juvenile estuary-dependent fishes will be important in discriminating between the direct effects of hypoxia on growth and the indirect effects mediated by habitat compression (Eby & Crowder, 2002), loss of prey resources (Pihl, 1994) and changes in predator dynamics (Breitburg *et al.*, 1994).

Many studies have characterized fish behaviour in relation to declining DO, and the results are highly context dependent. The rate of DO decrease (Moss & McFarland, 1970), previous acclimation history (Brady *et al.*, 2009), size and age (Bejda *et al.*, 1987; Bursleson *et al.*, 2001), predation risk (Sloman *et al.*, 2006) and species (Herbert & Steffensen, 2005, 2006) dictate the optimal behavioural strategy employed in any given situation. The strategies are generally characterized as either active or passive responses, and both strategies are frequently used by a given species over the course of exposure to declining DO. For instance, Dalla Via *et al.* (1998) described the temporal response in activity of sole *Solea solea* (L.) over a large range of oxygen saturation. As DO levels declined from 80 to 20% saturation, *S. solea* reduced activity (passive response). Severe hypoxia (20–6% saturation) resulted in a switch to anaerobic metabolism and only the most severe hypoxia (5% saturation) resulted in burst and panic-like swimming (active response). In contrast, Domenici *et al.* (2000) observed an increase in the swimming speed of Atlantic herring *Clupea harengus* L. during severe hypoxia (34–15% saturation) followed by a decrease in swimming speed and eventual school disruption. Domenici *et al.* (2000) suggested that the different strategies might be due to differences between species in hypoxia tolerance and overall activity level. Specifically, hypoxia-tolerant and relatively sluggish species might employ primarily the passive strategy, whereas hypoxia-intolerant and relatively active species might rely more on the active strategy (Domenici *et al.*, 2000).

Differences in behavioural responses of active and sluggish species represent an important distinction. Fishes that employ primarily a passive response to hypoxia would presumably remain in low oxygenated environments, choosing to save energy rather than escape. During severe diel-cycling hypoxia in relatively open systems

(i.e. tributaries or floodplains adjacent to open bay or river areas), the passive strategy might result in fishes being exposed to severe hypoxia or anoxia while higher DO conditions were within relatively close proximity. Success of this strategy is tightly linked to the spatial and temporal scale of the hypoxic zone. Few studies have compared sluggish *v.* active species using a standardized experimental design. Herbert & Steffensen (2005, 2006) recorded *C. harengus* (more active species) and Atlantic cod *Gadus morhua* L. (less active species) swimming speed as DO decreased, using almost identical methods. As expected, *C. harengus* displayed an active response whereas *G. morhua* displayed primarily a passive response. Interestingly, *C. harengus* swimming speed was only elevated when DO was unsteady (i.e. decreasing) and not during the steady states between DO decline steps. Similarly, during the initial DO decrease (unsteady DO), *G. morhua* increased swimming speed even though they were primarily passive responders. Herbert & Steffensen (2005, 2006) hypothesized that steady-state DO conditions indicate a spatially extensive hypoxic zone in which escape probability is low, and therefore, the optimal behavioural strategy is to stay; whereas unsteady DO conditions indicate a probability of escape sufficiently great to risk an active response. Behavioural responses of fishes to decreasing DO are probably tunable to changing conditions and modulated by previous exposure history.

Diel-cycling hypoxia presents unique challenges to juvenile estuary-dependent fishes because it varies at multiple temporal and spatial scales (Tyler *et al.*, 2009). At the within-day temporal scale, fishes can either stay as DO decreases (generally from 2 h before sunset to 2 h after sunrise) or attempt to escape. Within a season, the behavioural strategies of fishes result in acclimation to diel-cycling hypoxia and making daily temporary emigrations from otherwise preferred habitat. Variability in fish behaviour and in the spatial extent of hypoxia are the primary determinants of whether fishes are exposed to lethal or growth-limiting DO levels. Juvenile fishes are under strong selective pressure to grow during the nursery seasons (Houde, 1987, 1989; Sogard, 1997), and this energetic constraint intensifies the consequences of their behavioural decisions. Few studies have examined the swimming responses of juvenile estuary-dependent fishes to diel-cycling hypoxia (Brady *et al.*, 2009). The inclusion of additional species, particularly flatfishes, examined under the same behavioural protocol used in Brady *et al.* (2009) will ultimately shed light on the potential differential effects of hypoxia on species with different activity levels and behavioural strategies.

The overall objective of this study was to quantitatively characterize changes in swimming speed, angular correlation and expected displacement of juvenile *P. dentatus*, an estuary-dependent fish, in response to a complete diel DO cycle similar to that observed during mid-summer to late summer in the field. Additionally, some juvenile *P. dentatus* were acclimated (10 days) to a less severe diel DO cycle (DO cycling between 11.0 and 2.0 mg O₂ l⁻¹) common in late spring to early summer to examine the effect of acclimation on swimming behaviour and survival during exposure to the more extreme diel-cycling DO that occurs later in the summer. Interindividual variability in swimming response to diel-cycling DO was examined in acutely exposed (saturation-acclimated) and chronically exposed (diel-cycling hypoxia-acclimated) fish. Finally, the present results are compared with those of a more active species, *C. regalis* (Brady *et al.*, 2009), exposed to identical DO conditions.

MATERIALS AND METHODS

Paralichthys dentatus were collected from nursery areas in the Delaware coastal bays using an otter trawl. Fish were maintained in re-circulating aquaria at 20° C, 25 salinity, a 14L:10D photoperiod and fed frozen mysid shrimp *Mysis relicta ad libitum* for at least 10 days before observation. Food was withheld for 12 h before observation. During holding and observation periods, fish were maintained on a 30 mm thick layer of 0.1–0.5 mm grain size light-coloured sand in 25 salinity water. Fish were separated into three experimental groups before observation based on DO exposure during the 10 day holding period: control, saturation acclimated and diel-cycling hypoxia acclimated. Control and saturation-acclimated *P. dentatus* were exposed to saturated DO (c. 7.8 mg O₂ l⁻¹) conditions in individual tanks during the holding period. Diel-cycling hypoxia-acclimated *P. dentatus* were held in individual tanks and exposed to diel-cycling DO (2.0–11.0 mg O₂ l⁻¹) in a computer-controlled DO system (Greycay & Stierhoff, 2002). Minimum (2.0 mg O₂ l⁻¹) and maximum (11.0 mg O₂ l⁻¹) DO concentrations coincided with the beginning of the light (0700 hours) and dark (2100 hours) periods, respectively. DO treatments were chosen to closely approximate natural DO conditions in shallow, mid-Atlantic Bight estuaries (D'Avanzo & Kremer, 1994; Tyler *et al.*, 2009). Mean ± s.e. standard lengths (L_S) of *P. dentatus* for control, saturation-acclimated and diel-cycling hypoxia-acclimated experiments were 11.1 ± 0.5, 11.7 ± 0.8 and 15.0 ± 0.3 cm, respectively.

A video camera (Hitachi KP-MN; www.hitachi.com) attached to a frame grabber (PIXCI; www.epixinc.com) and associated image analysis software (XCAP; www.epixinc.com) was mounted 4 m above the tank (2.4 m diameter × 0.2 m depth) and used to record the position of individual *P. dentatus* every second. The dimensions of image pixels were calibrated before trials. The shallow depth minimized the effect of vertical movement on these 2D estimates. In preliminary trials, it was determined that fish swimming speed did not change significantly at the <1 s time scale and that partitioning the fish's path into 1 s intervals was a reasonable approximation of continuous swimming speed. Individual fish of the control and treatment groups were introduced into the tank (fish were never exposed to air) and acclimated for 1 h before a trial. *Paralichthys dentatus* typically buried in the sand incompletely, and therefore, there was enough contrast between the fish and the sand to determine their location. Control *P. dentatus* ($n = 10$) were observed in saturated conditions to determine whether behaviour changed over time without stimuli associated with DO change. Saturation-acclimated ($n = 13$) and diel-cycling hypoxia-acclimated ($n = 10$) fish were exposed to stepwise decreases in DO. Trials began at 7.0 mg O₂ l⁻¹ (c. 90% saturation; 18.9 kPa) and DO was decreased by 1.4 mg O₂ l⁻¹ (c. 22% saturation; 3.8 kPa) increments to 1.4 mg O₂ l⁻¹ (c. 22% saturation; 3.8 kPa), at which point DO was reduced to 0.8 mg O₂ l⁻¹ (c. 10% saturation; 2.2 kPa) and finally 0.4 mg O₂ l⁻¹ (c. 5% saturation; 1.1 kPa). Fish were exposed to each DO level for 30 min and transitions between levels lasted 3–5 min (Fig. 1). Subsequently, DO was raised in reverse fashion to examine swimming activity following hypoxia exposure. DO was measured with a YSI 5100 DO meter (www.ysi.com) and adjusted remotely by adding compressed nitrogen (decreasing DO) or oxygen (increasing DO) to an outer ring in the tank. The DO probe was located at the edge of the observation area. By measuring DO with four DO probes simultaneously before observation, it was determined that multiple pumps in the outer ring mixed water into the inner ring (2 m diameter observation area), ensuring homogeneous DO exposure.

Mean swimming speed and the distribution of changes in direction (turning angles) were averaged for each 0.5 h of constant DO exposure using the 1 s interval positioning data recorded by the camera. To examine swimming speed changes at a temporal scale finer than 30 min, 5 min averages were also calculated. A change of direction was calculated only for movements of at least 1 cm. From the distribution of turning angles, the angular mean and concentration around the mean were extracted. *Paralichthys dentatus* were equally likely to move left or right (balance tendency); therefore, the angular mean was essentially zero. Concentration of turning angles around the angular mean was calculated by taking the cosine of the turning angles [$r = \cos(\theta)$], which represents the angular correlation between successive turns. High angular correlation indicates a straight-line path ($r = 1$) and low angular correlation indicates random movement ($r = 0$). Since *P. dentatus* often swim along the edges of a circular tank, it should be noted that swimming in a circle can also result in high angular correlation. For example, a fish swimming clockwise in a circle making 10° turns to the right

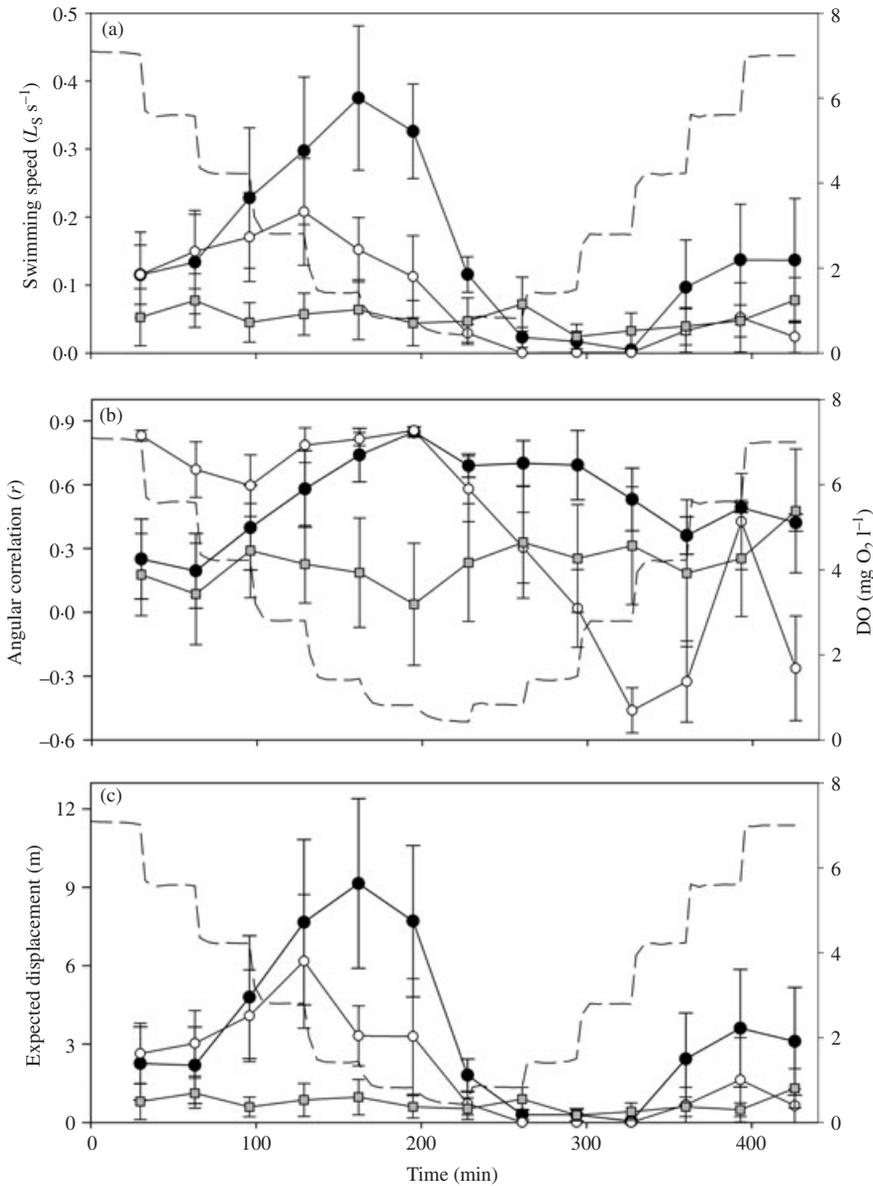


FIG. 1. Mean \pm S.E. (a) swimming speed in body lengths (standard lengths, L_S) s^{-1} , (b) angular correlation and (c) expected displacement (for each 30 min period) of saturation-acclimated (\bullet), diel-cycling hypoxia-acclimated (\circ) and control (\square) *Paralichthys dentatus* during the dissolved oxygen (DO) treatment period. Points show the mean response over the previous 30 min period. ---, DO exposure for saturation and diel-cycling hypoxia-acclimated fish.

would have an angular correlation of 1. Although observed angular correlation in a tank may not exactly reflect search patterns *in situ*, relative changes in angular correlation are suggestive of behavioural strategy changes that fish may utilize in the field. Step length and angular correlation between each second were used to compute expected displacement [$E(D)_{app}$] for

each half hour exposure period, based on the approximation proposed by Benhamou (2004): $E(D)_{\text{app}} = 0.886\lambda\{[n(1+r)](1-r)^{-1}\}^{0.5}$, where λ is step length, n is the number of steps (in this case the number of seconds) and r is angular correlation. The units of expected displacement are expressed in metres and were calculated for each 30 min observation period. This approximation was used to examine the influence of simultaneous changes in swimming speed and path straightness on the relative changes in each individual's expected displacement.

Control, saturation-acclimated, and diel-cycling hypoxia-acclimated trials were tested for the omnibus null hypothesis that no changes in swimming speed, angular correlation and expected displacement occurred over time using a one-way repeated measures ANOVA with Greenhouse–Geisser adjustment (Greenhouse & Geisser, 1959). Each 0.5 h period represented a factor (Maxwell & Delaney, 1990). For any trial that deviated from the omnibus null hypothesis, polynomial contrasts were fit to the difference scores to characterize the overall response.

Interindividual variability in swimming speed was examined using Kendall's coefficient of concordance (W) to test whether fish maintained their rank order through time. Friedman's χ^2 was obtained from W to test for statistical significance ($\alpha < 0.05$). A high Kendall's W in swimming speed means that fast individuals are so throughout a trial and *vice versa* for slower individuals. As interindividual variability declines, so does Kendall's W . In conjunction with Kendall's W , the coefficient of variation (c.v.), derived from $y\bar{x}^{-1}$ where $y = \text{s.d.}$ and $\bar{x} = \text{mean}$, in swimming speed was calculated to determine whether changes in concordance were linked to changes in swimming speed variability.

RESULTS

SATURATION *v.* DIEL-CYCLING HYPOXIA ACCLIMATION

Swimming speed, angular correlation and expected displacement of control *P. dentatus* (observed in saturated DO conditions) did not change significantly during the observation period (repeated measures ANOVA, $n = 10$; $P > 0.05$ for both swimming speed and angular correlation; Fig. 1). In contrast, hypoxia significantly altered several aspects of saturation-acclimated *P. dentatus* behaviour during DO treatment and recovery phases. Swimming speed of saturation-acclimated fish during DO treatment followed a quadratic relationship [repeated measures ANOVA, $n = 13$; $F = 4.336$, $P < 0.05$; Fig. 1(a)]. Swimming speed increased (active response) to a relative maximum at $1.4 \text{ mg O}_2 \text{ l}^{-1}$, a 3.5 fold increase over the speed observed at $7.0 \text{ mg O}_2 \text{ l}^{-1}$. Swimming speed remained elevated even at $0.8 \text{ mg O}_2 \text{ l}^{-1}$ where speed was still three-fold higher than at $7.0 \text{ mg O}_2 \text{ l}^{-1}$. Below $1.4 \text{ mg O}_2 \text{ l}^{-1}$, swimming speed decreased as DO decreased until DO was $0.4 \text{ mg O}_2 \text{ l}^{-1}$ and speed returned to the same level observed initially at $7.0 \text{ mg O}_2 \text{ l}^{-1}$. Swimming speed calculated at a 5 min resolution showed that *P. dentatus* had three distinct bouts of acceleration; at 2.8 , 0.8 and $0.4 \text{ mg O}_2 \text{ l}^{-1}$ (Fig. 2). The bout of increased swimming speed at $0.4 \text{ mg O}_2 \text{ l}^{-1}$ was interrupted by periods of extreme inactivity and represents a panic-like swimming response near anoxia.

Angular correlation of saturation-acclimated *P. dentatus* increased linearly with decreasing DO, increasing six-fold to a maximum of 0.82 at $0.8 \text{ mg O}_2 \text{ l}^{-1}$ [repeated measures ANOVA, $n = 13$; $F = 3.84$, $P < 0.05$; Fig. 1(b)]. Angular correlation (path straightness) was initially very low for saturation-acclimated *P. dentatus* at $7.0 \text{ mg O}_2 \text{ l}^{-1}$ ($r = 0.13$) and for control fish (observed in saturated DO conditions) throughout the test period ($r = 0.18$). Swimming of saturation-acclimated *P. dentatus* became six-fold more directed as DO decreased from 7.0 to $0.8 \text{ mg O}_2 \text{ l}^{-1}$. In addition to the marked increase in angular correlation, the c.v. was 26 times lower at

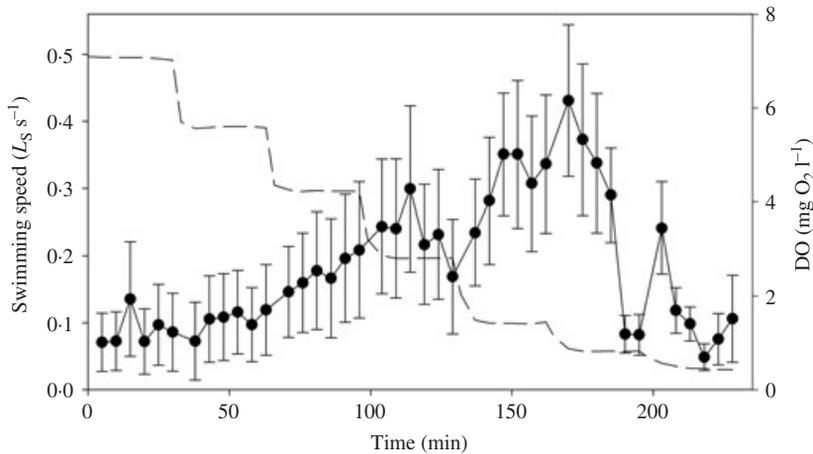


FIG. 2. Mean \pm s.e. swimming speed in body lengths (standard lengths, L_S) s^{-1} every 5 min of saturation-acclimated *Paralichthys dentatus* during the dissolved oxygen (DO) treatment period. Points show the mean response over the previous 5 min period. ---, DO exposure for saturation-acclimated fish.

0.8 mg $O_2 l^{-1}$ than at 7.0 mg $O_2 l^{-1}$ [Fig. 1(b)]. Saturation-acclimated *P. dentatus* appeared to reach the upper limit of their ability to adjust angular correlation. Concomitant increases in swimming speed and angular correlation led to a greater than four-fold increase in expected displacement at 1.4 mg $O_2 l^{-1}$ compared with expected displacement at 7.0 mg $O_2 l^{-1}$ (from 2 to 7 m) [Fig. 1(c)]. Even at 0.8 mg $O_2 l^{-1}$, expected displacement was 3.5 times greater than that of control *P. dentatus* at the same time in the test period.

Mean diel-cycling hypoxia-acclimated *P. dentatus* L_S was 3.3 cm longer than mean saturation-acclimated *P. dentatus* L_S . The average L_S and wet mass of four fish from each group overlapped and their swimming behaviour was compared to ensure that size differences did not affect the results of the overall comparison. The results of this analysis showed no difference in patterns from the analysis using all individuals, and therefore did not alter the conclusions. Diel-cycling hypoxia-acclimated *P. dentatus* displayed an active response to declining DO. Their response, however, was more modest and shorter lived than in saturation-acclimated fish. Swimming speed of diel-cycling hypoxia-acclimated *P. dentatus* increased only 1.8 fold compared with 3.5 fold for saturation-acclimated *P. dentatus* during DO decline [Fig. 1(a)]. Moreover, diel-cycling hypoxia-acclimated fish reached maximum swimming speed at 2.8 mg $O_2 l^{-1}$ instead of 1.4 mg $O_2 l^{-1}$; and during further DO decline, speed of diel-cycling hypoxia-acclimated fish returned to initial levels long before it did in saturation-acclimated fish. Angular correlation of diel-cycling hypoxia-acclimated *P. dentatus* showed little change over time [Fig. 1(b)], although it was higher than saturation-acclimated fish initially and remained so throughout most of the trial. Since both swimming speed and angular correlation were higher in diel-cycling hypoxia-acclimated fish during the beginning of the trial, expected displacement was also initially higher than for saturation-acclimated fish, but declined rapidly below 2.8 mg $O_2 l^{-1}$ [Fig. 1(c)].

Swimming speed of saturation and diel-cycling hypoxia-acclimated *P. dentatus* during the DO recovery phase both followed a quadratic pattern (repeated measures ANOVA, $n = 10$; $F = 7.1$, $P < 0.05$ and $F = 6.0$, $P < 0.05$, respectively). The difference between control *P. dentatus* behavioural variables and those of the exposed *P. dentatus* was used to assess recovery [Fig. 1(a)–(c)]. Both groups continued to decrease swimming speed during initial DO increase relative to control *P. dentatus*. In fact, only when DO ascended to $4.2 \text{ mg O}_2 \text{ l}^{-1}$ did diel-cycling hypoxia-acclimated fish reach the swimming speed of control *P. dentatus* (observed in saturated DO conditions) and saturation-acclimated *P. dentatus* increased their swimming speed beyond control levels [Fig. 1(a)].

Although diel-cycling hypoxia-acclimated *P. dentatus* did not change swimming speed substantially throughout the recovery period, they did change angular correlation. Diel-cycling hypoxia-acclimated fish made very few movements throughout the recovery period; however, when they did move, their movements were extremely uncorrelated as shown by the 180% decrease in angular correlation from 0.4 to $2.8 \text{ mg O}_2 \text{ l}^{-1}$ [Fig. 1(b)]. Although diel-cycling hypoxia-acclimated *P. dentatus* movement was random in relation to control *P. dentatus* following exposure to low DO, saturation-acclimated *P. dentatus* only gradually reduced angular correlation during DO recovery [Fig. 1(b)] from very directed to the average angular correlation of control *P. dentatus* during the same time period. Finally, the expected displacement of diel-cycling hypoxia-acclimated *P. dentatus* matched that of controls while saturation-acclimated *P. dentatus* steadily increased expected displacement as DO increased [Fig. 1(c)].

SURVIVORS v. NON-SURVIVORS

Twenty-three per cent of saturation-acclimated *P. dentatus* died during treatment, whereas no control or diel-cycling hypoxia-acclimated *P. dentatus* died in any experiment. All mortalities occurred at the end of exposure to $0.4 \text{ mg O}_2 \text{ l}^{-1}$, so those individuals did not contribute to analyses of the DO recovery phase. In addition to differences in survival, survivors and non-survivors also differed in their behavioural response to declining DO. Although survivors displayed the characteristic quadratic response to declining DO (repeated measures ANOVA, $n = 10$; $F = 4.41$, $P < 0.05$), non-survivors were generally inactive throughout the beginning of the trial, until DO decreased to $2.8 \text{ mg O}_2 \text{ l}^{-1}$ [Fig. 3(a)]. Indeed, even when they were most active, non-survivors were never faster than the initial swimming speed of surviving *P. dentatus* at $7.0 \text{ mg O}_2 \text{ l}^{-1}$ [Fig. 3(a)]. Although non-survivors were less active than survivors, their rate of increase in angular correlation (path straightness) with declining DO was very similar [Fig. 3(b)]. Ultimately, however, the effect of such low swimming speeds overwhelmed increasing angular correlation to result in a relatively modest increase in expected displacement (to *c.* 1 m) from 7.0 to $0.4 \text{ mg O}_2 \text{ l}^{-1}$ [Fig. 3(c)].

INTERINDIVIDUAL VARIABILITY

Changes in interindividual variability in swimming speed indicate that resumption of normal activity during DO recovery occurred slowly, if at all, for diel-cycling hypoxia-acclimated *P. dentatus*. Control *P. dentatus* (observed in saturated DO conditions) maintained significant rank order in swimming speed throughout the entire

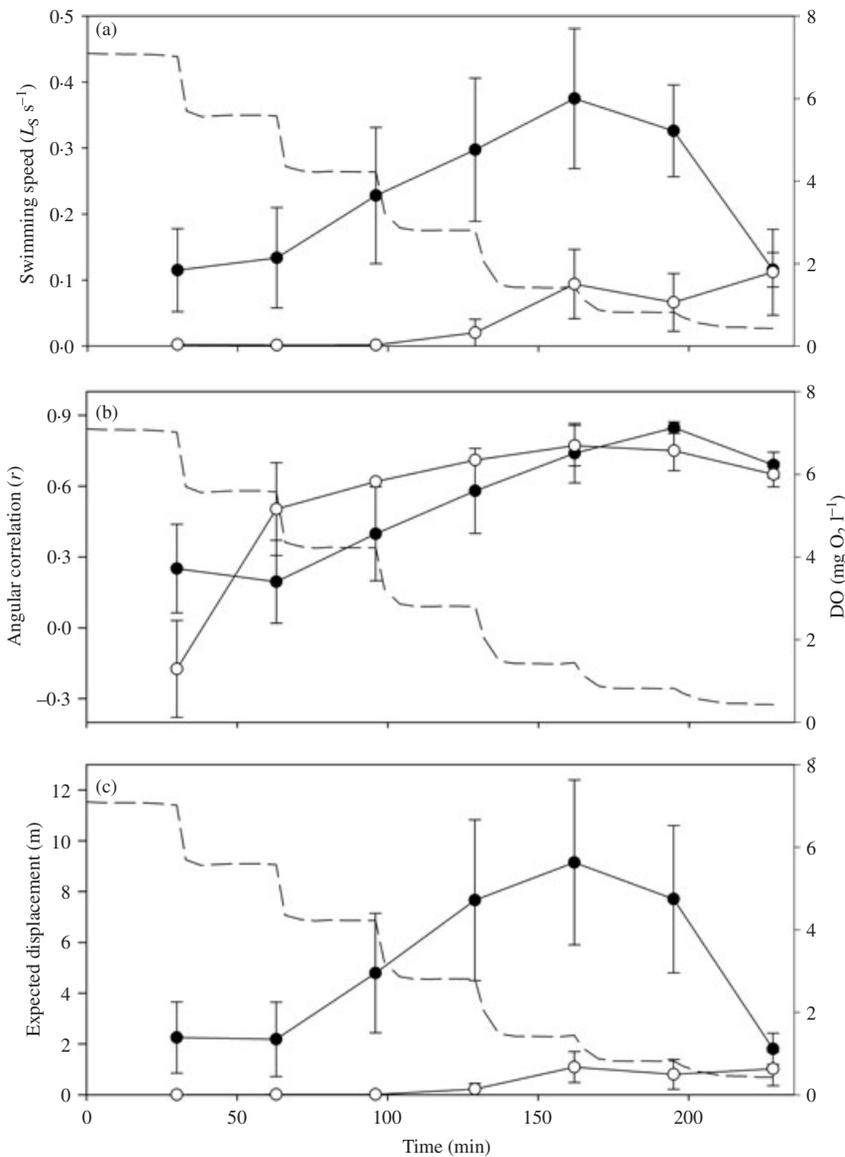


FIG. 3. Mean \pm s.e. (a) swimming speed in body lengths (standard lengths, L_S) s^{-1} , (b) angular correlation and (c) expected displacement (for each 30 min period) of surviving (●) and non-surviving (○) saturation-acclimated *Paralichthys dentatus* during the dissolved oxygen (DO) treatment period. Points show the mean response over the previous 30 min period. Non-surviving individuals died at the end of exposure to $0.4 \text{ mg O}_2 \text{ l}^{-1}$. ---, DO exposure.

test period (Friedman's χ^2 test; $n = 13$; $W_{\text{treat}} = 0.48$, $W_{\text{rec}} = 0.65$, $P < 0.001$) [Fig. 4(a), (b)]. In contrast, while both saturation [Fig. 4(c), (d)] and diel-cycling hypoxia-acclimated [Fig. 4(e), (f)] *P. dentatus* maintained significant rank order when DO was decreasing (Friedman's χ^2 test; $n = 7$; $W_{\text{treat}} = 0.54$, $W_{\text{treat}} = 0.54$,

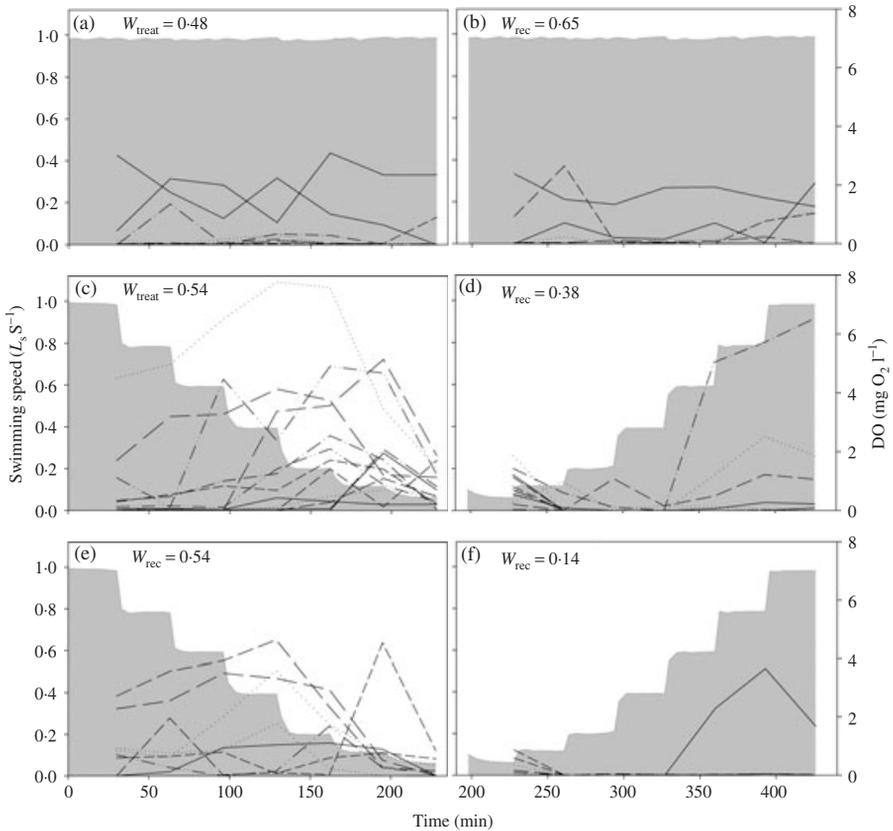


FIG. 4. Swimming speed in body lengths (standard lengths, L_S) s^{-1} of individual juvenile *Paralichthys dentatus* during the dissolved oxygen (DO) treatment and recovery periods. (a), (b) swimming speeds of individual control fish (observed in saturated DO conditions) during the first and second half of the test period, respectively. (c), (d) swimming speeds of individual saturation-acclimated fish during the DO treatment and recovery periods, respectively. (e), (f) swimming speeds of individual diel-cycling hypoxia-acclimated fish during the DO treatment and recovery periods, respectively. Kendall's coefficient of concordance (W_{treat} for the treatment period and W_{rec} for the recovery period) is included. The top of the grey areas indicate DO exposure level.

$P < 0.001$), both groups were much less concordant during the DO recovery period. In fact, although saturation-acclimated *P. dentatus* were only 58% as concordant (Friedman's χ^2 test; $n = 7$; $W_{\text{rec}} = 0.38$, $P < 0.01$) as control fish over the same time period, diel-cycling hypoxia-acclimated fish were even less concordant (only 21% of controls, Friedman's χ^2 test; $n = 7$; $W_{\text{rec}} = 0.14$, $P > 0.05$).

DISCUSSION

Saturation-acclimated *P. dentatus* showed an active response to decreasing DO. It is often assumed that flatfishes are generally inactive and that at least an initially passive response to declining DO would be expected because they are negatively buoyant

and incur an energetic cost just to lift off of the bottom (Duthie, 1982; Joaquim *et al.*, 2004). For instance, Dalla Via *et al.* (1998) found that *S. solea* decreased routine swimming activity as DO decreased. In the Dalla Via *et al.* (1998) study, DO decreased from 100% to *c.* 5% air saturation over the course of 2 h and then to 0% air saturation over the next 2 h. During the initial period of DO decline, the percentage of individual *S. solea* that exhibited panic behaviour actually increased, before all the individuals became paralysed at *c.* 0% air saturation. So, although it is assumed that flatfishes are generally passive responders to hypoxia, the initial panicked response reported for *S. solea* may be the more ecologically relevant response. The present study supports previous field studies (Szedlmayer & Able, 1993; Sackett *et al.*, 2007) that concluded *P. dentatus* are more active participants in their decisions regarding abiotic variation than previously thought. For example, Sackett *et al.* (2007) observed that tagged *P. dentatus* left the Mullica Estuary, New Jersey, during low DO events.

A comparison of the swimming responses of two juvenile estuary-dependent fishes (juvenile *C. regalis* in Brady *et al.*, 2009 and juvenile *P. dentatus* in the present study) that occupy similar habitats and were exposed to the same DO protocol in the laboratory reveals that many aspects of their behavioural response are actually similar. Of course, control *C. regalis* swimming speeds were an order of magnitude higher than control *P. dentatus* swimming speeds (Brady *et al.*, 2009). The shape of their swimming speed response curves was comparable, however, with two key differences: intensity and temporal scale of the reaction. *Cynoscion regalis* increased swimming speed by 42% with declining DO (Brady *et al.*, 2009), compared with an increase of 248% in *P. dentatus*. In addition, although *C. regalis* began decreasing swimming speed below 2.8 mg O₂ l⁻¹ (Brady *et al.*, 2009), *P. dentatus* did not decrease swimming speed until below 1.4 mg O₂ l⁻¹. The heightened intensity and duration of the *P. dentatus* response still resulted in expected displacement for *P. dentatus* being only 20% of that for *C. regalis* during their respective active responses (Brady *et al.*, 2009). *Paralichthys dentatus* and flatfishes generally start at such low swimming speeds that they have more latitude to increase swimming speed in response to poor environmental conditions. Additionally, since *P. dentatus* swim intermittently and with less directed movement than *C. regalis*, as is evident in the lower angular correlation of control *P. dentatus*, they also have more latitude to straighten out their swimming paths when in an escape mode. Ultimately, the scale of juvenile *P. dentatus* movement observed in the current study is much too low to allow them to make daily temporary emigrations from habitats affected by diel-cycling hypoxia, as has been reported for juvenile *C. regalis* (Tyler & Targett, 2007). Tyler & Targett (2007) found that juvenile *C. regalis* move *c.* 1.0 to 1.5 km down an estuarine tributary from the head of tide whenever DO dropped below *c.* 2.0 mg O₂ l⁻¹. *Paralichthys dentatus* moving in the same environment would routinely be overtaken by the hypoxic front moving from the head of tide down estuary unless assisted by tidal flow.

Since 23% of saturation-acclimated *P. dentatus* died during hypoxia treatment, it is tempting to conclude that the active response is risky. Indeed, Van Raaij *et al.* (1996) found that rainbow trout *Oncorhynchus mykiss* (Walbaum) surviving low DO treatment were those individuals that decreased swimming speed (passive response) in response to low DO. Surviving saturation-acclimated *P. dentatus*, however, were

actually more active than non-survivors. The relationship between stress and hypoxia-induced mortality may also be associated with timing. Non-surviving *P. dentatus* displayed a delayed active response, wherein they increased swimming speed only when DO decreased from 2.8 to 1.4 mg O₂ l⁻¹. Matching highly stressful conditions (DO <1.4 mg O₂ l⁻¹) with the height of the stress response (active response) may have significantly increased *P. dentatus* susceptibility to hypoxia-induced mortality. The survival differential may also reflect intraspecific hypoxia tolerance (genetic or larval exposure; Faust *et al.*, 2004) or inequalities in energetic partitioning (Billerbeck *et al.*, 2001).

Paralichthys dentatus acclimated to diel-cycling hypoxia exhibited a very different behavioural response than did *C. regalis* acclimated to the same conditions (Brady *et al.*, 2009). Diel-cycling hypoxia-acclimated *C. regalis* displayed no significant changes in swimming speed during DO treatment and had 46% lower swimming speed compared with control *C. regalis* (observed in saturated DO conditions; Brady *et al.*, 2009). In contrast, diel-cycling hypoxia-acclimated *P. dentatus* maintained an active response and showed no overall decrease in swimming speed compared with control *P. dentatus* (observed in saturated DO conditions) as DO decreased. An overall decrease in routine swimming speed may represent an important mechanism of diel-cycling hypoxia acclimation for *C. regalis* (Brady *et al.*, 2009). Many studies have demonstrated that reduction in locomotion is a common way for fishes to acclimate to low DO (Gracey *et al.*, 2001; Widmer *et al.*, 2006). The reason *P. dentatus* did not lower overall swimming speed compared with control *P. dentatus* and retained an active response to decreasing DO may be linked to the lower mobility of *P. dentatus*. *Cynoscion regalis* can save a significant amount of energy by reducing movement while *P. dentatus* do not have the same latitude. Duthie (1982) determined that the aerobic scopes of flatfishes are relatively small, <20% of the aerobic scope of sockeye salmon *Oncorhynchus nerka* (Walbaum) (Brett & Groves, 1979). Reducing activity represents less energy savings to flatfishes than roundfishes and may reduce the effectiveness or utility of hypometabolism as an acclimation mechanism. Recent field evaluations of *P. dentatus* movement in relation to hypoxia also support this conclusion. Tyler (2005) found that *P. dentatus* vacated tributary habitats before diel-cycling DO became severely hypoxic, whereas *C. regalis* made temporary emigrations away from hypoxia only to return later when DO levels rebounded. The retention of an active response in diel-cycling hypoxia-acclimated *P. dentatus* is not without consequence. Diel-cycling hypoxia-acclimated *P. dentatus* swam more randomly and were less active than saturation-acclimated fish during the DO recovery phase.

Behavioural plasticity gives populations more options for coping with capricious environments (Lomnicki, 1978; Wingfield, 2003). For the purposes of this study, interindividual variability was examined by calculating the maintenance of rank order in swimming speed. The level of interindividual variability in swimming speed of diel-cycling hypoxia-acclimated *P. dentatus* during the DO recovery phase was the lowest measured in any combination of acclimation or exposure. By maintaining an active response, diel-cycling hypoxia-acclimated *P. dentatus* appear to forgo the ability to recover in favour of short-term escape. Saturation-acclimated *P. dentatus* also experienced a reduction in maintenance of rank order, as hypoxia appears to force all the individuals to adopt a strategy of inactivity even as DO increases. This loss of interindividual variability may represent another indirect mechanism by which

hypoxia is detrimental to fishes. Morales & Ellner (2002) emphasize that determining the complexities of individual behaviour is the main challenge to scaling up movement patterns in fluctuating environments. For example, a reduction in the swimming speed of particularly active individuals in a population may exclude *P. dentatus* from taking advantage of favourable habitat farther afield.

The active swimming response in both saturation-acclimated and diel-cycling hypoxia-acclimated juvenile *P. dentatus* generally began when DO dropped from 5.6 to 4.2 mg O₂ l⁻¹, which is long before DO is lethal (LC90 = 1.8 mg O₂—Miller *et al.*, 2002) or even growth limiting at 20° C (3.5 mg O₂ l⁻¹, Stierhoff *et al.*, 2006). Claireaux *et al.* (1995) simultaneously characterized *G. morhua* physiological and behavioural responses to low DO. The results of the physiological experiments suggested that *G. morhua* could maintain oxygen consumption down to 9 kPa PO₂ (Claireaux *et al.*, 1995). Behavioural experiments demonstrated that *G. morhua* avoided oxygen conditions that may not necessarily be stressful based on physiological evidence (Claireaux *et al.*, 1995). Pre-emptive reactions in fishes may allow them to navigate habitats experiencing diel-cycling hypoxia, as well as systems that experience episodic wind-driven hypoxia events (Bell & Eggleston, 2005). Generally, open bay (Tyler, 2005) or ocean (Sackett *et al.*, 2007) sites and shallow water (Bell & Eggleston, 2005), with less volatile DO conditions, have been shown to offer juvenile *P. dentatus* some respite from hypoxic events in estuaries. So, although DO varies enough to limit the growth of juvenile *P. dentatus* in areas such as the Delaware coastal bays (Stierhoff *et al.*, 2009), process-level behavioural data are required to make the link between water quality and nursery habitat function. The results of the current study on the behavioural responses of *P. dentatus* to DO decline and recovery, even in individuals acclimated to hypoxia for 10 days, showed that *P. dentatus* exhibit a strong and pre-emptive avoidance response. Ultimately, the spatial extent and temporal duration of hypoxia are the primary determinants of the success of this behavioural strategy in avoiding direct effects on growth and mortality. In addition, the form of low DO exposure (in this case diel-cycling hypoxia) plays a large role in determining the behavioural strategy exhibited by fishes in general and laboratory experiments should employ DO conditions that accurately reflect those in the field to yield ecologically relevant results.

The authors greatly appreciate K. Stierhoff and R. Tyler for their perspective and comments during the course of this study. The authors also thank two reviewers for improving this paper. This research was supported by funding from the Delaware Sea Grant Program, NOAA, U.S. Department of Commerce, under grant no. NA03OAR4170011 (project R/F-23) to T.E.T.

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