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RESEARCH ARTICLE

Hypoxia tolerance of giant axon-mediated escape jetting in California market squid (*Doryteuthis opalescens*)

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ABSTRACT

Squids display a wide range of swimming behaviors, including powerful escape jets mediated by the giant axon system. For California market squid (*Doryteuthis opalescens*), maintaining essential behaviors like the escape response during environmental variations poses a major challenge as this species often encounters intrusions of cold, hypoxic offshore waters in its coastal spawning habitats. To explore the effects of hypoxia on locomotion and the underlying neural mechanisms, we made *in vivo* recordings of giant axon activity and simultaneous pressure inside the mantle cavity during escape jets in squid exposed to acute progressive hypoxia followed by return to normal dissolved oxygen (DO) concentration (normoxia). Compared with those in normoxia (>8 mg l^{-1} DO), escape jets were unchanged in moderate hypoxia (4 and 2 mg l^{-1} DO), but giant axon activity and associated mantle contractions significantly decreased while neuromuscular latency increased under severe hypoxia (0.5 mg l^{-1} DO). Animals that survived exposure to severe hypoxia reliably produced escape jets under such conditions and fully recovered as more oxygen became available. The reduction in neuromuscular output under hypoxia suggests that market squid may suppress metabolic activity to maintain sufficient behavioral output, a common strategy in many hypoxia-tolerant species. The ability to recover from the deleterious effects of hypoxia suggests that this species is well adapted to cope with coastal hypoxic events that commonly occur in Monterey Bay, unless these events become more severe in the future as climate change progresses.

KEY WORDS: Hypoxia, Squid, Escape response, Giant axon, Recovery

INTRODUCTION

A growing prevalence of upwelling-related hypoxia in coastal waters (Chan et al., 2008), paired with the shoaling and intensification of oxygen minimum zones (Stramma et al., 2010), presents a major challenge to many marine animals, particularly in the northeast Pacific Ocean, an area with the largest oxygen minimum zone (OMZ) on the planet (Gilly et al., 2013). In extreme cases, severe hypoxic events have led to mass mortalities of fish and invertebrates (Chan et al., 2008), habitat reduction (Stramma et al., 2011) and loss of biodiversity (Rabalais et al., 2002). However, variability of dissolved oxygen (DO) levels in less extreme scenarios can lead to more nuanced effects, influencing the behavior and physiology of individuals. Research on these effects has historically focused on benthic invertebrates and benthic fishes because of their distance from atmospheric oxygen and interaction with oxygen-poor sediments (Vaquer-Sunyer and Duarte, 2008). However, increasing occurrence of upwelling-driven hypoxia in the water column can also impact pelagic species, but the consequences of this remain largely unknown.

The California market squid (*Doryteuthis opalescens*) is a highly active pelagic squid that routinely migrates between normoxic conditions (>8 mg l^{-1} DO) and hypoxic waters (0.5–2 mg l^{-1} DO) that overlie the OMZ near Monterey Bay, CA, USA (Stewart et al., 2014). Oxygen availability is critical to locomotor performance in squid, and previous work on oxygen requirements in squid jetting has mainly focused on sustained swimming (O'Dor, 1982; Webber and O'Dor, 1985; Webber and O'Dor, 1986) or the role of DO in isolated nerve–synapse preparations (Bryant, 1958). However, the influence of environmentally relevant changes in DO on fast burst swimming *in vivo* has received little attention.

In squid, fast bursts of swimming are critical for avoiding predation or other undesirable encounters, and a primary example is the jet-propelled escape response, a powerful coordinated contraction of circular muscle fibers of the mantle. Controlling the escape jet are two parallel nerve pathways, the giant and non-giant axon systems, which can act separately or in concert to drive high-speed jetting (Otis and Gilly, 1990). Patterns of neuromuscular activity and the resulting jets are temperature dependent, and increased neural activity at low temperature in the non-giant axon pathway can augment escape responses by enhancing the magnitude of a mantle contraction (Neumeister et al., 2000). In contrast, the effects of hypoxia on escape jetting and the underlying neural mechanisms are unknown.

Previous research has examined the impacts of environmental hypoxia on the behavior and physiology of fishes, including their escape behaviors (reviewed in Domenici et al., 2013), but few studies have assessed an individual's ability to recover once oxygen becomes available again. The hypoxia tolerance of an organism includes both survival of hypoxia and recovery afterwards. The ability to recover from exposure to an environmental stressor, such as the lack of oxygen, could help shape the abundance and distribution of species, especially if it alters the success of crucial behaviors like escaping, foraging and reproducing.

In this study, we evaluated the hypoxia sensitivity and tolerance of a critical locomotor behavior in market squid, the jet-propelled escape response, and its underlying neural mechanisms. We found escape jets and associated neural mechanisms to be consistently insensitive to acute moderate hypoxia (4 and 2 mg l^{-1} DO) but hindered under severe hypoxia (0.5 mg l^{-1} DO). Individuals that survived exposure to severe hypoxia were able to fully recover as seawater was reoxygenated, and after return to normoxia (8 mg l^{-1} DO) their escape jets were indistinguishable from those before hypoxic stress.

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MATERIALS AND METHODS

Animals

Adult market squid, *D. opalescens* (Berry 1911), were collected by jigging on inshore spawning grounds in Monterey Bay, CA, USA, in June and July 2017 and June 2018 and transported to the laboratory at Stanford University's Hopkins Marine Station (Pacific Grove, CA, USA) in aerated seawater. Animals were maintained in a cylindrical holding tank (2.5 m diameter, 1 m height) for up to 2 weeks. The holding tank was plumbed with flow-through filtered seawater at ambient temperature and DO concentration (13–14°C; >8 mg l^{-1} DO) that was supplied by the Monterey Bay Aquarium (Monterey, CA, USA). Animals were fed twice a day with live goldfish or fathead minnows, as suggested by Hanlon (1990), and all specimens were kept in compliance with Stanford University institutional animal care guidelines. A total of 15 individuals were used in hypoxia experiments in 2017. Because a significant effect of hypoxia was found in the experimental group from 2017, 8 individuals were then used for control experiments in 2018. Although jigged squid were caught largely unharmed, any individuals exhibiting signs of senescence or injury were not used.

Experiments with restrained squid

Pressure inside the mantle cavity and *en passant* extracellular stellar nerve activity were recorded in live restrained animals as previously described (Neumeister et al., 2000; Otis and Gilly, 1990). Animals were anesthetized in 1.8% $MgCl_2$ in seawater at ambient temperature and DO until they were unresponsive to physical touch, and a small plug (5–10 mm in length and width) of mantle tissue over the right stellate ganglion was then surgically removed to reveal the hindmost or second-hindmost stellar nerve. Anesthetized animals were allowed to recover in aerated seawater for 20–25 min, after which they displayed normal jetting behavior.

Animals were restrained by affixing the midline of the dorsal mantle surface to an acrylic platform with cyanoacrylate cement. The platform was suspended in an insulated tank (58.4×33.0×31.8 cm) filled with seawater maintained between 7.7 and 8.3°C using an immersion chiller. DO levels were manipulated by bubbling either N_2 gas or air through a submerged micro-bubble diffuser (Pentair Aquatic Eco-Systems, Minneapolis, MN, USA). A small pump inside the tank circulated water to ensure temperature, DO and pH uniformity. Temperature, DO and pH were recorded at 30 s intervals during the experiment by a Hach HQ40d Multi unit with an LDO101 LED optode and PHC101 pH probe (Hach Company, Loveland, CO, USA). The pH ranged from 7.93 to 8.00 during the experiments.

Escape jets were elicited by a TT560 Speedlite camera flash (Neewer Technology Company, Shenzhen, China) administered in groups of five with 1 min between each flash. The flash triggered acquisition of signals for the pressure inside the mantle cavity and simultaneous stellar nerve activity via an analog-to-digital converter and interface (NI USB-6341, National Instruments, Austin, TX, USA) using commercially available software (LabVIEW SignalExpress, National Instruments). Signals were recorded at 10 kHz for 10.5 s starting 0.5 s prior to the stimulus. Video recordings (29.97 frames s^{-1}, Canon Powershot G9X, Canon Incorporated, Tokyo, Japan) of the response to the flash stimulus were made to document any notable behaviors not represented by pressure and neural recordings.

Pressure inside the mantle cavity was recorded using a pressure transducer (40PC006G, Honeywell, Morris Plains, NJ, USA) coupled to a syringe needle (20 gauge) filled with mineral oil and inserted through the mid-posterior region of the dorsal mantle. The voltage output of the pressure transducer was calibrated using a PLI-100 pressure injector (Medical Systems Corporation, Harvard Apparatus, Holliston, MA, USA).

Conventional extracellular recordings of nerve activity were made with a polypropylene suction electrode (Ag:AgCl wire) attached to a stellar nerve within 5 mm of its emergence from the stellate ganglion. Neural signals were sent through an AC-coupled amplifier (DP-301, Warner Instrument Corp., Hamden, CT, USA) that applied low-pass (3 kHz) and high-pass (0.1 Hz) filtering and an amplification of 1000 before acquisition by the analog-to-digital converter.

Experimental and control conditions

Test animals (N=15) were allowed to acclimate in the experimental tank for 30 min (8°C, 8 mg l^{-1} DO) and were then exposed to progressive decreases in DO to moderate hypoxia (4 and 2 mg l^{-1} DO) and then to severe hypoxia (0.5 mg l^{-1} DO) by bubbling N_2 into the experimental tank. Seawater in the tank was then incrementally reoxygenated to test recovery. Individuals spent 5 min at each new DO level before flash stimuli were initiated. A set of five flashes was given at normoxia and the two moderate hypoxia levels, and four sets of five flashes were given during severe hypoxia with 10 min between each set (Fig. 1).

Individuals spent up to 1 h under severe hypoxia. This duration was determined in separate experiments that identified the time for 50% of a group of N=8 restrained squid to stop respiring at 0.5 mg l^{-1} DO, as evidenced by lack of mantle movement. During the escape-response experiments with individual animals, if a squid died prior to 1 h of exposure to severe hypoxia, the experiment was terminated. If an individual stopped producing escape jets but was still respiring before 1 h of severe hypoxia, the water was immediately reoxygenated to 2 mg l^{-1}, and the rest of the recovery protocol was conducted.

Animals in control experiments (N=8) were exposed to an identical pattern of stimulation to that used for hypoxia experiments, but the seawater was aerated by a bubbler during the entire experiment to maintain DO at 8 mg l^{-1} (Fig. 1).

Data analysis

In-house MATLAB scripts synchronized data exported from LabVIEW SignalExpress with corresponding temperature, DO and pH recordings from the Hach probe based on time stamps. Pressure recordings were processed using a moving average of 501 points in MATLAB 2014b (The MathWorks Inc., Natick, MA, USA) as this method preserved the shape of the curve most closely. Neural signals were not smoothed for analysis.

Four parameters characterizing the neural and muscular components of an escape jet's magnitude and timing were used to quantify the response (Fig. 2). Escape jet magnitude was evaluated by calculating the peak amplitude of the pressure inside the mantle cavity and counting the number of giant axon spikes (1–3 spikes). The timing of the jet was assessed by the neural latency from the stimulus to the first giant axon spike and by the muscular latency from the first giant axon spike to the onset of the pressure rise. In each individual, the mean value of a given parameter was calculated for each set of five flash stimuli to yield a mean value at a specific DO concentration.

Two linear mixed-effects models accounting for repeated measures and unequal sample sizes evaluated the effect of hypoxia on escape jets compared with the response of control individuals. One model compared control individuals with all individuals exposed to progressive hypoxia at the first four DO concentrations before any individuals either died or stopped responding. The other

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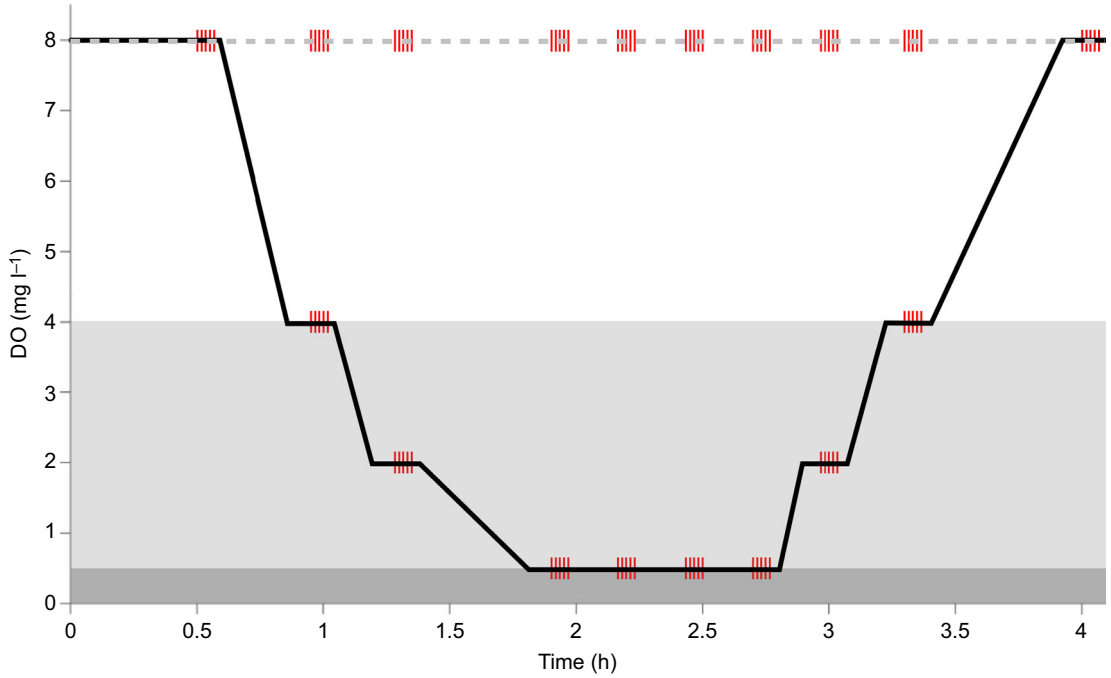

Fig. 1. Dissolved oxygen (DO) conditions for California market squid exposed to hypoxia or control experiments. Black line: hypoxia and reoxygenation (*N*=15); gray dashed line: normoxia control (*N*=8). Light shading indicates moderate hypoxia and dark shading represents severe hypoxia. All trials were conducted at 8°C. Flashes were administered in groups of 5 (red ticks) with a minute between each flash.

model assessed recovery by comparing control individuals with only those that continued responding throughout the entire hypoxia experiment. In both models, the predictors were DO category and experiment type, and individual was the random effect. Satterthwaite's method minimized Type 1 error in calculating *t*-values (Luke, 2017), and *P*-values (significance threshold of $P<0.05$) were computed from *t*-values output from the linear mixed-effects model.

RESULTS

Strobe flash stimuli elicited escape jets initiated by one to three giant axon spikes (Fig. 2), similar to results reported in previous studies (Neumeister et al., 2000; Otis and Gilly, 1990). The square root of pressure inside the mantle cavity is proportional to the speed of seawater exiting through the siphon during a jet (O'Dor, 1988), and the amplitude of the pressure transient is thus related to how quickly the squid can escape a threat. Larger pressure values are known to be associated with enhanced neural activity in both the giant (Otis and Gilly, 1990) and non-giant (Neumeister et al., 2000) axon systems.

Inter-individual variation in response to severe hypoxia

Individuals displayed one of three outcomes after exposure to severe hypoxia (0.5 mg l^{-1} DO) for up to 1 h (Table 1). Three individuals died during exposure, four individuals stopped producing escape jets before the end of the hour-long test period in severe hypoxia but resumed once the seawater was reoxygenated, and eight individuals produced escape jets during the entire exposure and recovery period. All individuals from these three groups showed a decrease in peak pressure and neural activity and an increase in neural and muscle latencies under severe hypoxia as described below.

Escape responses are hindered by severe hypoxia

Overall, moderate hypoxia (4 and 2 mg l^{-1} DO) had little measureable impact on escape jets [linear mixed-effects model; d.f.=9 for animals that died during exposure to severe hypoxia (pink), d.f.=10 for animals that stopped responding in severe hypoxia but resumed escape jets once the seawater was reoxygenated (blue), d.f.=14 for animals that continued responding throughout the experiment (black); $P>0.07$; Fig. 3]. In contrast, severe hypoxia (0.5 mg l^{-1} DO) significantly impacted all aspects of escape jets that were assessed (linear mixed-effects model; d.f.=9 (pink), d.f.=10 (blue), d.f.=14 (black); $P<0.04$; Fig. 3). Peak pressure decreased at least 2-fold on average across all experimental groups compared with that of control individuals at 0.5 mg l^{-1} DO (Fig. 3A), with the rate of pressure increase following a similar trend (not shown). Jets initiated by one giant axon spike (rather than two) dominated under severe hypoxia (Figs 2C and 3C). Both muscle and neural latencies increased under severe hypoxia for all groups compared with control animals (Fig. 3B,D).

Escape jets worsen throughout severe hypoxia but recover in normoxia

In the animals that recovered from the full hour of severe hypoxia (0.5 mg l^{-1} DO), all parameters used to characterize escape jetting remained affected during exposure (linear mixed-effects model;

Table 1. Inter-individual variation in outcomes after exposure to severe hypoxia (0.5 mg l^{-1} DO)

N	Outcome	Time spent in 0.5 mg l^{-1} DO (min)
3	Died during the 1 h exposure to 0.5 mg l^{-1} DO	10, 29, 29
4	Stopped producing escape jets before the end of the 1 h period at 0.5 mg l^{-1} DO but resumed once DO increased	7, 11, 41, 45
8	Continued producing escape jets throughout the entire experimental period	60

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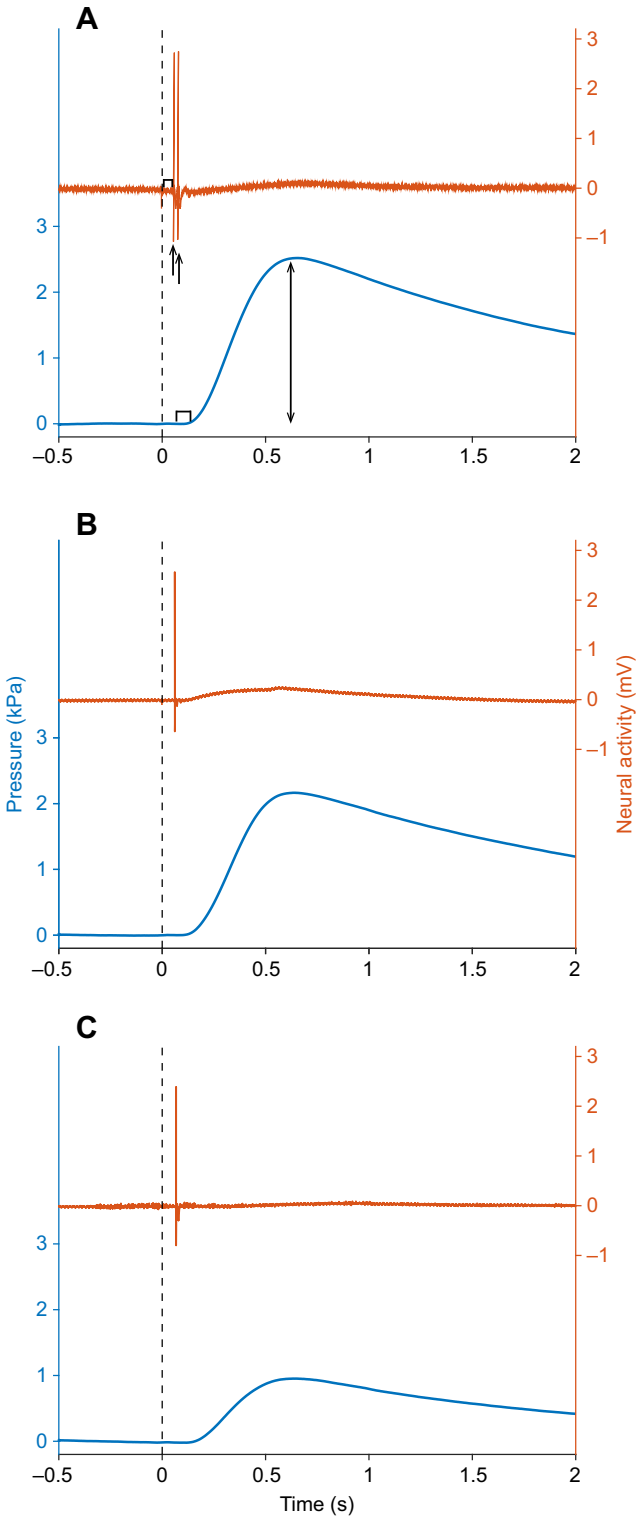

Fig. 2. Representative examples of pressure inside the mantle cavity (blue) and associated neural activity (orange) during escape jets under different DO conditions. Escape jets were produced in response to the flash stimulus (dashed line) at (A,B) 8 mg l^{-1} DO and (C) 0.5 mg l^{-1} DO in the same individual. The double-headed arrow in A shows how peak pressure was calculated relative to the pre-stimulus baseline. The baseline was computed as the mean of the values from 0.5 s before the stimulus until the stimulus at time 0, and was subtracted from pressure values. The single arrows indicate giant axon spikes in a stellar nerve. The square brackets show neural and muscular latencies. Neural latency is measured from the stimulus to the first giant axon spike, and muscular latency is measured from the first giant axon spike to the onset of the pressure rise.

d.f.=14; P<0.04; Fig. 4). Peak pressure incrementally decreased during severe hypoxia (Fig. 4A), and muscle latency increased (Fig. 4B). The number of giant axon spikes also decreased, but this difference was only significant for the third set of stimulations at 0.5 mg l^{-1} DO (linear mixed-effects model; d.f.=14; P=0.03; Fig. 4C). Neural latency remained significantly higher than control values under severe hypoxia, with a rapid initial increase followed by a plateau during the exposure rather than a gradual increase (Fig. 4D).

Once the seawater was reoxygenated, peak pressure gradually increased, and muscle latency gradually decreased, again incrementally (Fig. 4A,B). Values between experimental and control groups remained significantly different for the muscular component of the escape jet throughout the return to moderate hypoxia until normoxia was re-established at 8 mg l^{-1} DO (linear mixed-effects model; d.f.=14; P<0.001; Fig. 4A,B). Neural latency also decreased as more oxygen became available (Fig. 4D), but the change became non-significant at 4 mg l^{-1} DO, earlier than that for muscle latency. Although no significant difference was found between the giant axon activity of the two groups as DO increased from severe hypoxia, a qualitative increase in mean giant axon activity with DO was apparent (Fig. 4C).

DISCUSSION

We found that the impact of severe hypoxia (0.5 mg l^{-1} DO) on the magnitude and latency of the market squid's escape response was accompanied by corresponding changes in the underlying neural mechanisms. Market squid appear to exhibit considerable hypoxia tolerance, only showing adverse effects when DO was reduced to a lower level (0.5 mg l^{-1} DO) than the typical thresholds for hypoxia in marine organisms (2.8 mg l^{-1} DO: Diaz and Rosenberg, 1995; 2 mg l^{-1} DO: Vaquer-Sunyer and Duarte, 2008). Furthermore, 80% of the squid tested (12 of 15 individuals) survived exposure to severe hypoxia and about half of them withstood 0.5 mg l^{-1} DO for 1 h and were able to recover more or less completely as oxygen became available again. Escape jets produced when normoxia was re-established did not differ significantly from those recorded at the start of the experiment under normoxic conditions. The remaining 20% of experimental animals died during exposure to severe hypoxia.

Hypoxia tolerance through reduced neuromuscular activity

As hypoxia intensified, squid showed decreased pressure inside the mantle cavity and a reduced number of giant axon spikes as well as an increased latency to initiate a jet. The reduction in giant axon activity (number of spikes) and increase in neural latency match observations made in hypoxia-tolerant neurons of other animals, such as the painted turtle (*Chrysemys picta*), where neurons in the brain decreased firing rate and synaptic transmission through lowered membrane resistance at synaptic inputs, resulting in an energy saving until normoxia was re-established (Sick et al., 1993). Market squid may similarly suppress cell metabolism in neurons as a strategy for maintaining basic functionality in hypoxia until more oxygen becomes available. Hypoxia tolerance typically requires a combination of metabolic suppression to reduce ATP demand, strategies to deal with metabolite accumulation, and defense against deleterious free radicals as normoxia is re-established (Bickler and Buck, 2007). In contrast, hypoxia-sensitive neurons lack protective mechanisms to suppress activity and address metabolic end products, thus leading to damage and cell death (Hochachka and Lutz, 2001).

This potential energy-saving strategy must be viewed in the context of decreased performance in assessing the ecological impacts of hypoxia on escape responses in squid. A general

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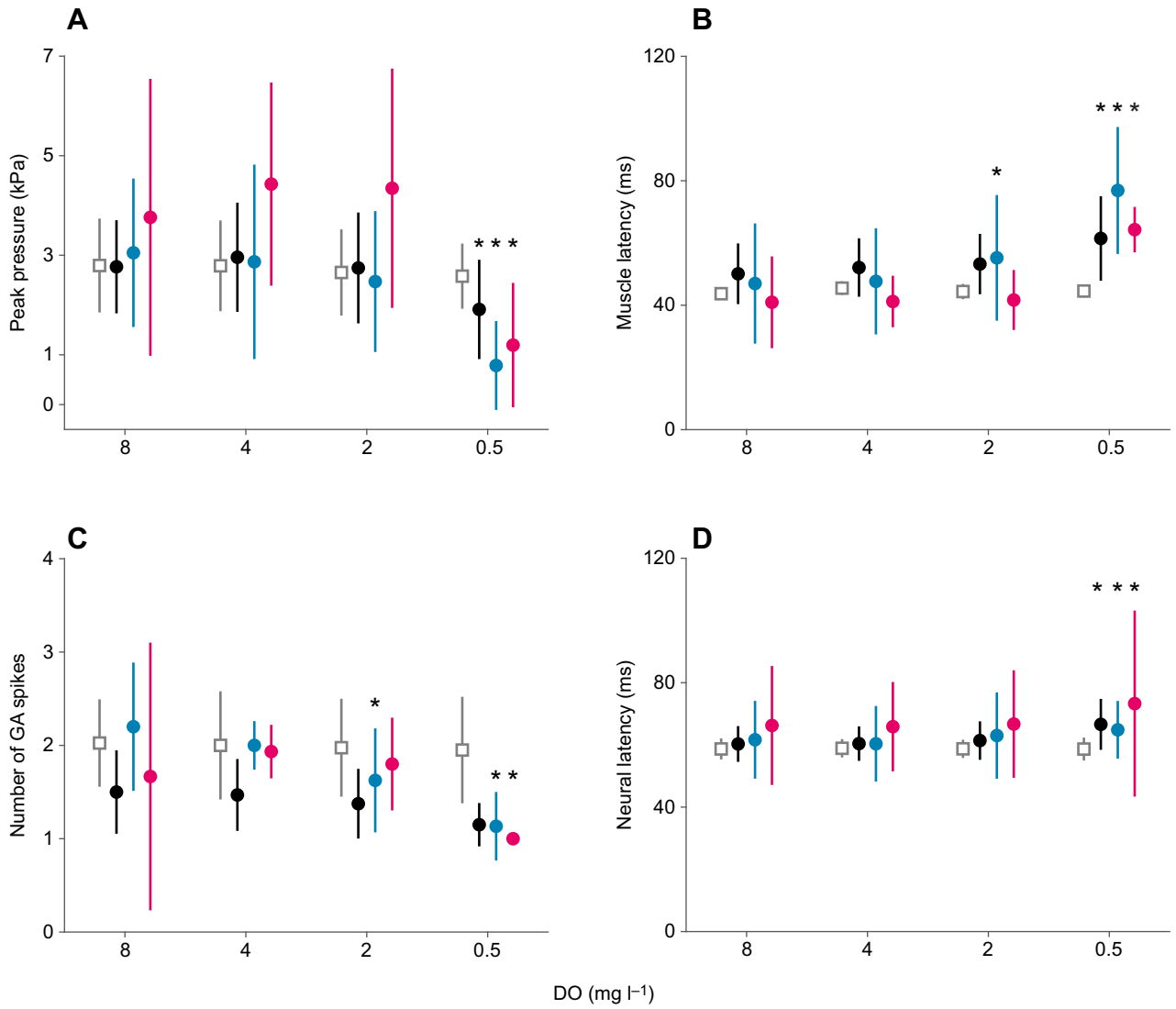

Fig. 3. Impacts of progressive hypoxia on escape jets. Escape jets characterized by (A) peak pressure, (B) muscle latency, (C) giant axon (GA) activity and (D) neural latency are not impacted overall by moderate hypoxia (4 and 2 mg l^{-1} DO) but are vulnerable to severe hypoxia (0.5 mg l^{-1} DO). Values are divided into experimental outcomes: control animals (open squares, *N*=8), animals that continued responding throughout the whole experiment (black circles, *N*=8), those that stopped responding in severe hypoxia but resumed escape jets once DO increased (blue circles, *N*=4), and those that died during exposure to severe hypoxia (pink circles, *N*=3). An asterisk indicates a significant change in the difference between the control and experimental group at that DO level compared with their difference at 8 mg l^{-1} DO (P<0.05 based on linear mixed-effects model). The significant result for the number of GA spikes at 2 mg l^{-1} DO is due to those differences going in opposite directions at 2 mg l^{-1} versus 8 mg l^{-1} DO. Means±95% confidence intervals are shown, and confidence intervals smaller than the size of the plotted point are not visible.

reduction in the number of giant axon spikes underlying the jet was associated with decreased muscular output under hypoxia, consistent with a general trend of this sort as previously reported (Otis and Gilly, 1990; Neumeister et al., 2000). In the present study, we found a decrease in pressure of about 25% to be associated with the difference between two giant axon spikes in control animals and one spike at 0.5 mg l^{-1} DO in squid that continued to respond throughout the entire experiment (Fig. 3A). Based on the analysis of O'Dor (1988), this decrease in pressure may result in a decrease of jet speed by about 13%, possibly increasing the chances of a failed escape jet. This reduction in pressure is similar to that reported in the studies cited above under normoxic conditions – about 30% at 15°C and 10% at 6°C, respectively (Otis and Gilly, 1990; Neumeister et al., 2000).

Although the change from two spikes to one can reasonably account for the decrease in pressure, muscular factors could also play a role in mantle pressure reduction. Circular muscles in the mantle that produce jets are composed of three distinct layers. The inner and outer layers, the superficial mitochondria-rich (SMR) layers, are aerobic and comprise about 25% of the mantle's mass. In contrast, the central mitochondria-poor (CMP) layer is much thicker and is characterized by high glycolytic activity and anaerobic capacity (Bone et al., 1995; Mommsen et al., 1981). Although the CMP layer has generally been assumed to be responsible for strong escape jetting (Gilly et al., 1996), both SMR and CMP layers appear to be active at slow and intermediate jetting speeds, and potentially at high speeds as well (Bartol, 2001). Neural inputs to aerobic SMR fibers have not been identified, but non-giant motor axons presumably innervate these fibers. This neural pathway is important in controlling escape responses to flash stimuli at low temperature (Neumeister et al., 2000), and impairment of muscular activity in aerobic SMR fibers might

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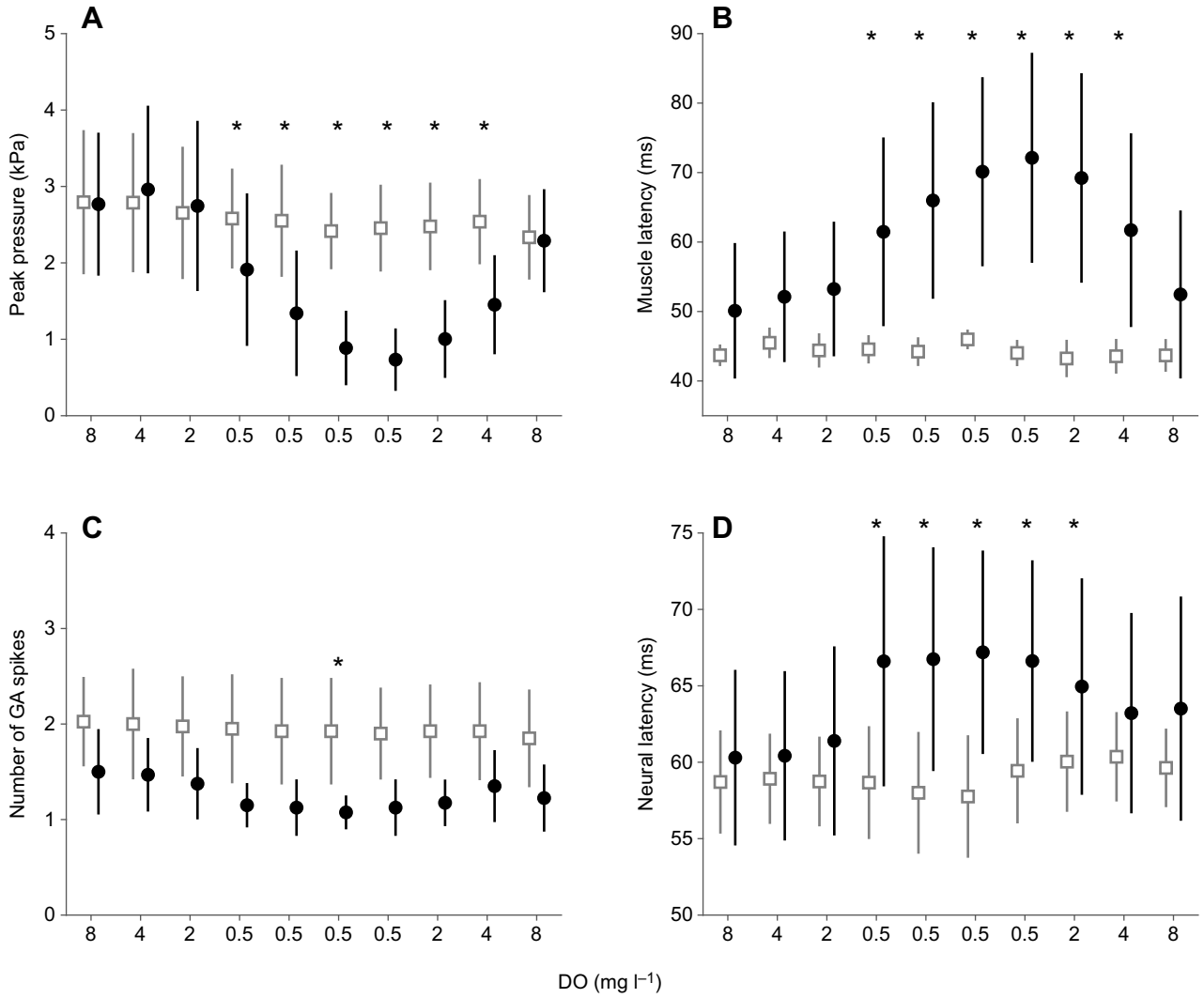

Fig. 4. Recovery of escape jets with reoxygenation. In animals that continued responding throughout the whole experimental period, escape jets, characterized by (A) peak pressure, (B) muscle latency, (C) giant axon activity and (D) neural latency, recover when oxygen is reintroduced following severe hypoxia (0.5 mg l^{-1} DO). An asterisk indicates a significant change in the difference between the control (open squares, *N*=8) and experimental (black circles, *N*=8) values at that DO level compared with their difference under starting conditions (8 mg l^{-1} DO; $P<0.05$ based on linear mixed-effects model). Means±95% confidence intervals are shown.

therefore underlie some of the hypoxia sensitivity of muscular performance during escape jetting.

Impairment of fast swimming by hypoxia in other species

We are unaware of any other laboratory studies on other cephalopod species regarding neuromuscular performance associated with fast jetting or escape responses and hypoxia. However, this issue has been considered in field studies of Humboldt squid (*Dosidicus gigas*) that used archival electronic tags to record depth and temperature at 1 Hz in areas of the Gulf of California where the oxygen concentrations throughout the water column were also measured. Maximum vertical velocity in the essentially normoxic surface zone (0–100 m depth; >8 mg l^{-1} DO) ranged from −2.0 to +2.7 m s^{-1}, but maximum velocity in the severely hypoxic OMZ (>250 m depth, <0.5 mg l^{-1} DO) was only −1.2 to +1.7 m s^{-1} (Gilly et al., 2012). Whether Humboldt squid are metabolically constrained by these conditions or whether they simply reduce jetting speed because fast jetting may not be ecologically relevant in the OMZ is unknown. Nonetheless, a reduction in fast jets under severe hypoxia appears to be a characteristic of Humboldt squid, a species that, like market squid, regularly encounters hypoxic environments.

Similar to the impact seen on market squid, hypoxia impairs escape responses in coastal fishes, such as golden grey mullet (*Liza aurata*) and European sea bass (*Dicentrarchus labrax*) (Lefrançois and Domenici, 2006; Lefrançois et al., 2005). In these species, the maximum speed and distance of escape responses decreased as DO fell to 1 mg l^{-1}, similar to the results reported here (Fig. 3A). However, escape responses in the two fish species were only successfully elicited 69% and 37% of the time, respectively, at 1 mg l^{-1} DO relative to normoxia, and neither species showed any change in latency from the time of stimulus presentation to onset of movement as DO dropped. These results differ from our results on squid, where responsiveness was more reliable under this level of hypoxia, but latency increased substantially (Fig. 4B,D). The contrast between fish and squid suggests there could be a tradeoff to preserve either reliable execution of escape responses or response latency in order to tolerate hypoxic stress. Examination of more species is needed to explore this idea.

Recovery from exposure to severe hypoxia

The ecological impact of hypoxia tolerance is more complex than simply maintaining performance during a lack of oxygen. Although the capacity for recovery from exposure to hypoxia has received attention in biochemical and metabolic studies, behavioral and organismal studies have traditionally focused only on the impact of decreasing oxygen availability (Domenici et al., 2013; Hermes-Lima and Zenteno-Savín, 2002). The few studies that do incorporate recovery focus on recovery at the ecosystem level, primarily on biodiversity and recolonization (Steckbauer et al., 2011; Vaquer-Sunyer and Duarte, 2008; Wu, 2002). An individual's physiology and behavior as a result of hypoxia tolerance or sensitivity is an important link between biochemical mechanisms and broad-scale ecology. Our study begins to address this link, but there is a clear need to consider more carefully the recovery of organismal functional and behavioral features after exposure to hypoxia or other environmental stressors.

Hypoxia and the ecology of market squid

Seemingly negative effects of severe hypoxia on the market squid's escape response may not be entirely detrimental in the context of their ecology. Insensitivity of escape jets to moderate hypoxia but vulnerability to severe hypoxia (Fig. 3) is consistent with a critical oxygen limit of 2 mg l^{-1} DO, below which a stable rate of oxygen uptake cannot be maintained (Burford et al., 2019). Furthermore, daytime field observations of immature market squid in offshore waters of the California Current System (CCS) show that market squid tend to inhabit a depth zone of ∼250–500 m that spans a DO range of 1.5–0.5 mg l^{-1} (Stewart et al., 2014). Our results indicate that squid were unimpaired at a DO concentration of ≥2.0 mg l^{-1}, and the daytime vertical distribution *in situ* would suggest that significant damage is unlikely at any concentration >0.5 mg l^{-1}. However, we did observe impairment of the escape response at 0.5 mg l^{-1}, and squid in the wild are occasionally seen at depths corresponding to this DO concentration (Stewart et al., 2014), suggesting that squid can tolerate severely hypoxic conditions for some amount of time and recover after ascending to a more oxygenated region of the water column. Our results are consistent with this idea. All experimental animals were able to withstand severe hypoxia for at least 5 min (some for at least 1 h) before escape jet production failed (Table 1). Thus, it is likely that individuals in the wild can spend at least an hour at depths characterized by severe hypoxia. Temperature in the CCS corresponding to the DO range of 0.5–1.5 mg l^{-1} is about 6.5–8.0°C (Stewart et al., 2014; Zeidberg and Robison, 2007), similar to the temperature used in our experiments.

Our experiments were performed on adult squid collected over spawning grounds in much shallower coastal waters (<50 m) in Monterey Bay. Conditions are generally normoxic in this area, but intrusions of moderately hypoxic seawater can occur in association with strong offshore upwelling events, although DO concentrations generally remain above 3 mg l^{-1} (Booth et al., 2012). Our results suggest that escape jetting of adult market squid in shallow coastal waters would not be impaired by such hypoxic intrusions, at least under present conditions. Other behaviors such as schooling and mating should be independently assessed in this regard.

As oceanic OMZs shoal and intensify with global climate change, upwelling events bringing intrusions of poorly oxygenated water into shallow depths will become more frequent in areas like Monterey Bay (Booth et al., 2012; Stramma et al., 2008). Although such developments could limit the market squid's spawning habitat, these animals may be well prepared for potential change because of their insensitivity to acute moderate hypoxia and ability to recover from stronger hypoxic exposure.

Definition of hypoxia

Based on the different levels of hypoxia tested in this study, our results provide an important foundation for evaluating hypoxic thresholds for market squid and expanding commonly accepted thresholds to include more pelagic species (Vaquer-Sunyer and Duarte, 2008). In their broad comparative analysis across seven phyla, Vaquer-Sunyer and Duarte (2008) identify 4.6 and 2 mg l^{-1} DO as critical biological thresholds for benthic fishes and invertebrates exposed to acute hypoxia. Market squid are pelagic and therefore more similar to fish than octopus, the only cephalopod considered in the 2008 study. This difference in life history combined with the market squid's ability to withstand hypoxia at 2 mg l^{-1} DO makes it difficult for them to fit into the established framework. Our results suggest that additional research on pelagic species, particularly those in the northeastern Pacific Ocean, is needed for a comprehensive understanding of the impact of hypoxia on coastal ecosystems in a time of OMZ expansion.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.H.L., W.F.G.; Methodology: D.H.L., W.F.G.; Software: D.H.L.; Validation: D.H.L.; Formal analysis: D.H.L.; Investigation: D.H.L.; Resources: D.H.L.; Data curation: D.H.L.; Writing - original draft: D.H.L.; Writing - review & editing: D.H.L., W.F.G.; Visualization: D.H.L.; Supervision: D.H.L., W.F.G.; Project administration: D.H.L., W.F.G.; Funding acquisition: D.H.L., W.F.G.

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