Aerobic performance of two tropical cephalopod species unaltered by prolonged exposure to projected future carbon dioxide levels

Blake L. Spady1,2, *, Tiffany J. Nay1, Jodie L. Rummer1, Philip L. Munday1 and Sue-Ann Watson1,3

1Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD 4811, Australia
2College of Science and Engineering, James Cook University, Townsville, QLD 4811, Australia
3Biodiversity and Geosciences Program, Museum of Tropical Queensland, Queensland Museum, Townsville, Queensland, 4810, Australia

*Corresponding author: Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD 4811, Australia. Tel: +61 4 5677 7883. Email: blake.spady@my.jcu.edu.au

Squid and many other cephalopods live continuously on the threshold of their environmental oxygen limitations. If the abilities of squid to effectively take up oxygen are negatively affected by projected future carbon dioxide (CO₂) levels in ways similar to those demonstrated in some fish and invertebrates, it could affect the success of squid in future oceans. While there is evidence that acute exposure to elevated CO₂ has adverse effects on cephalopod respiratory performance, no studies have investigated this in an adult cephalopod after relatively prolonged exposure to elevated CO₂ or determined any effects on aerobic scope. Here, we tested the effects of prolonged exposure (≥20% of lifespan) to elevated CO₂ levels (~1000 μatm) on the routine and maximal oxygen uptake rates, aerobic scope and recovery time of two tropical cephalopod species, the two-toned pygmy squid, Idiosepius pygmaeus and the bigfin reef squid, Sepioteuthis lessoniana. Neither species exhibited evidence of altered aerobic performance after exposure to elevated CO₂ when compared to individuals held at control conditions. The recovery time of I. pygmaeus under both control and elevated CO₂ conditions was less than 1 hour, whereas S. lessoniana required approximately 8 hours to recover fully following maximal aerobic performance. This difference in recovery time may be due to the more sedentary behaviours of I. pygmaeus. The ability of these two cephalopod species to cope with prolonged exposure to elevated CO₂ without detriment to their aerobic performance suggests some resilience to an increasingly high CO₂ world.

Keywords: Aerobic scope, cephalopod, CO₂ respirometry, ocean acidification, oxygen uptake

Introduction

Atmospheric carbon dioxide (CO₂) concentrations have increased from 280 ppm before the industrial revolution to over 400 ppm in the current day (Dlugokencky and Tans, 2018), a level that is higher than any time in the past 800,000 years (Lüthi et al., 2008). On the current emissions trajectory, it is projected that atmospheric CO₂ will exceed 900 ppm by the end of this century (Collins et al., 2013), increasing at a rate at least an order of magnitude faster than at any time in the past million years (Doney and Schimel, 2007). The partial pressure of CO₂ (pCO₂)
in the surface oceans is in approximate gas equilibrium with the atmosphere, meaning that CO_2_ concentrations in the oceans are increasing at approximately the same rate as the atmosphere (Doney, 2010). Furthermore, the oceans experience seasonal fluctuations in pCO_2_ and these fluctuations are projected to become amplified in the future due to the increased Revelle (buffer) factor of acidified seawater (McNeil and Sasse, 2016). The increase in average pCO_2_ along with the amplification of seasonal cycles of pCO_2_ indicates that marine organisms could experience CO_2_ levels >1000 μatm before the end of the century (McNeil and Sasse, 2016). This rapid increase of CO_2_ levels in the surface oceans could have a range of adverse effects on many marine species (Hoegh-Guldberg et al., 2007; Fabry et al., 2008; Doney et al., 2009; Kroeker et al., 2013; Clements and Hunt, 2015; Cattano et al., 2018).

The capacity to deliver sufficient oxygen to the tissues, in order to meet increasing demand, has been hypothesized to constrain the performance of marine species under climate change and ocean acidification (Fry and Hart, 1948; Pörtner and Farrell, 2008). Squid, the most active order of cephalopods, have high mass-specific oxygen consumption rates and blood with low oxygen-carrying capacity when compared to fishes (O’Dor and Webber, 1986; Shadwick et al., 1990). One of their most advantageous evolutionary adaptations, jet propulsion, allows them to rapidly escape predators and compete for food with carnivorous fishes (Hanlon and Messenger, 1996). However, this mode of locomotion is inherently inefficient, requiring a significant output of energy from their mantle muscles, which further increases their already high demand for oxygen (O’Dor, 1988a, 1988b). To satisfy such a high demand of oxygen, both at rest and during sustained swimming, squid must pump large amounts of blood and extract most (or all) of the oxygen from it during one cycle through the body, leaving little to no venous oxygen reserve (O’Dor and Webber, 1986; Wells et al., 1988; Rosa and Seibel, 2008). The combination of these factors contributes to many squid species living chronically at the threshold of their oxygen limitation (Pörtner, 2002). Furthermore, the oxygen-carrying capacity of cephalopod hemocyanin, which delivers the oxygen to tissues and cells, has been hypothesized to be highly sensitive to changes in pH (Bridges, 1995). These unique physiological traits may mean the respiratory capacity of squid is affected by rising CO_2_ levels in the ocean. However, models by Birk et al. (2018) suggest that climate change relevant CO_2_ levels may not alter squid blood oxygen binding to a degree that will affect aerobic performance. Whether the oxygen uptake capabilities of squid will be susceptible to elevated CO_2_ levels may depend on the level of sensitivity of their hemocyanin to changes in pH (e.g. Seibel, 2016; Birk et al., 2018).

The physiological performance of aquatic animals has often been linked to their aerobic scope, the difference between the routine oxygen uptake (MO_2Routine_ and maximal oxygen uptake (MO_2Max_ rates). Aerobic scope indicates the amount of oxygen available, beyond basal metabolic costs, for critical aerobic activities (Eliason et al., 2008; Pörtner and Farrell, 2008; Pörtner and Peck, 2010). The type of effect and magnitude of changes in aerobic scope resulting from elevated CO_2_ appear to be species specific. In fishes, for example, the effects of elevated CO_2_ on aerobic scope are highly variable, with different studies reporting a decrease (Munday et al., 2009), increase (Couturier et al., 2013; Rummer et al., 2013) or no effect on aerobic scope (Melzner et al., 2009a). Moreover, recent meta-analyses find no consistent effect of elevated CO_2_ on aerobic scope in marine fishes when all studies conducted to date are considered (Lefevre, 2016; Cattano et al., 2018; Hannan and Rummer, 2018). Although less studied, the reported effects of elevated CO_2_ on aerobic scope in molluscs are also variable. Elevated CO_2_ causes a reduced aerobic scope in the scallop, Pecten maximus (Schalkhauser et al., 2013), but no effect on the aerobic scope of the gastropod, Gibberulus gibberulus gibbosus (Watson et al., 2014; Lefevre et al., 2015). The effects of elevated CO_2_ on resting or routine oxygen consumption also varies among mollusc species. Elevated CO_2_ causes a depression in oxygen uptake rates in six bivalve species (Fernández-Reiriz et al., 2011; Wenguang and Maoxian, 2012; Navarro et al., 2013) and one gastropod species (Melatunan et al., 2011). However, other bivalve species respond to elevated CO_2_ with an increase in oxygen uptake rates (Beniash et al., 2010; Cummings et al., 2011). Given the variety of effects on respiratory performance observed in a range of mollusc taxa, most of those being sedentary species, it is difficult to predict how the more active cephalopod species will respond to elevated CO_2_.

In cephalopods, the effects of elevated CO_2_ on respiratory performance appear to be both species as well as life stage specific. Very high CO_2_ (~4000 μatm) had no effect on oxygen consumption in juvenile common cuttlefish, Sepia officinalis (Gutowska et al., 2008). In the same species, similarly high CO_2_ exposure (~3600 μatm) reduced oxygen uptake by ~20% during the late-stage embryo incubation period, although there was no effect at CO_2_ levels more closely resembling those that could occur in the ocean in the next 100 years (~1400 μatm) (Sigwart et al., 2016). Elevated CO_2_ (~1650 μatm) reduced oxygen uptake rates in late-stage embryos and newly hatched paralarvae of the squid Loligo vulgaris (Rosa et al., 2014). By contrast, MO_2Routine_ of adult bigfin reef squid (Sepioteuthis lessoniana) was not altered by moderately high CO_2_ levels (1586 μatm) (Hu et al., 2014), yet oxygen uptake rates were depressed by 40% when they were exposed to very high CO_2_ levels (4134 μatm) for 7 days (Hu et al., 2014). Juvenile jumbo squid, Dosidicus gigas, showed suppressed MO_2_ at elevated CO_2_ levels (estimated at ~1000 μatm) (Rosa and Seibel, 2008); however, with a longer acclimation to CO_2_ and the use of intermittent-flow respirometry rather than flow-through respirometry, there was no effect of elevated CO_2_ (1410 μatm) on the species (Birk et al., 2018). These results indicate a range of possible effects of elevated CO_2_ on the respiratory capacity of cephalopods that may be dependent on species and life stage. However, it also appears that prolonged exposure to
elevated CO₂ and using appropriate respirometry techniques may be important in establishing the physiological effects of rising ocean CO₂ levels on cephalopods.

In this study, we tested the effects of projected future CO₂ levels on the respiratory performance of two tropical cephalopod species. The two-toned pygmy squid (*Idiosepius pygmaeus*) has an average body length of 15 mm and inhabits shallow coastal waters from northern Australia to the South China Sea (Moynihan, 1983; Semmens et al., 1995). This species has a unique adhesive gland on the mantle, allowing it to attach to seagrass or flotsam where it can rest for extended periods (von Byern and Klepal, 2006). These seagrass habitats in which *I. pygmaeus* can be found have the potential for diel fluctuations in CO₂ concentrations between ~80 and 700 μatm (Chou et al., 2018), indicating that the species may be able to tolerate periods of elevated CO₂ conditions. The second species, the bigfin reef squid, is a larger and much more active species, found in tropical waters of up to 100 m, and individuals are often found feeding in coral reef habitats at night (Norman, 2003). These two species, while both commonly called ‘squid’, are in fact from two separate taxonomic orders. The bigfin reef squid is a true squid of the order Teuthida, whereas the pygmy squid is in the order Idiosepiida, which is more closely related to cuttlefish (Sepiida). The two species were subjected to a current-day control (396–440 μatm) or a projected future CO₂ level (997–1039 μatm) for 18 (pygmy squid) or 75 (bigfin reef squid) days. Pygmy squid and bigfin reef squid live for ~90 and 210 days, respectively; therefore, the treatment period was ~20 and 36% of the total lifespan of each species. We hypothesized that elevated CO₂ would reduce MO₂Max and aerobic scope in both cephalopod species, possibly due to the pH sensitivity of blood oxygen binding in some active squids (Bridges, 1995). To test this, we measured MO₂Max and MO₂Routine via intermittent-flow respirometry and then calculated aerobic scope (MO₂Max − MO₂Routine) in both the pygmy squid and bigfin reef squid after prolonged exposure to global change relevant levels of elevated CO₂.

**Materials and methods**

**CO₂ treatment systems**

Experiments were conducted using 8000 l recirculating seawater systems at James Cook University’s research aquarium in Townsville, Australia. CO₂ levels were set at (i) a current-day control (*I. pygmaeus*, 396 μatm; *S. lessoniana*, 440 μatm) and (ii) an upper end-of-century projection following RCP8.5 (elevated CO₂: *I. pygmaeus*, 1039 μatm; *S. lessoniana*, 997 μatm) (Collins et al., 2013). A pH control system (AT Control; Aqua Medic, Germany) dosed CO₂ into 3000 l sumps to achieve the desired pH level for each CO₂ treatment. pH on the National Bureau of Standards (NBS) scale (pHNBS) was measured daily (Seven2Go Pro; Mettler Toledo, Switzerland), and dosing set points were adjusted as necessary to maintain the target pCO₂ in each treatment. Equilibrated seawater from each system was delivered at a rate of 1.5 l min⁻¹ to tanks containing squid. Temperature was measured daily in each tank (Comark C26; Norfolk, UK).

Water samples were taken weekly to determine pH on the total scale (pH₇) by spectrophotometry (UVmini-1240; Shimadzu, Suzhou Instruments Co. Ltd, Kyoto, Japan) using m-cresol purple as an indicator dye (Dickson and Millero, 1987; Dickson et al., 2007). Comparison of pHNBS and pH₇ in the weekly sample were used to estimate daily pH₇ values. Total alkalinity was estimated weekly by Gran Titration (888 Titration; Metrohm AG, Switzerland) ensuring titration calibrations remained within 1% of certified reference material from Dr A.G. Dickson (Scripps Institution of Oceanography, refit by Dickson and Millero, 1987 and Dickson et al., 2007) for KHSO₄.

**Experimental animals**

Two-toned pygmy squid (wet mass, 0.25 ± 0.09 g; mantle length, 11.2 ± 1.7 mm; means ± SD) were collected by dip net (500 μm mesh) in March 2017 from Cleveland Bay in Townsville, Queensland, Australia (19°24’S, 146°82’E) and immediately transported to James Cook University, Townsville. Squid were maintained in round tanks (47Ø × 51H cm) filled with seawater to 67 l, at a maximum of five individuals per tank. Animals were provided with PVC pipe structures in the tanks as shelter. Animals were maintained at control conditions for 24 hours in holding tanks before being transferred to separate tanks of the same size that received a continuous flow of either control or elevated CO₂ treatment water. Glass shrimp (*Acetes sibogae australis*) were provided *ad libitum* to squid every morning. Squid were observed regularly feeding throughout the day, but food was withheld for 24 hours prior to experimentation to ensure a post-absorptive state that would maximize energy available for performance (Niimi and Beamish, 1974). Pygmy squid remained in either control (*n = 8*) or elevated CO₂ (*n = 10*) treatment for 18 days before respirometry trials. A treatment duration of 18 days was chosen for pygmy squid because their brief lifespans mean that mature animals could only be expected to live a maximum of 30 (females) to 45 (males) days post-capture (Jackson, 1988). This treatment duration represents ~20% of the total pygmy squid lifespan.

Bigfin reef squid (wet mass, 181.2 ± 39.9 g; mantle length, 148.7 ± 13.7 mm; means ± SD) were collected in June 2016 from the Townsville breakwater, Queensland, Australia. Animals were captured at night with a 2.5 cm mesh, round frame dip net and transported immediately to James Cook University, Townsville. Squid were kept individually in round tanks (47Ø × 51H cm) filled to 67 l. Individuals were kept at control conditions for 21 days before they were transferred
to separate tanks of the same size that received a continuous flow of either control or elevated CO2 treatment water. Bigfin reef squid were fed a variety of live food, including locally caught estuary glassfish (*Ambassis marianus*), juvenile flathead grey mullet (*Mugil cephalus*), as well as spiny chromis damselfish (*Acanthochromis polyacanthus*), twice daily. Feeder fish were maintained under control CO2 conditions prior to being offered to the squid. Bigfin reef squid remained in either control (*n* = 9) or elevated CO2 (*n* = 7) treatment for 75 days before their respirometry trials. In comparison to the pygmy squid, we chose a longer treatment duration for the bigfin reef squid because of their greater longevity. This treatment duration represents ~35% of their average 208-day lifespan in the wild (Walsh et al., 2002).

### Maximal and routine oxygen uptake measurements

Intermittent-flow respirometry (Clark et al., 2013) was used to determine both routine oxygen uptake (MO2Routine) and maximal oxygen uptake (MO2Max) for both cephalopod species. The MO2Max was established by using a standard exercise challenge immediately before placing the animal in the respirometry chamber. The use of a swim chamber would not have proved effective in determining MO2Max for either species. Pygmy squid tend to attach to the sides of the chamber with their unique adhesive mantle gland rather than swimming against the flow within a swimming chamber. Bigfin reef squid can unpredictably switch locomotory modes from fin undulations to jet propulsion, which can result in self-injury if they propel themselves against the chamber while swimming actively. Therefore, to achieve MO2Max for pygmy squid, animals were chased with a dip net in a small circular tank for 3 minutes, followed by a 15 second air exposure period (Roche et al., 2013; Rummer et al., 2016) immediately prior to introduction to the respirometry chamber. Pygmy squid (all individuals) were unable to continue jet escaping the dip net before the end of the 3-minute period due to exhaustion. The number of jets and ink discharges from each animal during the chase period were recorded. For the bigfin reef squid, however, due to potential injury reasons mentioned above, a different method was used to elicit MO2Max. Instead, bigfin reef squid were held in a large dip net and periodically lifted in and out of the water (10 seconds in/5 seconds out) for 3 minutes. On re-immersions into the water, squid would attempt to jet escape 0–4 times within the safety of the net. All squid ceased jetting before the end of the 3 minutes of periodic emersions. Bigfin reef squid were then subjected to a further 30-second air exposure period before being placed into respirometry chambers. The number of jets and ink discharges were also recorded.

The measurement period for intermittent-flow respirometry (time during which the flush pump was off) was determined as the minimum time required to ensure a steady slope representing the decline in O2 concentration of the chamber down to 75–80% air saturation over time (O2 uptake rate of the squid). The flush period (time during which the flush pump was on) was sufficient time for the O2 concentration of the chambers to be sufficiently replenished back to ~100%. Pygmy squid were tested in 20 ml chambers submerged in an aquarium with continuous delivery of water from their CO2 treatment system, with both flushing and recirculation pumps submerged and providing flow at 21.6 l h⁻¹. Preliminary experiments determined that MO2Routine of pygmy squid was reached in under 2 hours, so total trial time was set at 4 hours (Fig. S1). The measurement period was set at 150 seconds followed by a 60-second flushing period. Bigfin reef squid were tested in 6840 ml chambers also submerged in continuously replenished water from the CO2 treatment system of the squid being tested. The flush and recirculation pumps for these chambers delivered water at 400 l h⁻¹. Measurement periods for bigfin reef squid lasted 85 seconds, followed by a 300-second flush period; this cycle continued for a total 22-hour trial period. The squid to chamber volume ratio was between 1:20 and 1:50 for all animals and the O2 concentration did not fall below 80% air saturation during measurement periods in any of the chambers for either species (Swensen et al., 2016).

Temperature-compensated O2 concentration was continuously recorded (0.5 Hz) using oxygen-sensitive REDFLASH dye on contactless spots (2 mm) adhered to the inside of a cut glass pipette tube set within the recirculation pump loop and linked to a Firesting Optical Oxygen Meter (Pyro Science e.K., Aachen, Germany) via fibre-optic cables. Data were analysed in LabChart version 8.1.3 (ADInstruments, Colorado Springs, CO, USA), and MO2 (in milligrammes O2 per kilogramme of animal per hour) was calculated as the slope of the linear

---

**Table 1: Mean seawater data (± SD) for each species; total alkalinity and salinity values are from weekly measurements**

| Species       | CO2 treatment | Temperature (°C) | Salinity | pH(T) | Total alkalinity (μmol/kg SW) | pCO2 (μatm) |
|---------------|---------------|------------------|----------|-------|-------------------------------|-------------|
| *I. pygmaeus* | Control       | 28.1 (±0.3)      | 36.7 (±1.1) | 8.05 (±0.07) | 2373 (±55) | 396 (±76) |
|               | Elevated      | 28.1 (±0.4)      | 36.4 (±0.9) | 7.67 (±0.05) | 2228 (±63) | 1039 (±118) |
| *S. lessoniana* | Control       | 28.2 (±0.4)      | 36.3 (±0.4) | 7.99 (±0.05) | 2229 (±67) | 440 (±60) |
|               | Elevated      | 28.5 (±0.4)      | 36.0 (±0.7) | 7.69 (±0.04) | 2212 (±130) | 997 (±110) |
regression of oxygen concentration decline over time during the measurement period using the following equation:

$$\dot{M}O_2 = SV_{resp}M^{-1},$$

where $S$ is the slope (in milligrams of $O_2$ per litre per second), $V_{resp}$ is the volume of the respirometer minus the volume of the squid (in litres) and $M$ is the mass of the squid (in kilogrammes). The volume of respirometry chambers included the volume of the chamber as well as that of the recirculation tubing and pump. The value of $MO_{2Routine}$ was calculated by taking the average of the lowest 10% of $MO_2$, minus the background $O_2$ uptake, which was measured before and after each trial (assumed linear) (Rummer et al., 2016). The $MO_{2Max}$ was calculated by isolating the first five slopes into segments (each segment being 20% of the total measurement period) and selecting the highest rate of change found therein and ensuring that the $R^2$ of slopes was above 0.95. Aerobic scope for each individual was calculated by subtracting the $MO_{2Routine}$ value from the $MO_{2Max}$ value for that individual. To restrict background respiration to <5% of a squid’s $MO_{2Routine}$, chambers and pumps were rinsed with fresh water and 10% bleach solution after each trial and left to dry for 12 hours before being used again.

Statistical analyses

Generalized linear mixed models with Gaussian distributions were used to compare response variables ($MO_{2Routine}$, $MO_{2Max}$, aerobic scope and recovery time) for pygmy squid between CO$_2$ treatments, with the number of jets and number of inks included as fixed factors. A tank effect was not applicable for bigfin reef squid as they were always housed individually. A linear model with square root transformed data tested for differences in recovery times between the two squid species.

Results

Pygmy squid

Elevated CO$_2$ did not affect any of the traits measured for pygmy squid. Pygmy squid had a mean average $MO_{2Max}$ of $1164 \pm 57$ mg kg$^{-1}$ h$^{-1}$ (mean $\pm$ SE) under control and $1117 \pm 35$ mg kg$^{-1}$ h$^{-1}$ under elevated CO$_2$ ($\chi^2 = 0.112$, df = 14, $P = 0.738$) conditions. The $MO_{2Routine}$ was $341 \pm 33$ mg kg$^{-1}$ h$^{-1}$ under control and $418 \pm 47$ mg kg$^{-1}$ h$^{-1}$ under elevated CO$_2$ and was not significantly different between treatments ($\chi^2 = 2.667$, df = 14, $P = 0.102$) (Fig. 1A). The aerobic scope of pygmy squid was similar between CO$_2$ treatments, with an average of $746 \pm 52$ mg kg$^{-1}$ h$^{-1}$ at control compared to $776 \pm 49$ mg kg$^{-1}$ h$^{-1}$ under elevated CO$_2$ ($\chi^2 = 0.929$, df = 14, $P = 0.335$) (Fig. 2A). Recovery time of pygmy squid under both control and elevated CO$_2$ conditions lasted for just under 1 hour, averaging 55 minutes and 12 seconds ($\pm$10 minutes and 42 seconds) under control CO$_2$ and 53 minutes and 9 seconds ($\pm$11 minutes and 48 seconds) under elevated CO$_2$ conditions ($\chi^2 = 0.006$, df = 14, $P = 0.936$) (Fig. 3A). A power analysis revealed a power of 0.067 on the comparisons of aerobic scope in pygmy squid between CO$_2$ treatments.

Statistical analyses were performed with R statistical software (R Development Core Team, 2018). Residual analysis indicated that data met the assumptions of normality and homogeneity of variance. Power analyses were performed to determine the probability of finding a significant difference in aerobic scope given the sample size and effect size.

Figure 1: Routine (white boxes) and maximal oxygen uptake (grey boxes) of pygmy squid (A) and bigfin reef squid (B) from current day control and elevated CO$_2$ treatments; boxplots show the median and interquartile range and the minimum and maximum range of the data.
**Bigfin reef squid**

As observed in pygmy squid, elevated CO2 did not affect any of the traits measured for bigfin reef squid. The average $\dot{M}O_2^{\text{Max}}$ of bigfin reef squid under both control and elevated CO2 treatment levels were very similar at $1133 \pm 36 \text{ mg kg}^{-1} \text{ h}^{-1}$ and $1139 \pm 63 \text{ mg kg}^{-1} \text{ h}^{-1}$, respectively ($\chi^2 = 0.011$, df = 14, $P = 0.918$). Average $\dot{M}O_2^{\text{Routine}}$ measurements between treatments were also similar at $596 \pm 27 \text{ mg kg}^{-1} \text{ h}^{-1}$ in the control and $565 \pm 46 \text{ mg kg}^{-1} \text{ h}^{-1}$ in elevated CO2 ($\chi^2 = 0.588$, df = 14, $P = 0.443$) (Fig. 1B). The aerobic scope of bigfin reef squid averaged $509 \pm 41 \text{ mg kg}^{-1} \text{ h}^{-1}$ under control and $574 \pm 53 \text{ mg kg}^{-1} \text{ h}^{-1}$ under elevated CO2 conditions ($\chi^2 = 1.083$, df = 14, $P = 0.298$) (Fig. 2B). Recovery time in bigfin reef squid was very similar between treatments, lasting an average of 8 hours and 8 minutes (±47 minutes) under control and 7 hours and 43 minutes (±1 hour and 13 minutes) under elevated CO2 conditions ($\chi^2 = 0.144$, df = 14, $P = 0.704$) (Fig. 3B). The individual with the lowest aerobic scope (342 mg kg$^{-1}$ h$^{-1}$) also performed the fewest number of jets during the exercise protocol (11 jets). It is possible this individual, from the control treatment, did not achieve a true $\dot{M}O_2^{\text{Max}}$ resulting in the lowest aerobic scope. The recovery time of bigfin reef squid was significantly longer than that of pygmy squid by an average of 7 hours ($\chi^2 = 113.080$, df = 31, $P < 0.001$). A power analysis revealed a power of 0.146 on the comparisons of aerobic scope in bigfin reef squid between CO2 treatments.

![Figure 2: Absolute aerobic scope of pygmy squid (A) and bigfin reef squid (B) from current day control and elevated CO2 treatments; boxplots show the median and interquartile range and the minimum and maximum range of the data.](image)

![Figure 3: Recovery time that pygmy squid (A) and bigfin reef squid (B) required to reach a stable routine oxygen uptake rate following exhaustive exercise at current-day control and elevated CO2 treatment levels; boxplots show the median and interquartile range and the minimum and maximum range of the data (Note: the y-axis for pygmy squid ranges from 0–150 minutes while the y-axis for bigfin reef squid ranges from 0–800 minutes).](image)
Discussion

After extended exposure to elevated CO$_2$ levels, we found no significant changes to any measures of oxygen consumption in either the two-toned pygmy squid (I. pygmaeus) or the bigfin reef squid (S. lessoniana) when compared with current-day control CO$_2$ conditions. The responses observed here under elevated CO$_2$ differ from the reduction in MO$_{2\text{Max}}$ and MO$_{2\text{Routine}}$ observed in the jumbo squid (Rosa and Seibel, 2008) and the decrease in oxygen uptake rates observed in the common cuttlefish and European squid during late-stage embryo incubation (Rosa et al., 2014; Sigwart et al., 2016). However, these results are consistent with those of juvenile common cuttlefish (Gutowska et al., 2008) and experiments by Hu et al. (2014) in which bigfin reef squid showed no changes in MO$_{2\text{Routine}}$ under CO$_2$ levels of 1585 μatm after a 7 day exposure period. We predicted that elevated CO$_2$ would interfere with oxygen extraction causing a decrease in the MO$_{2\text{Max}}$, reducing aerobic scope. However, the recent estimation that CO$_2$ levels of ∼1000 μatm would cause a drop in squid hemocyanin-O$_2$ saturation by no more than 1.6% (Birk et al., 2018) suggests that squid may be able to cope with elevated CO$_2$ without costs to their oxygen uptake capabilities. Our results indicate that the respiratory physiology of the two species of tropical cephalopod studied here are likely to be resilient to realistic future CO$_2$ levels in the habitats they currently inhabit.

Based on our results and from studies previously conducted on other cephalopod species, it appears that elevated CO$_2$ elicits a range of aerobic responses in cephalopods, which may be life stage dependent. The reduced oxygen uptake observed during the embryonic period in some cephalopods at elevated CO$_2$ (Rosa et al., 2014; Sigwart et al., 2016) might be expected, as acid–base regulatory mechanisms in cephalopods often remain rudimentary until respiration switches from cutaneous (via skin) to branchial (via gills) (Hu et al., 2011b). Although little is known about the acid–base regulatory capabilities of pygmy squid, those of bigfin reef squid have been thoroughly investigated. Bigfin reef squid, among other cephalopods, have evolved ion regulatory epithelia in both the gills (Hu et al., 2011a, 2014) and skin cells (Hu et al., 2011b, 2013), which are effective in coping with acid-base disturbances.

Acid–base regulatory abilities are an indispensable trait in cephalopods, as well as in all animals, as there is a continuous natural confrontation with respiratory CO$_2$ that can cause extra- and intra-cellular pH disturbances (Robertson, 1949; Hu et al., 2013). Some cephalopods have been shown to have advanced acid–base regulatory machinery, comparable to that of fishes, and can effectively regulate their acid–base balance at high levels of CO$_2$ without compromising aerobic capacities (Gutowska et al., 2008, 2010). It appears that this may also be the case for both pygmy squid and bigfin reef squid, as MO$_2$ remains unchanged at CO$_2$ levels (∼1000 μatm) projected for the end of the century under the business as usual CO$_2$ emissions scenario. There could be changes in the allocation of resources at elevated CO$_2$, such as towards acid–base regulation, but this does not appear to affect the aerobic performance of squids. However, there may still be energetic costs at much higher CO$_2$ levels, because the extreme CO$_2$ treatment (4134 μatm) used by Hu et al. (2014) resulted in a 40% reduction in MO$_{2\text{Routine}}$ in bigfin reef squid.

The recovery times of pygmy squid and bigfin reef squid observed under control CO$_2$ conditions were noticeably different between species. While pygmy squid recovered in an average recovery time of less than 1 hour, bigfin reef squid took an average of ∼8 hours to return to their MO$_{2\text{Routine}}$. During trials, all pygmy squid were observed to use the adhesive gland on their mantle to remain attached to the wall of the respirometry chamber throughout the duration of the measurement period. In contrast, bigfin reef squid lack this gland and maintained a suspended position by the use of their undulating fins throughout the trials. This unique mechanism in pygmy squid grants the species a much greater capacity for rest than in the continuously swimming bigfin reef squid. This may help to explain why pygmy squid, under control conditions, overcame excess post-exercise oxygen consumption more rapidly than bigfin reef squid. Furthermore, the MO$_{2\text{Routine}}$ Values measured for pygmy squid are likely closer to true standard metabolic rates, i.e. maintenance costs, than those of bigfin reef squid due to their mode of life.

Most squid are negatively buoyant, and the requirements of being ‘at rest’ while remaining suspended in the water column still have considerable aerobic costs (Bartol et al., 2001). As bigfin reef squid recover from exercise, they remain relatively active in order to remain suspended in the water column. The species has large fins that run the full length of the mantle that can be used to maintain neutral buoyancy without the use of jetting. This allows them to spend, perhaps, a smaller fraction of their energy budget to maintain neutral buoyancy when compared to other squid species with proportionally much smaller fins, requiring fin use to be coupled with jetting (Hu et al., 2014). While this decoupled option of swimming with fin undulations alone in bigfin reef squid is more efficient than in more powerfully swimming pelagic squid species, pygmy squid can attach to a piece of seagrass or other benthic structure where they may truly rest, thus avoiding spending extra energy during recovery. This mode of rest could also be very beneficial to pygmy squid because lower mantle ventilation pressures in squid, such as those during rest, as opposed to those during active swimming, lead to higher oxygen extraction rates (Melzner et al. 2006).

It is worth noting that the average values of MO$_{2\text{Routine}}$ values in bigfin reef squid under control CO$_2$ levels observed by Hu et al. (2014) were markedly higher, by ∼70%, than those observed here. The MO$_{2\text{Routine}}$ values in this experiment were determined during a 22-hour trial period, whereas the previous experiment determined MO$_{2\text{Routine}}$ during a 20–30 minute measurement period. The results from this experiment indicate that bigfin reef squid experience excess post-exercise oxygen consumption for ∼8 hours after exhaustive
exercise. While Hu et al. (2014) did not exercise the squid in their experiments, it is likely that a measurement period of 20–30 minutes may not have allowed for a true $\dot{M}$O$_2$Routine measurement due to the handling stress and introduction to the respirometry chamber (Keys, 1930; Svendsen et al., 2018). Nevertheless, considering all animals were handled and introduced to the chambers in the same way, the comparisons among CO$_2$ treatments from the previous experiment are still useful. Furthermore, there were comparable results upon exposure to similar elevated CO$_2$ levels, causing unaltered $\dot{M}$O$_2$ in both the former experiments and in those that were performed here.

In this study, we used a relatively prolonged exposure to elevated CO$_2$ (>20% of the animals lifespan) along with intermittent-flow respirometry to determine the effects of elevated CO$_2$ on cephalopod MO$_2$. In juvenile jumbo squid, D. gigas, elevated CO$_2$ levels (estimated at 1410 μatm) suppressed MO$_2$Max by ~30% and MO$_2$Routine by ~20% (Rosa and Seibel, 2008). However, with a longer acclimation to CO$_2$ and the use of intermittent-flow respirometry rather than flow-through respirometry, there was no effect of elevated CO$_2$ on S. lessoniana depending on the length of exposure. This suggests that future studies should use exposure times that are long enough for individuals to overcome any short-term effects of elevated CO$_2$ as well as consider intermittent-flow respirometry methods (e.g. Steffensen, 1989). Future studies should also consider additional environmental changes, such as elevated temperature, which could potentially interact with elevated CO$_2$ to affect oxygen consumption in unexpected ways. Multifactorial experiments will be important to gain a more complete understanding of the effects of climate change and ocean acidification on cephalopod physiology. Furthermore, power analyses revealed that the sample size of this study was low considering the variation among individuals. A larger sample size would be needed to investigate more subtle effects of elevated CO$_2$ that may not have been detected in this study.

Our findings contribute to increasing evidence suggesting a level of tolerance to elevated CO$_2$ in adult cephalopods, both among species and among the traits being tested. It has been suggested that the active, high-energetic lifestyle, along with occurrences of natural hypercapnia during the course of embryonic development, constitutes factors that pre-adapt cephalopods to cope with elevated CO$_2$ levels (Melzner et al., 2009b). While elevated CO$_2$ has been shown to illicit negative physiological responses in some cephalopod species (e.g. Kaplan et al., 2013; Sigwart et al., 2016), other studies have observed no adverse effects of elevated CO$_2$ on the same traits in other species (e.g. Gutowska et al., 2010; Birk et al., 2018). Physiological stress from elevated CO$_2$ during the early ontogeny of animals has been described as the ‘true bottleneck’ through which species must pass in order to successfully tolerate future elevated CO$_2$ oceans, even for species that demonstrate tolerance in other traits (Melzner et al., 2009b). Therefore, it will be important for future studies to investigate the effects of elevated CO$_2$ on cephalopods during their embryonic and paralarval development, especially in species that show no adverse effects of elevated CO$_2$ in other physiological traits as adults, such as those studied here.

### Conclusions

This study shows that MO$_2$Max, MO$_2$Routine, aerobic scope and recovery time of two tropical cephalopod species are unaltered following prolonged exposure to elevated CO$_2$. In warmer tropical latitudes, where the energy requirements of cephalopods are higher, altered aerobic performance could be particularly consequential. However, it appears that CO$_2$ levels projected for the end of this century will not adversely affect the respiratory performance of either the two-toned pygmy squid or bigfin reef squid. The unaltered aerobic performance of these ecologically distinct cephalopod species, from separate taxonomic orders, after prolonged exposure to elevated CO$_2$ indicates that a wide range of cephalopod species may have the aerobic capacity to cope with an increasingly CO$_2$-rich ocean. However, as some previous studies have observed negative effects of elevated CO$_2$ on respiratory performance of some cephalopod species, it will be important to understand the potentially species-specific and life stage-specific effects, which can have critical implications for the structure of marine ecosystems in the future.

### Acknowledgements

This study followed animal ethics guidelines at James Cook University (JCU Animal Ethics Number: A2189). Collection permits from the Queensland Department of Agriculture, Fisheries and Forestry were obtained to collect all species used in this study (Permit Number: 170231). The authors thank the technical staff at James Cook University Marine and Aquaculture Facilities Unit (Ben Lawes, Andrew Thompson and Simon Wever) for logistical support and assistance. Thanks to Shannon McMahon and Donald Warren for help with animal capture as well as the Munday Lab group for general help and advice. The assistance of Erin Weir was also greatly appreciated during this study.

### Authors’ contributions

All authors contributed towards writing the manuscript. B.L.S., S.-A.W. and T.J.N. designed the experiments. B.L.S. conducted the experiments, maintained experimental facilities and animals and conducted the carbonate chemistry analysis. B.L.S. analysed and interpreted the data. All authors have read and approved of the final manuscript.
Cummings V et al. (2011) Ocean acidification at high latitudes: potential effects on functioning of the antarctic bivalve Laternula elliptica. PLoS One 6: e16069.

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res A 34: 1733–1743.

Dickson AG, Sabine CL, Christian JR (2007) Guide to Best Practices for Ocean CO₂ Measurements. In Vol Vol 3. North Pacific Marine Science Organization, Sidney, p. 191.

Dlugokencky E, Tans P (2018) Trends in atmospheric carbon dioxide. Retrieved from NOAA/ESRL. http://www.esrl.noaa.gov/gmd/ccgg/trends/global.html.

Doney SC (2010) The growing human footprint on coastal and open-ocean biogeochemistry. Science 328: 1512–1516.

Doney SC, Balch W, Fabry V, Feely R (2009) Ocean acidification: a critical emerging problem for the ocean sciences. Oceanography 22: 16–25.

Doney SC, Schimel DS (2007) Carbon and climate system coupling on timescales from the Precambrian to the Anthropocene. Annu Rev Environ Resour 32: 31–66.

Ellison EJ, Clark TD, Hague MJ, Hansson LM, Gallagher ZS, Jeffries KM, Gale MK, Patterson DA, Hinch SG, Farrell AP (2008) Differences in thermal tolerance among sockeye salmon populations. Science 1861: 1–4.

Fabry V, Seibel BA, Feely RA, Orr J (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J Mar Sci 65: 414–432.

Fernández-Reiriz MJ, Range P, Álvarez-Salgado XA, Labarta U (2011) Physiological energetics of juvenile clams Ruditapes decussatus in a high CO₂ coastal ocean. Mar Ecol Prog Ser 433: 97–105.

Fry FEJ, Hart JS (1948) The relation of temperature to oxygen consumption in the goldfish. Biol Bull 94: 66–77.

Gutowska MA, Melzner F, Langenbuch M, Bock C, Claireaux G, Pörtner HO (2010) Acid-base regulatory ability of the cephalopod Sepia officinalis in response to environmental hypercapnia. J Comp Physiol B 180: 323–335.

Gutowska MA, Pörtner HO, Melzner F (2008) Growth and calcification in the cephalopod Sepia officinalis under elevated seawater pCO₂. Mar Ecol Prog Ser 373: 303–309.

Hanlon RT, Messenger JB (1996) Cephalopod Behaviour. Cambridge University Press, New York.

Hannan KD, Rummer JL (2018) Aquatic acidification: a mechanism underpinning maintained oxygen transport and performance in fish experiencing elevated carbon dioxide conditions. J Exp Biol 221: jeb154559.

Hoeegh-Guldberg O et al. (2007) Coral reefs under rapid climate change and ocean acidification. Science 318: 1737–1742.

Hu MY, Guh Y, Stumpp M, Lee J, Chen R, Sung P, Chen Y (2014) Branchial NH₄⁺-dependent acid–base transport mechanisms and energy metabolism of squid (Sepioteuthis lessoniana) affected by seawater acidification. Front Zool 11: 55.

Hu MY, Lee JR, Lin LY, Shih TH, Stumpp M, Lee MF, Hwang PP, Tseng YC (2013) Development in a naturally acidified environment: Na⁺/H⁺-
exchanger 3-based proton secretion leads to CO₂ tolerance in cephalopod embryos. *Front Zool* 10: 51.

Hu MY, Tseng Y-C, Stumph M, Gutowksa MA, Kiko R, Lucassen M, Melzner F (2011a) Elevated seawater PCO₂ differentially affects branchial acid-base transporters over the course of development in the cephalopod *Sepia officinalis*. *Am J Physiol Regul Integr Comp Physiol* 300: R1100–R1114.

Hu MY, Tseng Y, Lin L, Chen P, Charmantier-daures M, Hwang P, Melzner F (2011b) New insights into ion regulation of cephalopod molluscs: a role of epidermal ionocytes in acid-base regulation during embryogenesis. *Am J Physiol Regul Integr Comp Physiol* 301: R1700–R1709.

Jackson GD (1988) The use of statolith microstructures to analyse life-history events in the small tropical cephalopod *Idiosepius pygmaeus*. *Fish Bull* 87: 265–272.

Kaplan MB, Mooney TA, McCorkle DC, Cohen AL (2013) Adverse effects of ocean acidification on early development of squid (*Doryteuthis pealei*). *PloS One* 8: e63714.

Keys AB (1930) The measurement of the respiratory exchange of aquatic animals. *Biol Bull* 59: 187–188.

Kroeker KJ, Kordas RL, Crim R, Hendriks IE, Ramajo L, Singh GS, Duarte MC, Gattuso JP (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Glob Chang Biol* 19: 1884–1896.

Lefevre S, Watson S-A, Munday PL, Nilsson GE (2015) Will jumping snails prevail? Influence of near-future CO₂, temperature and hypoxia on respiratory performance in the tropical conch *Gibberulus gibberulus gibbosus*. *J Exp Biol* 218: 2991–3001.

Lüthi D et al. (2008) High-resolution carbon dioxide concentration record 650,000–800,000 years before present. *Nature* 453: 379–382.

McNeil BI, Sasse TP (2016) Future ocean hypercapnia driven by anthropogenic amplification of the natural CO₂ cycle. *Nature* 529: 383–386.

Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RN (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol Oceanogr* 18: 897–907.

Melatunno S, Calosi P, Rundle SD, Moody AJ, Widdicombe S (2011) Exposure to elevated temperature and PCO₂ reduces respiration rate and energy status in the periwinkle *Littorina littorea*. *Physiol Biochem Zool* 84: 583–594.

Melzner F, Bock C, Pörtner HO (2006) Temperature-dependent oxygen extraction from the ventilatory current and the costs of ventilation in the cephalopod *Sepia officinalis*. *J Comp Physiol B* 176: 607–621.

Melzner F, Göbel S, Langenburch M, Gutowksa MA, Pörtner HO, Lucassen M (2009a) Swimming performance in Atlantic Cod (*Gadus morhua*) following long-term (4–12 months) acclimation to elevated seawater PCO₂. *Aquat Toxicol* 92: 30–37.

Melzner F, Gutowska MA, Langenburch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M (2009b) Physiological basis for high CO₂ tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* 6: 2313–2331.

Moyrinhan M (1983) Notes on the behavior of *Idiosepius pygmaeus* (Cephalopoda; Idiosepiidae). *Behaviour* 85: 42–57.

Munday PL, Crawley NE, Nilsson GE (2009) Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. *Mar Ecol Prog Ser* 388: 235–242.

Navarro JM, Torres R, Aculia K, Duarte C, Manriquez PH, Ladries M, Lagos NA, Vargas C, Aguilar V (2013) Impact of medium-term exposure to elevated pCO₂ levels on the physiological energetics of the mussel *Mytilus chilensis*. *Chemosphere* 90: 1242–1248.

Niimi AJ, Beamish FWH (1974) Bioenergetics and growth of largemouth bass (*Micropterus salmoides*) in relation to body weight and temperature. *Can J Zool* 52: 447–456.

Norman MD (2003) *Cephalopods: A World Guide*. ConchBooks, Hackenheim.

O’Dor RK (1988a) Limitations on locomotor performance in squid. *J Appl Physiol* (1985) 64: 128–134.

O’Dor RK (1988b) The forces acting on swimming squid. *J Exp Biol* 137: 421–442.

O’Dor RK, Webber DM (1986) The constraints on cephalopods: why squid aren’t fish. *Can J Zool* 64: 1591–1605.

Pierret D, Lewis E, Wallace DWR (2006) MS Excel program developed for CO₂ system calculations. (Oak Ridge, TN, USA, ORNL/CDIAC-105a: Carbon Dioxide Information Analysis Center). Oak Ridge National Laboratory, U.S. Department of Energy.

Pörtner HO (2002) Environmental and functional limits to muscular exercise and body size in marine invertebrate athletes. *Comp Biochem Physiol A Mol Integr Physiol* 133: 303–321.

Pörtner HO, Farrell AP (2008) Physiology and climate change. *Science* 322: 690–692.

Pörtner HO, Peck MA (2010) Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. *J Fish Biol* 77: 1745–1779.

R Development Core Team (2018) R: a language and environment for statistical computing. Vienna, Austria. http://www.R-project.org/.

Robertson JD (1949) Ionic regulation in some marine invertebrates. *J Exp Biol* 26: 182–200.

Roehe DG, Binning SA, Bosiger Y, Johansen JL, Rummer JL (2013) Finding the best estimates of metabolic rates in a coral reef fish. *J Exp Biol* 216: 2103–2110.

Rosa R, Seibel BA (2008) Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. *Proc Natl Acad Sci USA* 105: 20776–20780.

Rosa R, Trubenbach K, Pimentel MS, Boavida-Portugal J, Faleiro F, Baptista M, Dionisio G, Calado R, Pörtner HO, Repolho T (2014)
Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (Loligo vulgaris). J Exp Biol 217: 518–525.

Rummer JL, Binning SA, Roche DG, Johansen JL (2016) Methods matter: considering locomotory mode and respirometry technique when estimating metabolic rates of fishes. Conserv Physiol 4: doi:10.1093/conphys/cow008.

Rummer JL, Stecyk JAW, Couturier CS, Watson S-A, Nilsson GE, Munday PL (2013) Elevated CO2 enhances aerobic scope of a coral reef fish. Conserv Physiol 1: doi:10.1093/conphys/cot023.

Schalkhausser B, Bock C, Stemmer K, Brey T, Pörtner HO, Lannig G (2013) Impact of ocean acidification on escape performance of the king scallop, Pecten maximus, from Norway. Mar Biol 160: 1995–2006.

Seibel BA (2016) Cephalopod susceptibility to asphyxiation via ocean incalescence, deoxygenation, and acidification. Physiol 31: 418–429.

Semmens JM, Moltschaniwskyj NA, Alexander CG (1995) Effect of feeding on the structure of the digestive gland of the tropical sepioid Idiosepius pygmaeus. J Mar Biol Assoc UK 75: 885–897.

Shadwick RE, Dor KO, Gosline JM (1990) Respiratory and cardiac function during exercise in squid. Can J Zool 68: 792–798.

Sigwart JD, Lyons G, Fink A, Gutowska MA, Murray D, Melzner F, Houghton JDR, Hu MY (2016) Elevated pCO2 drives lower growth and yet increased calcification in the early life history of the cuttlefish Sepia officinalis (Mollusca: Cephalopoda). ICES J Mar Sci 73: 970–980.

Steffensen JF (1989) Some errors in respirometry of aquatic breathers: how to avoid and correct for them. Fish Physiol Biochem 6: 49–59.

Svendsen MBS, Bushnell PG, Steffensen JF (2016) Design and setup of intermittent-flow respirometry system for aquatic organisms. J Fish Biol 88: 26–50.

Svendsen MBS, Johansen JL, Bushnell PG, Skov PV, Norin T, Domenici P, Steffensen JF, Abe A (2018) Are all bony fishes oxygen regulators? Evidence for oxygen regulation in a putative oxygen conformer, the swamp eel Synbranchus marmoratus. J Fish Biol 94: 178–182.

von Byern J, Klepal W (2006) Adhesive mechanisms in cephalopods: a review. Biofouling 22: 329–338.

Walsh LS, Turk PE, Forsythe JW, Lee PG (2002) Mariculture of the loliginid squid Sepioteuthis lessoniana through seven successive generations. Aquaculture 212: 245–262.

Watson S-A, Lefevre S, McCormick MI, Domenici P, Nilsson GE, Munday PL (2014) Marine mollusc predator-escape behaviour altered by near-future carbon dioxide levels. Proc Biol Sci 281: 1–8.

Wells MJ, Hanlon RT, Lee PG, DiMarco FP (1988) Respiratory and cardiac performance in Lolliguncula brevis (Cephalopoda, Myopsida): the effects of activity, temperature and hypoxia. J Exp Biol 36: 17–36.

Wenguang L, Maoxian H (2012) Effects of ocean acidification on the metabolic rates of three species of bivalve from southern coast of China. Chinese J Oceanol Limnol 30: 206–211.