Effects of increasing pCO_2 on life history traits and feeding of the littoral mysid *Praunus flexuosus*

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Abstract

Mysids, an important food web component in the littoral zone of coastal waters, have been neglected so far in ocean acidification research. Juveniles of the littoral mysid *Praunus flexuosus* were exposed in the laboratory to four pCO_2 levels (530, 930, 1200, 1600 μatm) for 5 weeks. In addition, juveniles were provided with two different food levels during the experiment. High pCO_2 did not affect survival, but delayed moulting. Juvenile growth decreased and inter-moult period between the last moulting events increased with increasing pCO_2 at low but not at high food supply, suggesting that high food availability is needed to prevent these negative effects of elevated pCO_2. However, small individual juveniles showed lower feeding rates at high pCO_2 compared to the control after prolonged exposure, suggesting decreased activity likely due to impaired metabolism. The subtle negative effects of elevated pCO_2 on life history traits observed in this study suggests that *P. flexuosus* probably has to adapt to counteract adverse effects of predicted high pCO_2, especially when food is limiting.

Key-words: climate change, ocean acidification, high CO_2, crustacean zooplankton, mysids, exposure experiments, survival, moulting

Introduction

The seawater of the world's oceans is slowly changing its carbonate chemistry due to an increasing uptake of anthropogenically produced CO_2, thereby changing the bicarbonate buffering system and reducing the pH (Caldeira and Wickett 2003; Gattuso and Hansson 2011). This phenomenon called 'ocean acidification' (OA) can have adverse effects on marine biota depending on phylogeny (Doney et al. 2009; Kroeker et al. 2013; Wittmann and Pörtner 2013) and thus on ocean ecosystem functioning (Nagelkerken and Connell 2015). Heavily calcifying organisms, such as coccolithophores, corals, molluscs, or echinoderms, are often negatively affected by elevated CO_2 concentrations (pCO_2) in seawater (Kroeker et al. 2013).

Marine crustaceans could be more tolerant to OA than the aforementioned groups (Kroeker et al. 2013; Wittmann and Pörtner 2013), even though negative effects of high pCO_2 on life history traits have been reported for certain species (reviewed in Kurihara 2008; Whiteley 2011). Not surprisingly, OA research on crustaceans focused mainly on specific taxa or groups, such as copepods (Whiteley 2011; Lewis et al. 2013), due to their abundance and central role in pelagic food webs, or commercially valuable crustaceans such as crabs, shrimps, or lobster (Kurihara et al. 2008; Long et al. 2013; Small et al. 2016). However, there is still a lack of studies and taxonomic coverage in crustacean OA research compared to other taxonomic groups, preventing robust general interpretations of the effects of high pCO_2 on crustaceans (Kroeker et al. 2013). This calls for studies that investigate potential effects of changing pH/pCO_2 on other important, but so far neglected, crustacean groups such as mysids.

Crustaceans can adjust physiologically to OA-related changes in seawater carbonate chemistry. Such adjustments are important because increasing environmental pCO_2 can cause decreasing haemolymph pH, which would compromise protein function and oxygen supply (Melzner et al. 2009; Whiteley 2011). Thus, crustaceans need to compensate acid-

base imbalances of their body fluids to keep physiological processes within functional limits (Melzner et al. 2009). Studies on decapod crustaceans have revealed that acid-base balance is closely associated with iono- and osmo-regulation, because these processes share the same mechanisms (e.g. Truchot 1981; Whiteley et al. 2001). Strong iono- and osmo-regulators are likely better in compensating disruptions in haemolymph pH due to their well-developed ion exchange mechanisms and thus, these species are likely less vulnerable to increasing environmental pCO_2 (Whiteley 2011). Though, continuous adjustments in acid-base balance are metabolically costly, thereby reducing energy availability for other energetically costly processes such as growth and reproduction (Seibel and Walsh 2003; Pörtner and Farrell 2008). However, the increasing energetic demands with increasing pCO_2 can be met by sufficient food acquisition and thus, high food supply may mitigate adverse effects of high pCO_2 on growth, reproduction, or calcification (Thomsen et al. 2013; Towle et al. 2015; Ramajo et al. 2016).

Mysids, shrimp-like crustaceans, are common and abundant in coastal areas (Mauchline 1980) and are important food web components that can link benthic and pelagic systems (Lehtiniemi and Nordström 2008). Mysids are omnivorous, feeding on phytoplankton, zooplankton, and detritus (Mauchline 1980; Lehtiniemi and Nordström 2008) and are important food items for many fish species (Thiel 1996; Nissling et al. 2007). Female mysids are characterized by a brood pouch (marsupium) in which the entire development of the embryo and larvae takes place (Mauchline 1980). A ‘clutch’ of embryos is deposited in the marsupium and larvae develop synchronously until juveniles are released within a short time span (Mauchline 1980).

We used *Praunus flexuosus* in laboratory exposure experiments as one of the larger littoral mysid species (adults up to 26 mm). *P. flexuosus* is abundant in North-European coastal waters, especially in the littoral zone among macrophytes (Mauchline 1980), and can tolerate large changes in salinity and temperature (Vlasblom and Elgershuizen 1977;

McLusky 1979). The global average in ocean surface pH is predicted to decrease by 0.4 to 0.7 for 2100 and 2300, respectively, under high greenhouse gas emission scenarios (Caldeira and Wickett 2003; RCP8.5, IPCC 2013). Besides the average decrease in ocean pH, near shore littoral zones can also experience reduced pH due to riverine freshwater input, upwelling processes or biological activity (Hofmann et al. 2011). The current surface pH of the investigated *P. flexuosus* population (caught in a western Norwegian fjord) ranges seasonally from around 8.05 to 8.25 (Omar et al. 2016), but even higher variability can be expected in shallow inshore waters on a daily, tidal, and seasonal basis (e.g. Feely et al. 2008; Wootton et al. 2008). Thus, *P. flexuosus* may have evolved a well-developed acid-base regulation system to withstand strong natural variations in pH/pCO_2.

As far as we are aware, there have been no published studies to date that report effects of changing pH/pCO_2 on mysids. To assess potential effects of near future and extended OA scenarios on mysids, we exposed juvenile *P. flexuosus* to four pCO_2 levels (530, 930, 1200, and 1600 μatm) and associated pH (~8, 7.7, 7.6, 7.5). Freshly released juveniles (cohort hatched in the laboratory from field-caught adult females) were exposed for 5 weeks to avoid studying acute stress responses to elevated pCO_2 imposed by short-term exposure. We used juveniles in the experiment to investigate a potentially vulnerable life stage and explored survival, moulting, length, mass, and feeding rates. Additionally, juveniles were kept under two different food regimes (high and low food supply) to investigate whether food availability can help compensate for potentially adverse effects of high pCO_2 exposure. We hypothesise adverse effects of increasing pCO_2 on the measured biological response variables. Further we hypothesise that these potentially adverse effects are stronger under food limitation and that feeding rates are higher under high pCO_2 to satisfy enhanced energetic demands.

Materials and methods

Collection of mysids

Adult mysids of the species *Praunus flexuosus* were collected on 8th and 9th June 2013 using a small dip net (opening 25 cm diameter, ca. 1.5 mm mesh size) in the littoral zone at Glesvær (N 60° 12.42', E 5° 3.01') 15 km north west of the Austevoll Research Station of the Institute of Marine Research, Bergen, Norway. The mysids were transported to the Austevoll Research Station and distributed to several tanks (~40 L) containing ambient seawater to allow for acclimation to laboratory conditions (~2 weeks). During acclimation, adult mysids were fed *Artemia salina* nauplii (henceforth *Artemia*) *ad libitum* and commercial shrimp food (EZ-Larva, Zeigler Bros. Inc., USA). Females were identified visually by the marsupium (larvae containing brood pouch). The mature females released juveniles synchronously at the beginning of the acclimation period. After 2 weeks, shortly before the next hatching event was expected, 48 females were gently transferred individually to jars (1 L, clear polycarbonate) containing ~700 mL seawater using a tea strainer. Juveniles that had hatched within a 24 hr period were collected from 20 females and pooled (to avoid effects of potential differences in terms of maternal provisioning). From this pool, juveniles were distributed randomly to the experimental jars (5 juveniles per jar, see below and Fig. 1) using a wide-mouth pipette before the start of the pCO_2 exposure experiment. The mysids were kept under dim light in a 16:8 h light:dark cycle.

Preparation of different seawater pCO_2 levels

Seawater of different pCO_2 levels was prepared according to Sperfeld et al. (2014). An acidic stock solution (pH~5.8) was produced by releasing CO_2 gas into a seawater tank. The acidic stock solution was mixed with ambient seawater (obtained at 150 m depth, pH~7.94) in three additional mixing tanks to prepare seawater with elevated pCO_2 levels (Fig. 1a). Different amounts of acidic stock solution were added to the mixing tanks using dosage pumps that were controlled by pH-electrodes and pre-set pH transmitters. The pH

transmitters were programmed to open magnet valves above pre-set pH values of 7.5, 7.6 and 7.75, which allowed flow of acidic stock solution into the mixing tanks. The water of different pCO_2 from the mixing tanks were pumped in closed circuits to four header tanks that were mounted above the exposure tanks/jars used for experiments (Fig. 1a), classifying our experimental set-up as B4 design with clumped segregation according to Cornwall and Hurd (2016). An equal water level in mixing and header tanks was controlled by floatation valves. The circulation between the mixing and header tanks was much higher than the drain from the header to the exposure tanks to ensure stable pCO_2 levels (see also Sperfeld *et al*. (2014) for further details about the 'ocean acidification facility' at the Austevoll Research Station).

The investigated *P. flexuosus* population probably experienced an *in situ* pH of around 8.05 to 8.25 depending on season (Omar et al. 2016). To simulate decreases in predicted near future pH of 0.4 for year 2100 and decreases in extended future pH of 0.7 for year 2300 (Caldeira and Wickett 2003; RCP8.5, IPCC 2013), mysids were exposed to four different pCO_2 levels: ~530 μatm (pH 7.94, ambient seawater), ~930 μatm (pH 7.72), ~1200 μatm (pH 7.61), and ~1600 μatm (pH 7.50) (see Table 1 for detailed carbonate chemistry characteristics).

Experimental set-up and protocol

The experiment started with a total of 240 juvenile mysids (mean ± SD, total length: 4.4 ± 0.24 mm, n = 28, individual dry mass: 0.078 ± 0.003 mg, n = 28 distributed among 3 aluminium micro dishes), i.e. with five individuals per experimental jar (Fig. 1b; 4 pCO_2 levels × 12 jars × 5 juveniles = 240). Additional jars with juveniles had been kept at ambient and the highest pCO_2 level to conduct short-term feeding trials at the end of the experiment (see below). Juveniles were kept in experimental jars (1 L, clear polycarbonate) with a flow-through of filtered seawater (O_2 > 85% saturation) of the different pCO_2 levels

164 at ambient temperature (~12.5°C). Twelve experimental jars, with a circular opening at the
165 side (4.5 cm diameter) that was covered by a plankton net of 150µm mesh size, were
166 placed into three tanks (~40 L) of a given pCO_2 level (Fig. 1b, see Sperfeld *et al*. 2014 for
167 further details about the set-up). The tanks were constantly supplied with the seawater of
168 different pCO_2 (flow rate ~25 L h^{-1}) and served as a surrounding water matrix for the
169 experimental jars to stabilize their temperature (~12.5°C). Each jar was constantly supplied
170 with the treatment seawater through a silicone tube (6 mm diameter) with a small pipette
171 tip at the end (inflow). The circular opening at the side that was covered by the plankton
172 net served as outflow into the surrounding matrix water of the tanks. Each experimental jar
173 contained approximately 700 mL of seawater (controlled by the water level in the tanks)
174 with a water exchange of approximately every half hour (flow rate ~1.4 L h^{-1}). Water
175 exchange between individual jars within a tank was very unlikely due to the strong
176 unidirectional water flow out of each jar. We considered the 40 L tank as the replication
177 unit (i.e. n = 3 per pCO_2 level), because jars within one tank could be more similar to each
178 other in terms of non-treatment variables (e.g. temperature, bacterial communities) than
179 jars in different tanks (Cornwall and Hurd 2016). However, potential non-treatment effects
180 imposed by the mixing or header tanks were very unlikely due to the strong flow through
181 character of our experimental facility, diminishing potential bacteria influence or
182 temperature variability as well as ensuring reliably stable pCO_2 levels throughout the
183 experiment (see also Suppl. Mat., Fig. S1).

184 We investigated the interactive effects of elevated pCO_2 levels and limiting food
185 availability to explore the combined effects of multi-stressor scenarios. Because adverse
186 effects of high pCO_2 exposure may only become apparent under food limitation (Thomsen
187 et al. 2013), we offered juveniles two different food supply levels. Half of the jars were
188 supplied with high amounts of *Artemia* (*ad libitum*, high food supply), and the remaining
189 jars were supplied with half of the *Artemia* (limiting amounts, low food supply). This

resulted in 2 jars per replicate/tank of both the high and low food supply treatment per pCO_2 level (Fig. 1b). Food supply was also adjusted to juvenile growth; at high food supply, juveniles were fed daily with 120 *Artemia* per juvenile at the beginning, 160 *Artemia* per juvenile after 7 days, and 200 *Artemia* per juvenile after 17 days (densities of 0.171, 0.229, and 0.286 *Artemia* mL^{-1}, respectively). At low food supply, juveniles were fed daily with half of the amounts, 60 *Artemia* at the beginning, 80 *Artemia* after 7 days, and 100 *Artemia* after 17 days per juvenile (densities of 0.086, 0114, and 0.143 *Artemia* mL^{-1}, respectively). We used short-term (24 h) enriched *Artemia* nauplii (enriched with Larviva Multigain, France) to provide a highly nutritious food for the growth of juveniles. The juveniles grew well by increasing their length in average 2 fold (15-20 fold increase in mass) within the 5 weeks of the exposure experiment, which is in the range of *P. flexuosus* growth observed in a previous laboratory study (Winkler and Greve 2002). The juveniles reached a length of approximately 7 to 10 mm at the end of the experiment and thus were within good margins before maturation (15 mm at 15°C; Winkler and Greve 2002). Even though the low food treatment did not provide the juveniles with low food *sensu stricto* (i.e. juveniles showed good growth), the results indicated that the low food regime still imposed food limitation compared to the high food regime.

Before feeding, experimental jars were inspected daily for dead individuals and moults to determine survival and moulting frequency, respectively. The inter-moult period of juveniles (i.e. the number of days between consecutive moult events) was calculated from the mean moulting time of all individuals in a jar. This was possible as juveniles moulted relatively synchronized during the first 5 moults. Debris on the bottom of the jars was removed regularly using a pipette. The experiment was terminated after 38 days (~5 weeks) and the surviving individuals were immediately measured for their total length using a stereo microscope and subsequently transferred to pre-weighted aluminium micro

dishes for measuring dry mass using an electronic microbalance (Mettler Toledo UMX2, ±1 µg) after drying for at least 48 hr at 60°C.

Short-term feeding trials

Short-term feeding experiments were conducted on 3 consecutive days at the end of the experiment using juveniles from additional jars that had been kept on high food supply at ambient (530 μatm) and the highest (1600 μatm) pCO_2 level (Fig. 1b). Individual juveniles (5-6 per pCO_2 level and day, overall n = 16 per pCO_2) were transferred into small jars filled with 30 mL seawater of the respective pCO_2 level. 30 *Artemia* were added to each jar (start of feeding, density of one *Artemia* mL^{-1}). After 3-4 hours feeding time, juveniles were removed from the feeding jars and immediately measured for their total length and subsequently transferred to pre-weighted aluminium micro dishes for later dry mass measurements (see above). Remaining *Artemia* (dead and alive) were counted and short-term feeding rates (i.e. number of *Artemia* eaten per individual and hour) were determined. Even though most *Artemia* were dead after the short feeding period (in average ~95%), they were still available as food as the juvenile mysids did also feed on non-moving *Artemia* at the bottom of the jars (personal observation). Thus, to avoid overestimation of mysid feeding rates, we counted uneaten dead *Artemia* as surviving prey.

Carbonate chemistry analysis

Water samples were taken from the experimental (40 L) tanks frequently (i.e. 17 times during the 38 days of the experiment; see also Suppl. Mat., Fig. S1) and immediately analysed for pH and total alkalinity (A_T). pH (total scale) was measured using the spectrophotometric technique (U-2900 spectrophotometer, Hitachi, Japan) with m-cresol purple as indicator dye (Clayton and Byrne 1993). Samples in cuvettes were temperature controlled to approach *in situ* temperatures. The small deviations between measurement

241 temperature (t1) and *in situ* temperature (t2) were corrected using the following equation
242 (Gieskes 1969):

243 $$pH_{t2}\ (in\ situ) = pH_{t1} + 0.0114*(t1 - t2)$$

244 A_T was analysed by potentiometric titration (Dickson et al. 2007) using an alkalinity
245 titrator (Radiometer TIM 840 titration manager, Titralab, Germany). Certified reference
246 material provided by Andrew Dickson (Scripps Institution of Oceanography, San Diego,
247 USA; Batch 114) was used to control for uncertainty in A_T measurements.
248 Salinity and temperature were measured using a conductivity meter (Cond 340i,
249 WTW, Germany). Samples for analyses of silicate and phosphate concentrations were
250 preserved in chloroform and measured spectrophotometrically after molybdenum blue
251 reaction using an Skalar autoanalyser (Grasshoff 1965).
252 Carbonate chemistry parameters were calculated using the program CO2sys, version
253 2.1 in Microsoft Excel (Pierrot et al. 2006), with the standard set of carbonate system
254 equations and constants of Mehrbach et al. (1973) after refit of Dickson and Millero
255 (1987). The input variables A_T, salinity, phosphate, and silicate were used as mean values
256 (Table 1) due to low variability and no shift during the experiment.
257
258 ***Data analyses***
259 All statistical analyses and tests of their assumptions were performed using the
260 statistical software R, version 3.2.5 (R Core Team 2016). To account for the B4 design of
261 our CO_2-manipulation system, mixed effect models with exposure tank number (ET-id) as
262 random effect were used to incorporate some source of variance (potentially caused by
263 non-treatment effects) from tank identity (Cornwall and Hurd 2016).
264 Survival was analysed with mixed effects Cox models (Therneau et al. 2003) using the
265 'coxme' and 'survival' package (Therneau 2012). Food regime and pCO_2 level were used
266 as fixed effects, and individual was nested within jar to account for the nested design of

multiple individuals per jar. Likelihood ratio tests were used to test for differences among survival curves (pCO_2 used as factorial variable) and Holm corrected *P*-values were given for multiple comparisons.

Linear mixed effects (LME) models were applied to the other response variables using the 'lme4' package with the random effect as described above. Food regime and pCO_2 level were used as fixed effects and mean values of response variables per jar were used in the models (dry mass was log-transformed to meet assumptions). To test for significance of fixed effects, analysis of variance tables of type III with Satterthwaite approximation for degrees of freedom (df) were used, resulting occasionally in non-integer df. Tukey contrasts following LME models were used for multiple comparisons to identify significant differences among treatments. Differences in feeding rates of juveniles between the two tested pCO_2 levels were analysed using individual mass or length as continuous variable (i.e. as fixed effect covariate to account for size differences) and measurement day as random effect.

Results

Carbonate chemistry

Mixing of ambient seawater with varying quantity of the highly CO_2-enriched seawater resulted in clearly distinct and elevated pCO_2 levels of approximately 930, 1200, and 1600 μatm in the exposure tanks (Table 1; Fig. S1). The measured pH-values in the CO_2-enriched tanks matched well the pH transmitter settings. The pCO_2 of ambient seawater with a calculated average of approximately 530 μatm (Table 1) was higher than the atmospheric equilibrium (i.e. 380 μatm), probably because seawater used in this study originated from deeper depth (150 m).

Survival and moulting

293 The juveniles showed low mortality during the 5 weeks of the experiment (survival
294 ~70%; Fig. 2). Survival was not significantly different among pCO_2 levels when low and
295 high food treatments were combined as well as when low and high food treatments where
296 analysed separately (Table 2; Fig. S2).
297 The juveniles moulted synchronously 5 times during the exposure experiment with
298 clear breaks between the moulting events (Fig. 3a). Very high synchrony was observed
299 during the first three moults, whereas the moulting interval spread more at the fourth and
300 fifth moult (Fig. 3a). A higher number of moults was observed in the ambient control
301 treatment (530 μatm) compared to the CO_2-enriched treatments at the third, fourth, and
302 fifth moulting event (Fig. 3a; Fig. S3; LME model with CO_2-enriched treatments pooled;
303 3^{rd} moult: $F(1,10.7) = 8.75$, $P = 0.013$; 4^{th} moult: $F(1,11.8) = 6.47$, $P = 0.026$; 5^{th} moult:
304 $F(1,11.3) = 8.22$, $P = 0.015$). This resulted in a higher cumulative number of moults in the
305 control treatment at the end of the experiment compared to the CO_2-enriched treatments
306 (Fig. 3b; $F(3,48) = 7.12$, $P = 0.0005$). These patterns in moulting were very similar in both
307 food treatments (Fig. S4) and the cumulative number of moults was not affected by food
308 regime (Table 3).
309 The inter-moult period increased with the age of juveniles from ~6 days for the first
310 moulting event to ~8 days between the 4^{th} and 5^{th} moult (Table 4). Inter-moult periods
311 were not significantly different among pCO_2 levels up to the fourth moulting event (Table
312 4). However, the inter-moult period between the fourth and fifth moult increased with
313 increasing pCO_2 by almost a day (Table 3, Table 4), and this increase was driven by a
314 significant increase (~1.2 d) in the low but not in the high food treatment (Table 4).
315
316 ***Growth***
317 The food supply regime and pCO_2 level had an effect on mean total length and mean
318 dry mass of the juveniles (Fig. 4, Table 3). In average, juveniles were larger and heavier at

high food compared to low food supply, with the effect having a larger magnitude for dry mass than for total length (increase of ~5% in length and ~20% in mass; Fig. 4). The pCO_2 effect is mainly driven by the smaller length (8% decrease) and lower mass (21% decrease) at 1600 μatm compared to 530 μatm in the low food treatment (Tukey contrasts following LME model, total length: $P = 0.0187$; $\log_{10}$(dry mass): $P = 0.0185$). In fact, total length and individual dry mass decreased with increasing pCO_2 in the low food treatment (continuous pCO_2 as fixed effect in LME model, total length: $F(1,24) = 6.92$, $P = 0.015$; $\log_{10}$(dry mass): $F(1,24) = 6.46$, $P = 0.018$), whereas there was no change with increasing pCO_2 in the high food treatment (continuous pCO_2 as fixed effect in LME model for total length and $\log_{10}$(dry mass), $F(1,12) < 0.2$, $P > 0.70$).

Feeding rates

Feeding rates of juveniles, measured in short-term trials after 5 weeks of exposure to ambient and high pCO_2, were affected by pCO_2 after accounting for varying size of individuals (Fig. 5; LME model with day as random effect, pCO_2: $F(1,29.2) = 17.44$, $P = 0.0002$, individual dry mass as covariate: $F(1,30.2) = 9.20$, $P = 0.0049$, mass × pCO_2: $F(1,29.2) = 8.96$, $P = 0.0056$). Smaller individuals that were exposed for 5 weeks to high pCO_2 showed lower feeding rates compared to similar sized individuals that were exposed to ambient pCO_2 (Fig. 5; see also significant interaction term above). Similar results were observed when using total length for accounting for varying individual size (Fig. S5).

Discussion

In our laboratory exposure experiment, using juvenile specimens of the littoral mysid *P. flexuosus*, we observed some subtle direct effects of increasing pCO_2 on the measured biological response variables. These negative effects were mainly caused by the highest applied pCO_2 level (1600 μatm), suggesting that this coastal mysid species will not suffer

dramatically from predicted near future changes in pCO_2. However, the observed adverse effects of increasing pCO_2 on moulting frequency and inter-moult period suggest an increased selection pressure on moulting as key trait of crustacean life history as these animals need to moult for successful growth and reproduction.

It has been postulated that the natural variability in pCO_2 organisms experience may determine their sensitivity or resilience to future OA conditions (Lewis et al. 2013). We exposed *P. flexuosus* to a wide range in pCO_2 levels, as this mysid species inhabits the littoral zone of coastal waters, which can be subject to recurring large pCO_2 fluctuations both diurnally and seasonally (e.g. Feely et al. 2008; Wootton et al. 2008). Our investigated coastal mysid species may have evolved well-developed acid-base regulation systems to tolerate such natural pCO_2 fluctuations. It has been shown in the laboratory that *P. flexuosus* can tolerate large changes in salinity (Vlasblom and Elgershuizen 1977; McLusky 1979) associated with hyper/hypo-osmotic regulation (McLusky and Heard 1971; McLusky 1979). These findings together with the wide distribution of *P. flexuosus* across waters differing strongly in salinity and temperature, from coastal Atlantic to the Baltic Sea (Mauchline 1980), suggests that this mysid species has a high acclimation and/or adaptation potential to varying environmental conditions including changes in pCO_2. However, it remains to be further tested whether this high acclimation and/or adaptation potential is indeed beneficial for long-term changes in pCO_2 as imposed by OA.

Effects of pCO_2 on survival and moulting

The survival of juvenile mysids in our experiment was not affected by pCO_2 during the 5 weeks of exposure. This indicates no direct lethal effect of pCO_2 on *P. flexuosus* within the range tested and fits to the growing evidence that crustacean survival in average is not reduced significantly by OA-relevant increases in pCO_2 (Kroeker et al. 2013). However, moulting frequency was adversely affected by pCO_2. Juveniles moulted more often in the

ambient control treatment compared to all elevated pCO_2 levels, indicating that even relatively small increases in pCO_2 can have adverse effects on moulting of this early life stage. The reduced moulting in all elevated pCO_2 levels was evident only from the 3rd moulting event onwards. We also observed an increasing inter-moult period with increasing pCO_2 between the 4th and 5th moult, but not between earlier moults. These results suggest that adverse effects on some biological response variables become apparent only after prolonged exposure to elevated pCO_2, or alternatively, that later moulting is more sensitive to elevated pCO_2 than earlier moulting.

P. flexuosus may have a strong buffering mechanism for maintaining osmotic and ionic balance in the case of changing pCO_2 due to its strong hyper/hypo-osmotic regulation capability (McLusky and Heard, 1971; McLusky, 1979; Whiteley 2011). Though, moulting could be impaired directly by slight changes in the animal's acid-base status at elevated pCO_2, influencing the activity of enzymes involved in the moulting process, as enzymes are maximally active only within a narrow pH range (Harvey and Ferrier 2011). An impairment of moulting involved enzymes due to lower pH could explain the negative effects on moulting frequency and inter-moult period observed in our study. Alternatively, moulting could be negatively affected at elevated pCO_2 due to insufficient energy availability, as moulting is an metabolically demanding process (e.g. Chan et al. 1988).

Effect of varying food supply on the responses to elevated pCO_2

The inter-moult period of juveniles observed in our study (6 to 9 days) increased with increasing age in accordance to previously described values for *P. flexuosus* (Winkler and Greve 2002). Inter-moult period of crustaceans is known to vary strongly with temperature (e.g. Buchholz 2003), but can also depend to some extent on other external factors such as varying food supply (Buchholz 1991; Qiu et al. 1997). We kept temperature constant but varied food supply in our experiment and found that later inter-moult periods increased

significantly with increasing pCO_2 only within the treatments supplied with low food. A similar pattern was also observed for the size and mass of juveniles at the end of the experiment, i.e. the growth of juveniles decreased with increasing pCO_2 at low food supply but not at high food supply. The results on inter-moult period and growth suggest that higher food supply can be used by the animals to compensate the adverse effects of high pCO_2 exposure. In accordance with this, it has been observed that high food availability can outweigh adverse effects of high pCO_2 exposure in juvenile *Mytilus edulis* (Thomsen et al. 2013). Similarly, the Caribbean coral *Acropora cervicornis* can maintain ambient growth rates at elevated pCO_2 via increased feeding when supplied with sufficient food (Towle et al. 2015).

Elevated pCO_2 levels can impose increasing maintenance costs, e.g. caused by acid-base homeostatic regulation, thereby reducing the energy available for other energetically costly processes such as growth (Seibel and Walsh 2003; Pörtner and Farrell 2008). These increasing energetic demands at elevated pCO_2 can be met by sufficient food acquisition (Thomsen et al. 2013; Ramajo et al. 2016), thus masking potentially adverse effects that would become visible only during periods of food scarcity. Organisms in natural environments are often confronted with strong variations in food availability, highlighting the need for studies that vary food supply together with stressors such as elevated pCO_2 as done in the current experiment on juvenile mysids. Unfortunately, not much is known about food limiting conditions of *P. flexuosus* in nature. The range of food availability and diversity in the littoral zone is broad and in a study investigating *P. flexuosus* and other littoral mysids in the Baltic Sea it is assumed that food was well available (Lehtiniemi and Nordström 2008). However, considering seasonal variation in food availability, food limitation of *P. flexuosus* cannot be excluded, especially in the rather oligotrophic western North Atlantic, making some populations potentially more vulnerable to predicted future changes in pCO_2.

Feeding rate responses to high pCO_2 after prolonged exposure

The minor negative effect on juvenile feeding rates observed after 5 weeks of exposure to elevated pCO_2 is in contrast to our hypothesis of higher feeding rates at high pCO_2 to meet increased energetic demands. Increased feeding rates, for instance, have been observed for the copepod *Centropages tenuiremis* after short-term exposure (4 days) to OA-relevant pCO_2 with concomitantly increased respiration rates (Li and Gao 2012). Likewise, feeding rates and metabolism increased for freshly *in situ*-caught Antarctic krill at elevated pCO_2 in a short-term (<48 h) exposure experiment (Saba et al. 2012), suggesting also an immediate response to meet energetic demands. Our feeding trials however, were conducted with juveniles kept for a longer time period in the laboratory (5 weeks on high food supply) and animals may not sustain the observed short-term adjustments in feeding and associated metabolic rates over longer time periods. Smaller juvenile mysids showed lower feeding rates at high pCO_2 compared to the control at the end of the experiment, suggesting that the prolonged stress slowed down activity probably by impairing metabolic functions (Pörtner and Farrell 2008; Sokolova et al. 2012). This is supported by a study on juvenile European lobster, *Homarus gammarus*, which showed reduced metabolic rates as well as reduced food consumption after 5 weeks exposure to OA-relevant pCO_2 (Small et al. 2016). Thus, prolonged exposure to elevated pCO_2 could have weakened the capability of some individuals to compensate for increased energetic costs by increased feeding, which would ultimately reduce growth. However, we did not observe reduced growth in the treatment with high food supply, where length and mass at the end of the experiment did not decrease with increasing pCO_2. Also in the juvenile European lobster study, inter-moult growth was not lower in the near future OA-treatment (1100 μatm) compared to the control (Small et al. 2016). However, with feeding rates measured only at the end of the experiment, we cannot exclude that juvenile mysids

compensated for increased energetic demands earlier during exposure. Measuring feeding rates across the whole duration of experiments can reveal if and how long organisms mitigate adverse effects of elevated pCO_2 by increased feeding rates.

Conclusions and suggestions for future studies

In our experiments we tested only for direct effects of increasing pCO_2 on biological response variables, but indirect effects via changes in food quality (Rossoll et al. 2012) or species interactions (Diaz-Pulido et al. 2011; Kroeker et al. 2012) may also affect mysid populations in nature. In the present study, we observed some sub-lethal, but potentially important effects of elevated pCO_2 on life history traits of juvenile mysids, suggesting that *P. flexuosus* probably has to adapt to counteract adverse effects of predicted future changes in pCO_2. However, *P. flexuosus* occurs in seawater of very different environmental conditions (Mauchline 1980) and is also the only non-native marine zooplankton species successfully established on the east coast of North America (Ruiz et al. 2011), suggesting a high adaptation potential of this probably highly phenotypically plastic species to changing environmental conditions. Thus, *P. flexuosus* may not suffer dramatically under future OA conditions, but future studies should explore both phenotypic plasticity and the capacity of *P. flexuosus* to adapt over multiple generations (Reusch 2014; Sunday et al. 2014). This could be done by investigating populations from waters differing in natural pCO_2 exposure or by transgenerational studies that require longer-term exposure experiments spanning at least two generations (e.g. Thor and Dupont 2015). Additional studies should also explore the role of pCO_2 variability, because coastal waters, which this littoral species inhabits, can be subject to recurring large fluctuations in pCO_2 both diurnally and seasonally (e.g. Feely et al. 2008; Wootton et al. 2008). We recommend that the pCO_2 range applied in experimental studies should exceed the current natural pCO_2 variation of waters inhabited by *P. flexuosus*. Future studies using mysids should also concomitantly investigate

475 metabolic rates and acid-base status to reveal their capabilities in adjusting physiologically
476 to both short- and long-term changes in pCO_2. A growing number of studies, including this
477 one, indicate the importance of food supply in physiological responses and acclimation to
478 elevated pCO_2, suggesting that variations in food availability should be included in
479 predictions of organisms' responses to future OA scenarios.
480
481 **Acknowledgements**
482 We thank Øyvind Tønnessen and Inger Semb Johansen for technical assistance as well
483 as the editor and three anonymous referees for valuable comments on earlier drafts of this
484 manuscript. This work is a contribution to the ocean acidification research activities at the
485 Institute of Marine Research, Bergen, Norway. Erik Sperfeld acknowledges the
486 International IGB Fellowship Program of the Leibniz-Institute of Freshwater Ecology and
487 Inland Fisheries (IGB, Berlin, Germany) for partial financial support.
488
489 **Compliance with ethical standards**
490 All authors declare they have no conflict of interest. All applicable international,
491 national, and/or institutional guidelines for the care and use of animals were followed.
492
493 **Electronic supplementary material**
494 Figure S1. Measured pH and corresponding pCO_2 during the course of the experiment.
495 Figure S2. Survival curves of juvenile *P. flexuosus* exposed to different pCO_2 levels at both
496 low and high food supply.
497 Figure S3. Average number of moults of juvenile *P. flexuosus* for the five moulting events
498 observed in the experiment.
499 Figure S4. Absolute and cumulative moult number of juvenile *P. flexuosus* over time in the
500 treatments of low and high food supply.

Figure S5. Short-term feeding rates (number of *Artemia salina* nauplii eaten per hour) depending on the total length of individual juvenile *P. flexuosus* after exposure of 5 weeks to ambient pCO_2 (530 μatm) or 1600 μatm.

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666

Figure 1

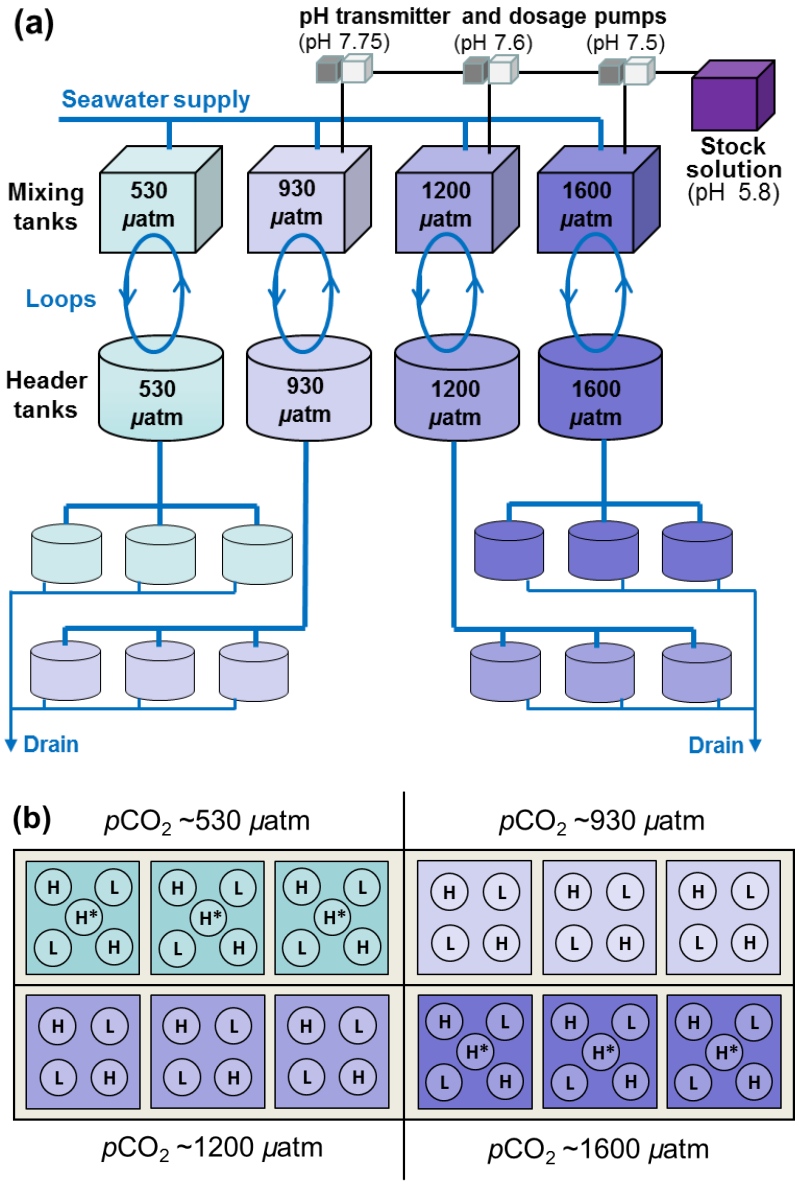

Fig 1 Schematic of (a) the CO_2 manipulation set-up and (b) detailed experimental set-up used in the exposure experiment. Juvenile *Praunus flexuosus* have been exposed to 4 different pCO_2 levels (530, 930, 1200, 1600 µatm) at constant temperature (~12.5°C) in a flow-through system for 5 weeks. The experiment started with 5 individuals per experimental jar (indicated by small circles). Mysids in half of the jars were supplied with high food (H) or low food (L), resulting in 2 jars of both the high and low food treatment per exposure tank as the replication unit per pCO_2 level (i.e. n=3). Asterisks indicate additional jars containing juvenile mysids that have been used in feeding trials after 5 weeks exposure. Experimental jars were placed in tanks providing a surrounding water matrix with the same pCO_2 level (indicated by quadrats, three tanks per pCO_2 level) and there was a unidirectional flow of water through the jars into the surrounding water matrix.

Figure 2

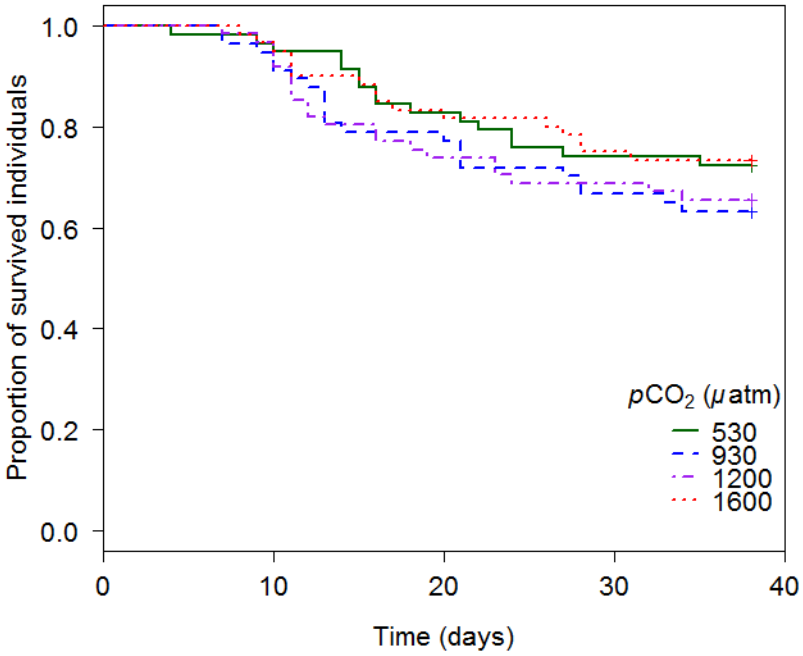

Fig 2 Survival curves of juvenile *P. flexuosus* exposed to different pCO_2 levels (low and high food combined, see Fig. S2 for graphs of both low and high food treatments).

Figure 3

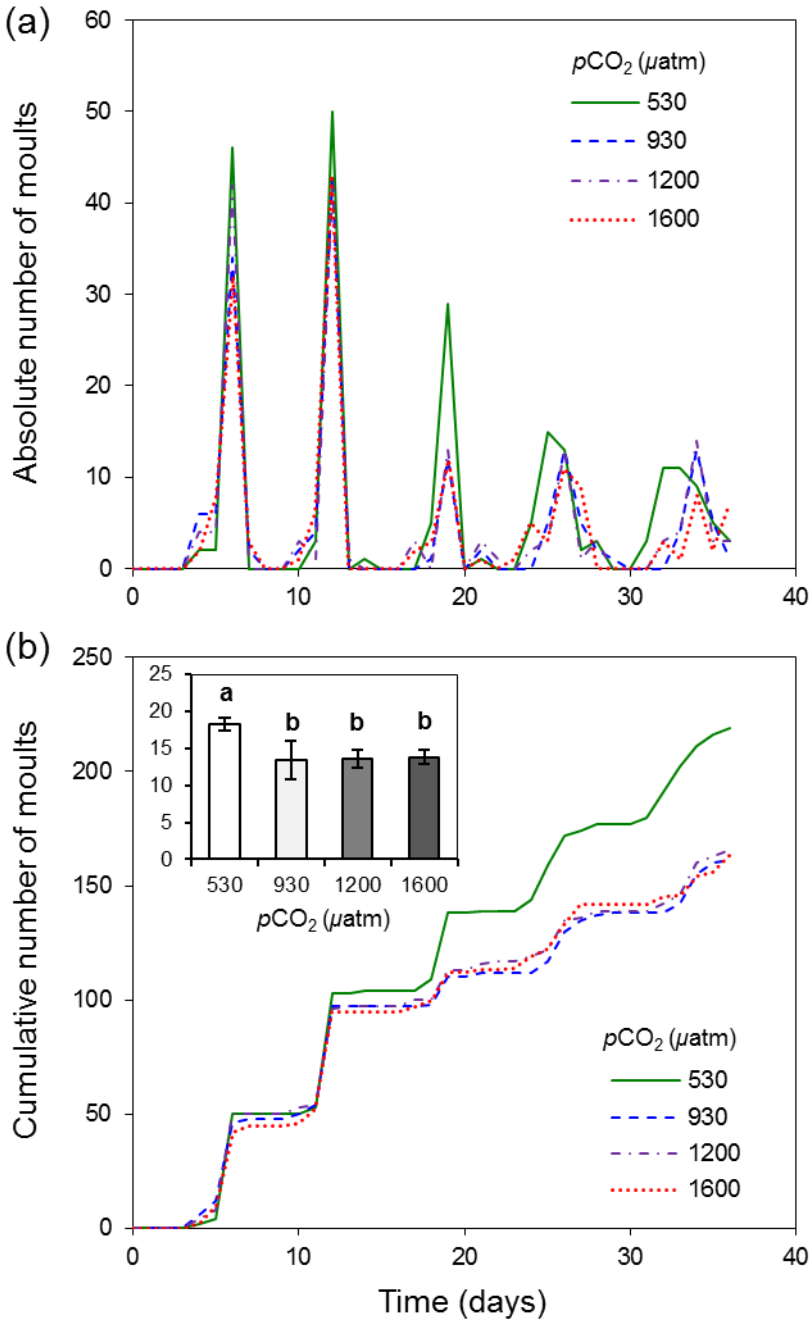

Fig 3 (a) Absolute moult number and (b) cumulative moult number of all juvenile *P. flexuosus* individuals over time (low and high food combined, see Fig. S4 for graphs of both low and high food treatments). Inlet in (b) shows the mean ± SD (n=3) cumulative number of moults per pCO_2 level at the end of the experiment (different letters indicate significant differences among pCO_2 levels, Tukey contrasts, $P < 0.05$).

Figure 4

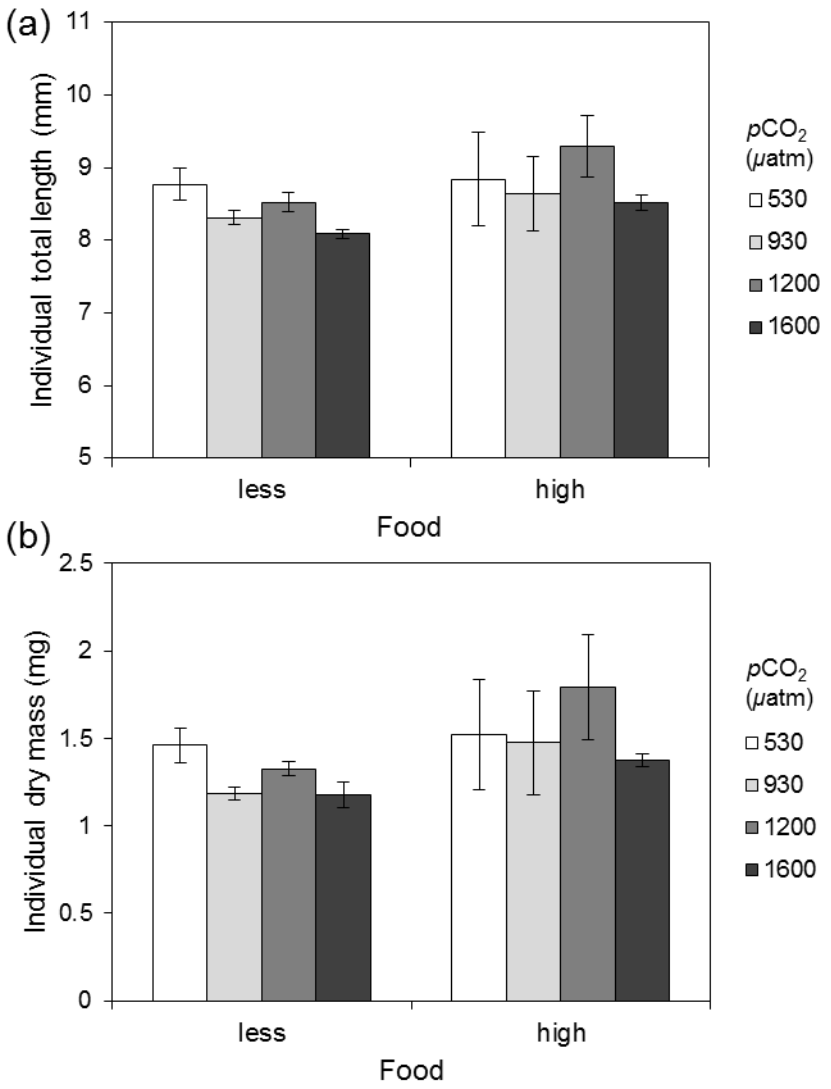

Fig 4 Mean ± SD (n=3) for (a) total length (mm) and (b) dry mass (mg) of juvenile *P. flexuosus* after exposure of 5 weeks to different pCO_2 levels in the treatments of low and high food.

Figure 5

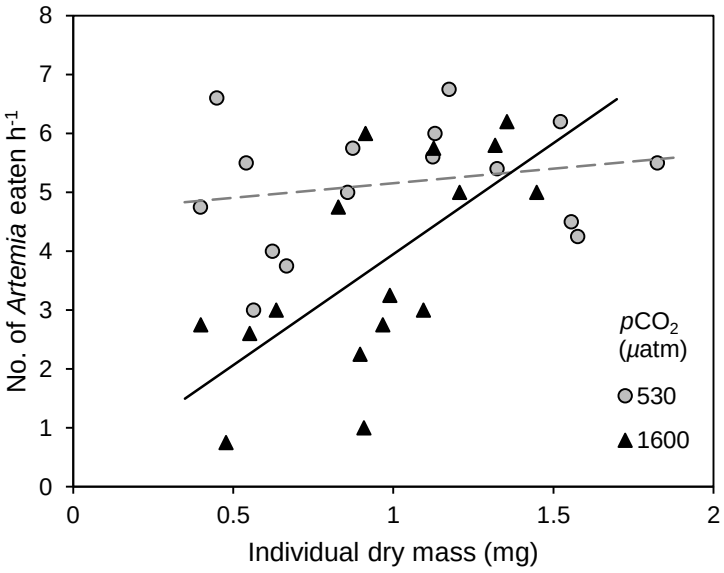

Fig 5 Short-term feeding rates of juvenile *P. flexuosus* after exposure of 5 weeks to 530 μatm (ambient) or 1600 μatm (high) pCO_2 depending on dry mass of individual mysids (see Fig. S5 for feeding rates depending on individual total length). Feeding rates were measured as number of *Artemia salina* nauplii eaten per hour and determined on 3 consecutive days at the end of the juvenile experiment using 5-6 individuals per day from additional juveniles kept at high food supply.

Table 1. Carbonate chemistry of experimental seawater in exposure tanks.

pH transmitter setting	8.0 (amb.)	7.75	7.6	7.5
Temperature (°C)	12.4 ± 0.1	12.5 ± 0.2	12.6 ± 0.2	12.6 ± 0.2
pH (total scale)	**7.94** ± 0.01	**7.72** ± 0.01	**7.61** ± 0.01	**7.50** ± 0.02
*p*CO2 (μatm)	**525.0** ± 14.1	**925.6** ± 27.6	**1208.8** ± 28.8	**1588.6** ± 83.4
C_T (μmol kg^{-1})	2160.4 ± 4.0	2242.0 ± 3.8	2276.5 ± 3.6	2311.1 ± 7.1
HCO_3^- (μmol kg^{-1})	2019.0 ± 5.8	2128.6 ± 4.5	2167.3 ± 3.8	2199.6 ± 6.1
CO_3^{2-} (μmol kg^{-1})	120.2 ± 2.3	76.1 ± 1.8	60.5 ± 1.5	47.6 ± 2.5
CO_2 (μmol kg^{-1})	21.2 ± 0.5	37.3 ± 1.1	48.7 ± 1.3	63.9 ± 3.5
ΩCa	2.86 ± 0.06	1.81 ± 0.04	1.44 ± 0.04	1.13 ± 0.06
ΩAr	1.83 ± 0.04	1.16 ± 0.03	0.92 ± 0.02	0.72 ± 0.04

Average values ± SD (n = 17) calculated using CO2sys (see Materials and Methods for details) with measured temperature, pH, total alkalinity (A_T, 2319.2 ± 8.3 μmol kg^{-1}, n = 6), salinity (35.04 ± 0.06‰, n = 17), phosphate (1.39 ± 0.64 μmol kg^{-1}, n = 6), and silicate (5.89 ± 0.08 μmol kg^{-1}, n = 6) as input variables. Approximate pCO_2 values (highlighted in bold) were used to indicate pCO_2 levels of this study (i.e. 530, 930, 1200, and 1600 μatm).

Table 2. Statistical results of mixed effects Cox models with tank-id as a random effect and food regime and pCO_2 level as fixed effects (individuals nested within jar).

	χ^2	df	*P*
juvenile mysids high and low food			
pCO_2	2.88	3	0.411
food	0.032	1	0.857
pCO_2 × food	5.82	3	0.121
low food			
pCO_2	1.89	3	0.596
high food			
pCO_2	7.58	3	0.056

Table 3. ANOVA type III results for fixed effects of linear mixed models fitted to the cumulative number of moults at the end of the experiment, inter-moult period between the fourth and fifth moult, mean total length (mm), and mean dry mass (µg) with pCO_2 (µatm) and food regime as fixed effects (and tank-id as a random effect).

	df (num,denum)	*F*	*P*	
	Cumulative number of moults			
pCO_2	3,48	7.42	0.0004	***
food	1,48	0.02	0.88	
pCO_2 × food	3,48	0.68	0.57	
	Inter-moult period 4^{th}-5^{th} moult			
pCO_2 (cont.)	1,7	10.6	0.014	*
food	1,25.9	0.90	0.351	
pCO_2 × food	1,26.5	2.63	0.117	
	Total length			
pCO_2	3,48	4.54	0.007	**
food	1,48	9.92	0.003	**
pCO_2 × food	3,48	1.43	0.247	
	$\log_{10}$(dry mass)			
pCO_2	3,48	3.80	0.016	*
food	1,48	16.01	0.0002	***
pCO_2 × food	3,48	1.59	0.203	

Note that pCO_2 was set to a continuous fixed effect in the model fit with inter-moult period and not factorial as in the other model fits. Degrees of freedom (df) are calculated using Satterthwaite approximations and thus can be non-integers. Significant effects are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Table 4. Intermoult periods (in days) observed in the experiment using juvenile *P. flexuosus* exposed to different pCO_2 (μatm) levels.

pCO_2 (μatm)	to 1st	1st-2nd	2nd-3rd	3rd-4th	4th-5th
			low food		
530	6.96 ± 0.07	6.00 ± 0.00	6.95 ± 0.20	6.45 ± 0.25	7.59 ± 0.22*
930	5.68 ± 0.16	6.10 ± 0.07	7.14 ± 0.25	7.14 ± 0.75	7.92 ± 0.12*
1200	5.77 ± 0.23	6.28 ± 0.20	7.13 ± 0.22	7.67 ± 1.33	8.54 ± 0.65*
1600	5.75 ± 0.54	6.16 ± 0.39	6.92 ± 0.15	7.18 ± 0.16	8.77 ± 0.88*
			high food		
530	5.84 ± 0.15	6.16 ± 0.15	6.86 ± 0.43	6.93 ± 1.21	7.64 ± 0.46
930	5.66 ± 0.09	6.24 ± 0.19	7.44 ± 0.51	6.85 ± 0.60	7.60 ± 0.15
1200	5.70 ± 0.26	6.07 ± 0.21	7.07 ± 1.78	6.25 ± 1.16	7.92 ± 0.12
1600	5.74 ± 0.13	6.03 ± 0.04	6.23 ± 0.04	7.02 ± 1.44	7.89 ± 0.79
			low and high food combined		
530	5.90 ± 0.10	6.08 ± 0.07	6.91 ± 0.23	6.69 ± 0.66	7.61 ± 0.21*
930	5.67 ± 0.12	6.17 ± 0.12	7.35 ± 0.33	6.92 ± 0.23	7.76 ± 0.01*
1200	5.73 ± 0.23	6.17 ± 0.05	7.12 ± 0.72	6.96 ± 1.21	8.14 ± 0.09*
1600	5.75 ± 0.29	6.09 ± 0.19	6.72 ± 0.29	7.25 ± 0.56	8.39 ± 0.64*

Average values ± SD (n=3) are given; numbers in parentheses indicate number of observations/jars. * indicates a significant increase along the pCO_2 gradient (pCO_2 as continuous variable low food: $F(1,19) = 11.15$, $P = 0.0035$; low and high food combined: $F(1,41) = 10.89$, $P = 0.0020$).