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Elevated pCO_2 does not impair performance in autotomised individuals of the intertidal predatory starfish *Asterias rubens* (Linnaeus, 1758)

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HIGHLIGHTS

- Ocean acidification research requires further understanding on the interactions with other stressors
- We examined the combined effects of pCO_2 and arm autotomisation on *Asterias rubens*
- Neither stressor affected mortality, growth, arm regeneration, righting time or arm calcium content
- Lipid content in the pyloric caeca increased in response to elevated pCO_2
- *A. rubens* appears unaffected by short-term exposure to pCO_2 levels predicted for 2100

ABSTRACT

The impacts of ocean acidification remain less well-studied in starfish compared to other echinoderm groups. This study examined the combined effects of elevated pCO_2 and arm regeneration on the performance of the intertidal predatory starfish *Asterias rubens*, as both are predicted to come at a cost to the individual. A two-way factorial experiment (~400 µatm vs ~1000 µatm; autotomised vs non-automised individuals) was used to examine growth rates, lipid content (pyloric caeca and gonads), and calcium content (body wall) in both intact and regenerating arms, as well as subsequent effects on rate of arm regeneration, righting time (behaviour) and mortality over 120 days. Autotomised individuals tended to show lower (not significant), survival and growth. Elevated pCO_2 had no effect on mortality, body growth, arm regeneration, righting time or arm calcium content. Lipid content was higher in the pyloric caeca, but not in the gonads, in response to elevated pCO_2 irrespective of autotomisation. The results of the study suggest that adult *A. rubens* remain unaffected by increased pCO_2 and/or arm autotomy for 120 days, although longer term experiments are necessary as the results indicated that survival, growth and calcification may be impaired with longer-term exposure to elevated pCO_2.

Key-words: Global change; Water chemistry; Ocean Acidification; Asteroidea; growth; regeneration, Righting Time Response.

1. Introduction

The acceleration of the release of anthropogenic carbon dioxide (CO_2) into the atmosphere and consequential absorption by the oceans has resulted in reductions in surface seawater pH and carbonate saturation (Ω) over the last 250 years (Doney et al., 2009; Feely et al., 2009). Since 2005, there has been considerable global research effort to predict how marine organisms, particularly those that calcify, will respond to the changes in seawater chemistry known as ocean acidification. Application of the RCP8.5 'business as usual' scenario, which predicts increases of 1000+ µatm pCO_2 and subsequent reductions in seawater pH by a further 0.3-0.4 pH units by the end of the century (IPCC, 2014), has generally indicated negative biological and ecosystem effects (e.g. Browman, 2016; Kroeker et al., 2010, 2013; Nagelkerken and Connell, 2015). Although, as stated by Browman (2016), a broader perspective is required as there are many gaps in our knowledge about compensatory responses, such as calcification, adaptive responses and the interaction between simultaneous environmental changes, as well as the influence of life stages, season and nutritional condition. To date, it has been demonstrated that sensitivities to elevated pCO_2 concentrations vary among taxa, species and populations (Kroeker et al., 2013; Przeslawski et al., 2015; Whiteley, 2011; Wittman and Pörtner, 2013). It is thought that species living in more environments that experience greater fluctuations in pH and temperature (e.g. vents, coastal shelf sea and the intertidal) may be more resilient to future environmental change due to their pre-adapted physiological capacities and plasticity (Collard et al., 2013; Hendriks et al., 2010; Whiteley et al., 2018).

Studies on the response of Echinodermata to near-future pCO_2 conditions have tended to focus on echinoid sea urchins and stellate and ophiuroid brittlestars (e.g. Dupont et al., 2008, 2010b; Hu et al., 2014; Kroeker et al., 2010, 2013; Rodríguez et al., 2017; Ross et al., 2015; Suckling et al., 2015; Wood et al., 2008; 2010; 2011). Stellate asteroid starfish have received less attention until recently (e.g. Appelhans et al., 2012; 2014; Hu et al., 2018; Keppel et al., 2015), despite their important ecological role as carnivores in benthic marine ecosystems where they drive

keystone predatory pressure and community structure (Calil et al., 2009; Freeman et al., 2001; Kayal el al., 2012; Menge, et al., 1999). Asteroids are structurally different from ophiuroids in that the arms have external skeletal support, and contain both gonads and pyloric caeca (digestive organs and nutrient reserves). Moreover, asteroids have a remarkable capacity to regenerate their arms after autotomy of up to 75% of their body mass in order to avoid predators, to replace damaged or infected appendages, or to recover from fishing disturbance (Lawrence, 2010; Ramsay et al., 2001b). Autotomy in most asteroid families is a common feature taking place along a single plane proximal to the disc resulting in the loss of an entire arm including body wall and viscera, amounting to a loss of 20% of their capacity to store nutrients and reproduce (Lawrence, 2010; Schram et al., 2011). The loss of an arm can compromise nutrient uptake by affecting the ability of the starfish to move and capture prey (Ramsay et al., 2001a). Regeneration can also come at a cost, due to the synthesis of new tissues, leading to a diversion of energy away from growth, nutrient stores and reproduction in the remaining, intact arms (Díaz-Guisado et al., 2006; Lawrence and Larrain, 1994; Ramsay et al., 2001a). Changing environmental conditions, such as elevations in seawater pCO_2 have also been shown to reduce rates of growth and development during early life history stages, attributed to the costs associated with compensatory adjustments in body fluid pH and calcification. For instance, growth rates are restricted by elevated CO_2 in the larvae of sea urchin *Strongylocentrosus* species (Stumpp et al., 2011, 2013), and in juvenile and larval stages of *A. rubens* (Appelhans et al., 2012, 2014; Hu et al., 2018; Keppel et al., .2015). These studies suggest competing demands for energy reserves for arm regeneration in starfish under conditions of elevated pCO_2, although the combined effects of autotomy and elevated pCO_2 (780 μatm) had no effect on the rate of arm regeneration or energy resources in adult tropical starfish *Luidia clathrata* after 97 days (Schram et al., 2011). As compensatory responses to elevated CO_2 vary substantially among species within the same taxa (Dupont et al., 2010b; Kroeker et al., 2010; Stumpp et al., 2012), and as tropical species do not appear to augment their metabolic rates as much as temperate species in terms of adjustments to high CO_2 (Kelley and Lunden, 2017), we

were interested in establishing whether arm regeneration is more sensitive to elevated CO_2 in temperate adult starfish species.

The purpose of the current study was to examine the combined effects of experimental autotomy in the common temperate starfish, *Asterias rubens*, exposed to the RCP8.5 pCO_2 levels predicted for 2100 (~1000 µatm; IPCC, 2014). *A. rubens* is widely distributed in the northeast Atlantic Ocean from the mid-shore down to 650 m (Pearse et al., 1987) and is an important and voracious predator of benthic epifauna, such as barnacles, mussels and oysters, sometimes competing for food with commercial fish species (Anger et al., 1977; Sloan and Aldridge, 1981). We exposed adult *A. rubens* to elevated pCO_2 in a two-way factorial design (ambient vs elevated pCO_2; non-autotomised vs autotomised individuals) for 120 days. We were especially interested in the effects of these treatments on the opposing demands of increased compensation for elevated pCO_2 and the costs associated with regeneration, in a laboratory-based situation where the food availability was standardised across treatments. To this end, we investigated rates of growth in both intact and regenerating arms, and examined associated changes in an important energy resource (lipids), which can constitute 30% of the dry weight of the pyloric caeca (Lawrence, 2010). We also determined changes in calcium content of the body wall of both intact and regenerating arms to assess whether calcification rates follow similar responses to those observed in ophiuroid brittlestars, such as *Amphiura filiformis* where elevated pCO_2 increased calcium content of the regenerating arm (Wood et al., 2008). Finally, we observed righting responses to assess any subsequent effects on the behaviour or ability of the starfish to respond to challenging and stressful situations (e.g. Lawrence and Cowell, 1996). Overall, the study aimed to improve our understanding of whether an asteroid species living in rock pools from the mid-shore downwards and exposed to variable pCO_2, but also with a remarkable ability to regenerate body parts, is susceptible to elevated pCO_2, and whether there are any repercussions to energy resources and performance.

2. Materials and methods

2.1. Sampling, experimental design and acidification of natural seawater

Sixty common starfish *Asterias rubens* L. were collected in February 2015 from the intertidal zone in the Menai Strait, North Wales, UK (53º 14'N, 04º 09'W), placed in plastic buckets with seawater and transported back to the School of Ocean Sciences (SOS) at Bangor University (UK). In the laboratory, the starfish were placed in a stock tank (120 x 60 x 30cm) supplied with flow-through seawater pumped onshore from the Menai Strait to acclimatize to the experimental conditions (salinity of 32, 12°C, pH 8.0, 12h Light: 12h Dark photoperiod). After 21 days acclimatisation, the radius of the arm opposite the madreporite of each starfish was measured (±1mm) using Vernier callipers and each animal was blotted dry with absorbent paper towel, weighed (± 0.01g) and placed individually into an experimental 3.5 L plastic jar (with holes all across the surface to allow flow-through of water) within the stock tank for a further 7 days acclimatisation. During acclimatisation and subsequent experimental exposure, starfish were fed *ad libitum* with live blue mussel *Mytilus edulis*. Thirty individuals (15 per treatment) had one arm autotomised, opposite to the position of the madreporite, along the single autotomy plane located at the base of the arm by applying pressure half way down the arm with a pair of pliers (Loh and Todd, 2012). Loss of haemolymph from the wound is minimal using this method due to rapid muscle contraction and clotting to form a wound epidermis (Hernroth et al., 2010).

The elevated pCO_2 treatment used was based on the reduction of 0.3-0.4 pH units (the 'business-as-usual' IPCC scenario) expected to occur by the end of the 21st century (IPCC, 2014). Starfish were exposed to one of two pCO_2 levels: ambient pCO_2 (~400 µatm) and elevated pCO_2 (~1000 µatm). Ambient pCO_2 was achieved by bubbling air into a mixing tank (350 L) and elevated pCO_2 was achieved in a separate mixing tank by controlling the flow of a gas mixture of air and pure CO_2 via gas line restrictors and flow meters as detailed by Findlay et al. (2008). Water from the mixing tanks was pumped into two separate header tanks (100 L), one for each treatment, and from each header tank into the holding tanks (three per treatment) by gravity before running to

147 waste. Temperature was controlled via an in-line thermostatic heater balanced against a chiller
148 (details in Whiteley et al., 2018). Seawater parameters were measured following the methods
149 outlined in Suckling et al. (2014) and Whiteley et al. (2018). In summary, pH, salinity and
150 temperature were measured daily using a multi-parameter water analyser (Mettler Toledo
151 SevenGo) and a pH probe (LE pH Electrode LE438-IP67) calibrated with NIST (National Institute
152 of Standards and Technology) certified pH buffer solutions twice weekly. Phosphate and silicate
153 were sampled monthly via GFF filtered seawater (60 mL) and analysed using a flow injection auto-
154 analyser (Lachat 8500). Seawater (100 mL) samples were collected in borosilicate glass bottles
155 and TCO_2 was immediately analysed (Ciba-Corning TCO_2 Analyser, Olympic Analytical, UK).
156 These parameters were then used to calculate pCO_2 and carbonate saturation states (Ω) using
157 CO_2SYS (Pierrot et al., 2006). Over the 120-day experimental period, all the seawater parameters
158 measured (pH, salinity and temperature) were stable in both pCO_2 treatments showing controlled
159 laboratory conditions (Table 1). Control seawater was supersaturated with respect to calcite and
160 aragonite while high pCO_2 seawater was supersaturated with respect to calcite but under saturated
161 with respect to aragonite (Table 1).

162 Acclimatised starfish were allocated to one of four treatments (15 starfish per treatment) in
163 a fully orthogonal design: pCO_2 (~400 µatm; ~1000 µatm) and arm autotomisation (autotomised;
164 non-autotomised). Three replicate holding tanks were used for each treatment, each with 5
165 autotomised and 5 non-autotomised starfish (Figure 1). At the end of the experiment (120 days),
166 the starfish were dissected and the pyloric caeca and gonads from all the arms pooled and weighed
167 to the nearest 0.01g. Samples from the pyloric caeca and gonadal tissues and from the arms were
168 retained for analysis of lipid (see section 2.3) and calcium content (see section 2.4), respectively,
169 and stored at –20°C until analysis.

170

171 *2.2. Mortality, growth, arm regeneration and behaviour*

Mortality, growth, arm regeneration and righting behaviour were determined for all starfish on days 0, 30, 60, 90, and 120 days of the experimental exposures. Mortality was checked daily and expressed as mean cumulative mortality per replicate per 30 day time period. To evaluate body growth and arm regeneration, starfish were removed from the holding tanks, blotted dry with a paper towel for 30s, and immediately weighed (± 0.01g). The starfish were then placed on a laminated sheet of 1 mm graph paper, photographed and the length of all arms measured using ImageJ (Abramoff et al., 2004). Regeneration capacity was calculated by dividing the length of the regenerating arm by the length of the longest arm at each experimental time. The 'righting time response' (RTR) was tested before measuring body mass and arm regeneration to prevent stressing the individuals. Starfish were placed upside down with the aboral surface downwards in an experimental aquarium maintained at the appropriate experimental conditions and the time taken to return the oral face to the substrate was recorded (Held and Harley, 2009; Joly-Turquin et al., 2009; Kleitman, 1941; Lawrence and Cowell, 1996). RTR was calculated as: RTR = 1,000/righting time (s; Watts and Lawrence 1990) where a higher RTR value indicates that the individual rights itself more quickly.

2.3. Lipid energy content

Lipid content of the pooled pyloric caeca and gonads collected from each of six randomly-selected starfish per treatment was determined using the sulpho-phospho-vanilline method modified by Torres et al. (2007). Tissue subsamples (20 mg) were homogenized in ice cold 400 µl deionized water, and extracted in a 40 µl subsample by addition of 180 µl chloroform:methanol solution (2:1). After centrifugation at 4°C for 20 min at 14,000 rpm (Multispeed refrigerated centrifuge, RK121R, ALC), the lower chloroform phase was decanted and warmed at 60°C for approximately 40 min. After cooling, 200 µl of concentrated sulphuric acid was added and samples were incubated at 95°C for 10 min. A sub-sample of 20 µl was pipetted in triplicate into a microplate and 300 µl 8 mM phospho-vanillin added. Samples were incubated for 40 minutes at

198 room temperature in the dark and then measured in a microplate reader (Multiscan FC, Thermo
199 Scientific) at a wavelength of 530 nm using cholesterol as standard. The lipid content in the pyloric
200 caeca and gonad samples for each starfish was converted into energy equivalents using an enthalpy
201 of combustion of 39.5 kJ g^{-1} (De Coen and Janssen, 1997), and expressed as mass-specific values
202 (kJ mg^{-1} wet mass) and as whole-tissue values (kJ $tissue^{-1}$).
203
204 *2.4. Calcium content*
205 At the end of the experiment calcium content was measured in the arm(s) of six randomly-
206 selected starfish per treatment. Samples were taken from the left arm opposite to the position of
207 the madreporite of all individuals and from the regenerating arm of the autotomised animals. Prior
208 to sampling, each arm to be sampled was rinsed with distilled water, blotted dry, and approximately
209 30 mg of intermediate part of arm (including ectoderm, calcareous plate and peritoneal epithelium
210 tissues) was removed by dissection. Each sample was weighed (± 0.01g), dried in an oven at 60°C
211 for 24 hours and reweighed before digestion in 400 ml 70% nitric acid at 42°C overnight. The total
212 calcium content in each sample was then determined in a 4 ml aliquot (x100 dilution) using a flame
213 photometer (Sherwood Scientific Flame Photometer Model 410) and expressed as µmol Ca^{2+} mg^{-1}
214 dry mass.
215
216 *2.5 Statistical analysis*
217 All data were tested for normality (Shapiro-Wilk test) and homogeneity of variance
218 (Levene's test) and data that did not meet these requirements were log-transformed [Body mass =
219 log(x); RTR = log(x+1)] prior to parametric statistical analysis. Body mass and RTR were analysed
220 using a Linear Mixed Model (LMM) with pCO_2, autotomy and time as fixed factors and tank and
221 individual (id) as random factors in the model $P \sim time + autotomy + pCO_2 + time*autotomy +$
222 $time*pCO_2 + autotomy*pCO_2 + time*autotomy*pCO_2 + (1 | id) + (1 | tank)$. Cumulative mortality
223 was assessed using the same three fixed factors in the LMM with tank as a random factor. Relative

growth was assessed visually by calculating the formulae [final grams wet mass – initial grams wet mass)/initial grams wet mass × 100] (Gooding et al., 2009). Arm regeneration was analysed using a similar LMM but with two fixed factors (pCO_2 and time) and tank/id as random factors. As arm regeneration was expressed as proportional data, it was arcsine-square-root transformed prior to statistical analysis (Sokal and Rohlf, 1995). All LMMs were run in R (R Core Team, 2017) using the lme4 package (Bates et al., 2015) as fully saturated models (i.e. including all interactions between fixed effects) and the best model, as determined by AIC model selection (using the 'dropterm' function) was selected. In addition, the final model was checked by visual inspection of the residuals plot and the Q-Q normal plot. Mass-specific and whole-tissue lipid energy content in the pyloric caeca and gonads, and calcium content of the intact and regenerating arms, were analysed using a two-way (pCO2 and autotomy) ANOVA as LMM was not appropriate given the sample sizes (i.e. two individuals per treatment replicate) and a single terminal measurement. The calcium content of the regenerating arms was compared between the two pCO_2 treatments using a Student's t test. ANOVA and t-test analyses were conducted in SPSS v25.

3. Results

3.1. Mortality

Mortality increased with time (LMM, $p<0.001$; Table 2; Figure 2) with 20 starfish dead after 120 days. There was a significant interaction between autotomy and time (LMM, $p=0.009$; Table 2; Figure 2) with cumulative mortality higher amongst autotomised starfish (13 vs 7 starfish; Figure 2) although this was not significant ($p=0.41$). The LMM indicated a large tank effect on mortality (Table 2; see Figure 2). Individuals exposed to higher pCO_2 showed a tendency of 33% reduction in cumulative mortality (8 vs 12 starfish) at the end of the experiment than individuals reared at ambient values.

3.2. Body growth and arm regeneration

All starfish increased in body mass during the experiment (LMM, $p<0.001$; Table 2; Figure 3A) but autotomised individuals tended to have lower body mass over time than non-autotomised starfish (LMM, $p=0.078$; Table 2), even at the end of the experiment after partial arm regeneration. However, autotomized starfish reared under low pCO_2 showed faster rates of relative growth from day 60 onwards (Figure 3B).

The rate of arm regeneration of starfish was relatively continuous over the 120 day exposure period (ca. 2.5% per week; LMM, $p<0.001$; Table 2), with no overall effect of pCO_2 or its interaction with time (Table 2; Figure 4). After 120 days, average regeneration was *ca.* 42% of the size of the longest arm (Figure 4).

3.3. Behaviour (Righting Time Response – RTR)

The results of the LMM indicated that there was no effect of time, autotomy or pCO_2 on the righting time responses (RTR) of starfish (all $p>0.05$; Table 2). RTR values tended to be higher among autotomised individuals at ambient pCO_2 on days 30 and 60 but this was due to high variability in RTR values for individuals in one of the replicate tanks for this treatment (Figure 5). Apart from this, the four treatments all showed comparable ranges of individual RTR values on the five measurement times (Figure 5).

3.4. *Lipid content*

Lipid energy content in pyloric caeca were higher (ranging from 2.10 ± 0.10 to 3.01 ± 0.15 kJ mg^{-1}) than in the gonads (ranging from 1.14 ± 0.12 to 1.61 ± 0.14 kJ mg^{-1}) in all treatments (ANOVA; tissue (pCO_2*autotomy): $F_{5,136}=45.03$, $p<0.001$; pCO_2: $F_{1,136}=5.74$, $p=0.018$; autotomy: $F_{1,136}=0.003$, $p=0.957$) (Figure 6A). The total lipid energy content estimated in the pyloric caeca (ranging from 7.22 ± 1.73 kJ to 12.98 ± 1.13 kJ $tissue^{-1}$) was also higher than for the gonad (ranging from 0.23 ± 0.11kJ to 0.56 ± 0.19 kJ $tissue^{-1}$) in all treatments (ANOVA; tissue (pCO_2*autotomy): $F_{5,40}=35.01$, $p<0.001$; pCO_2: $F_{1,40}=4.52$, $p=0.04$; autotomy: $F_{1,40}=0.001$, $p=0.983$) (Figure 6B).

Lipid energy content in the pyloric caeca (kJ mg^{-1} wet mass) and the whole tissue (kJ $tissue^{-1}$) were higher in the elevated pCO_2 treatment when compared to ambient conditions, with no effect of autotomy (see Tukey test results in Figure 6A and B, respectively). There was no effect of pCO_2 or autotomy on the lipid energy content in the gonads, expressed as either mass-specific or total tissue energy equivalents (see Tukey test results in Figure 6A and B, respectively).

3.5. Calcium content

The mean calcium content of the intact arms was not affected by pCO_2 ($F_{1,20}$=0.70; p=0.41) and autotomy ($F_{1,20}$=0.51; p=0.48) and revealed a non-significant interaction between them ($F_{1,20}$=1.30; p=0.27) (Figure 7A). The calcium content of the regenerating arms was also not affected by pCO_2 (t_{10}=0.76; p=0.47), although the average values in individuals reared at higher pCO_2 (5.89 ± 6.12 μmol Ca^{2+} mg^{-1} dry mass) were 33% lower than those reared at ambient pCO_2 (8.84 ± 6.52 μmol Ca^{2+} mg^{-1} dry mass) (Figure 7B).

4. Discussion

The present study demonstrates that near future elevations in seawater pCO_2 with associated changes in seawater carbonate chemistry had little effect on the rate of regeneration of autotomised arms and righting time in adult *A. rubens.* Similar responses were observed in the tropical starfish, *Luidia clathrata* exposed to ~780 μatm pCO_2 (pH 7.8) for a slightly shorter exposure time (97 days; Schram et al., 2011). In contrast, exposure of the brittlestar *Amphiura filiformis* to lower seawater pH levels (pH 6.8, 7.3 or 7.7; 40 days) caused autotomized arms to regenerate faster and to acquire more calcium compared with controls (pH 8.0) (Wood et al., 2008). However, seawater pH of 7.3 and associated undersaturated carbonate conditions had little effect on regeneration in another species of brittlestar, *Ophiura ophiura*, over a 6 week period (Wood et al., 2010), but a significant negative effect on arm regeneration in a polar species, *Ophiocten sericeum* (20 day exposure; Wood et al., 2011). Such variable responses can be attributed to the capacity of species to

compensate physiologically for the changes in seawater carbonate chemistry (Melzner et al., 2009; Whiteley, 2011). The capacity for acid-base regulation and biomineralisation in adult echinoderms is species-specific and appears to be greater in those species normally exposed to natural CO_2 fluctuations (Byrne and Przeslawski, 2013; Dupont et al., 2010a). Echinoderms are generally poor compensators in terms of buffering changes in body fluid pH via acid-base regulation but sea urchins appear to be better than sea stars (Collard et al., 2013; Dupont and Thorndyke, 2012; Stump et al., 2012). Compensatory responses, however, may come at a cost and lead to a depletion in energy reserves (Wittman and Pörtner, 2013). Overall, the lack of an effect of elevated pCO_2 on rates of regeneration in *A. rubens* could be related to the fact that all animals were fed regularly during the experiment ensuring that energy reserves were sufficient to fuel tissue accumulation and any compensatory responses (Lawrence, 2010). The longer term effects of both elevated pCO_2 exposure and repeated autonomy events remain unknown, especially under conditions of limited food availability where reduced energy intake may limit the potential for compensation. Studies examining the effects of elevated seawater pCO_2 on *A. rubens* have used *ad-libitum* feeding regimes (Appelhans et al., 2012, 2014; Hu et al., 2018; Keppel et al., 2015; this study) and performance under nutrient limitation remains unknown.

By the end of the exposure period, the energy available via the lipid reserves in the pyloric caeca were higher under elevated rather than ambient pCO_2, but lipid energy content in the gonads was unaffected. In echinoderms, lipids accumulated from the diet are stored mainly as triglycerides in the pyloric caeca (Oudejans and van der Sluis, 1979b; Prowse et al., 2008) and are an important energy store, especially for reproduction. The size of both the pyloric caeca and the gonads varies in asteroid echinoderms over the reproductive cycle with gametogenesis occurring in *A. rubens* in September-March and spawning in April-June (Oudejans et al., 1979; Oudejans and van der Sluis, 1979a; Vevers, 1949). Previous studies have reported that lipids are translocated from the pyloric caeca to the gonads during their development in asteroid starfish (Lawrence and Lane, 1982; Oudejans and van der Sluis, 1979a; 1979b; Raymond et al., 2004; Rubilar et al., 2008). However,

328 the accumulation of lipid reserves in the pyloric caeca during elevated pCO_2 exposure in *A. rubens*
329 in the current study could signify a shift in energy balance between cellular energy availability and
330 energy consumption (De Coen and Janssen, 1997). It is possible that starfish exposed to elevated
331 pCO_2 were experiencing metabolic depression either to conserve energy reserves, as observed in
332 the intertidal starfish *Parvulastra exigua* exposed to a pCO_2 of ~750 µatm (pH 7.8; McElroy et al.,
333 2012), or as a response to the failure to maintain internal acid-base homeostasis (Pörtner, 2008),
334 although juvenile *A. rubens* show no reduction in metabolic rate at low pH (Appelhans et al., 2014;
335 Collard et al., 2013). However, it is also possible that regular feeding of the starfish enabled them
336 to accumulate sufficient energy stores to support the costs of regeneration during exposures to near
337 future pCO_2 levels of ~1000 µatm.

338 In the present study, growth of *A. rubens* expressed as changes in body mass was not
339 affected by pCO_2 or autonomy. Similarly, Schram et al. (2011) reported growth in adult tropical
340 starfish *Luidia clathrata* was unaffected by similar levels of pCO_2 exposure (pH 7.8, pCO_2 780
341 µatm) after 97 days following autotomy. In contrast, previous studies on adult *A. rubens* have
342 reported reduced growth rates with increasing pCO_2 (Appelhans et al., 2012; Keppel et al., 2015),
343 however, these differences may be attributable to differences in experimental design and/or
344 conditions between studies. Appelhans et al (2012) found no difference in growth at 650 (pH 8.06)
345 and 1250 µatm (pH 7.84) after 70 days (as seen in our study after 60 days; see Figure 3) at 13°C
346 but reduced growth at 3500 µatm (pH 7.36). Keppel et al. (2015) reported reduced growth after 70
347 days at pH 7.9 compared to pH 8.1 for adult *A. rubens* reared at 24°C (at the upper end of the
348 thermal tolerance for the species) compared to 20°C. Appelhans et al. (2014) reported pCO_2-
349 dependent differences in growth [650 > 1150 > 3500 µatm (pH 7.85 > 7.64 > 7.17)] of juvenile *A.*
350 *rubens* in a long-term experiment (39 weeks at 9°C) but not until after 18 weeks of
351 experimentation, although a follow-up short-term experiment observed differences in growth after
352 6 weeks under similar pCO_2 conditions at 13°C. In contrast, Gooding et al. (2009) report a positive
353 effect of increased pCO_2 on the growth of juvenile intertidal starfish, *Pisaster ochraceus*. This was

attributed to the lack of a continuous calcified skeletal structure in this species, decreasing the relative amount of calcified tissue requiring the involvement of expensive re-calcification mechanisms during high CO_2 exposure. Clearly the effect of elevated pCO_2 on growth rates is variable among echinoderms in general, and among studies on the same species but may also be a result of differences in experimental protocols.

The degree of calcification of the regenerating arms was also unaffected by elevations in pCO_2 in the present study as has been observed in previous ocean acidification studies on *A. rubens* (Appelhans et al., 2014; Keppel et al., 2015) and the tropical starfish *Luidia clathrata* (Schram et al., 2011). In contrast, the starfish *Pisaster ochraceus* exposed to pCO_2 levels of 780 µatm (pH 7.79/12°C and 7.82/15°C) for 70 days showed a decline in calcified material compared to 380 µatm (pH 7.85//12°C and 7.88/15°C) (Gooding et al., 2009), while the brittlestar *Amphiura filiformis* exposed to acidified water at pH 7.7, 7.3 and 6.8 for 6 weeks exhibited elevated calcium carbonate content in the arms compared to pH 8.0 (Wood et al., 2008). Therefore, although it appears that energy allocation into growth and calcification of the regenerating arms varies between echinoderm taxa, the evidence indicates that these processes can be maintained in *A. rubens* exposed to near future elevated pCO_2 conditions. This is important since impaired regeneration of autotomised limbs could have ecological consequences resulting from impaired feeding and locomotion of the affected individuals (Barrios et al., 2008; Díaz-Guisado et al., 2016; Dominguez et al., 2016; Shaeffer, 2016).

In summary, the results of the present study indicate that the combined stressors of increased pCO_2 and autotomy did not affect survival, body growth, arm regeneration, righting time or calcium content of adult individuals of the intertidal predatory starfish *Asterias rubens* during a 120 day exposure period. However, higher concentrations of lipids in the pyloric caeca under increased pCO_2 suggest that reproductive investment may be compromised with long-term exposure. In addition, there was a tendency for mortality to be higher amongst autotomised starfish and calcification to be lower in starfish exposed to increased pCO_2. Therefore, we recommend that

future studies consider experiments with chronic or longer exposure than the present study to elucidate the capacity of *A. rubens* to cope with climate-driven environmental change.

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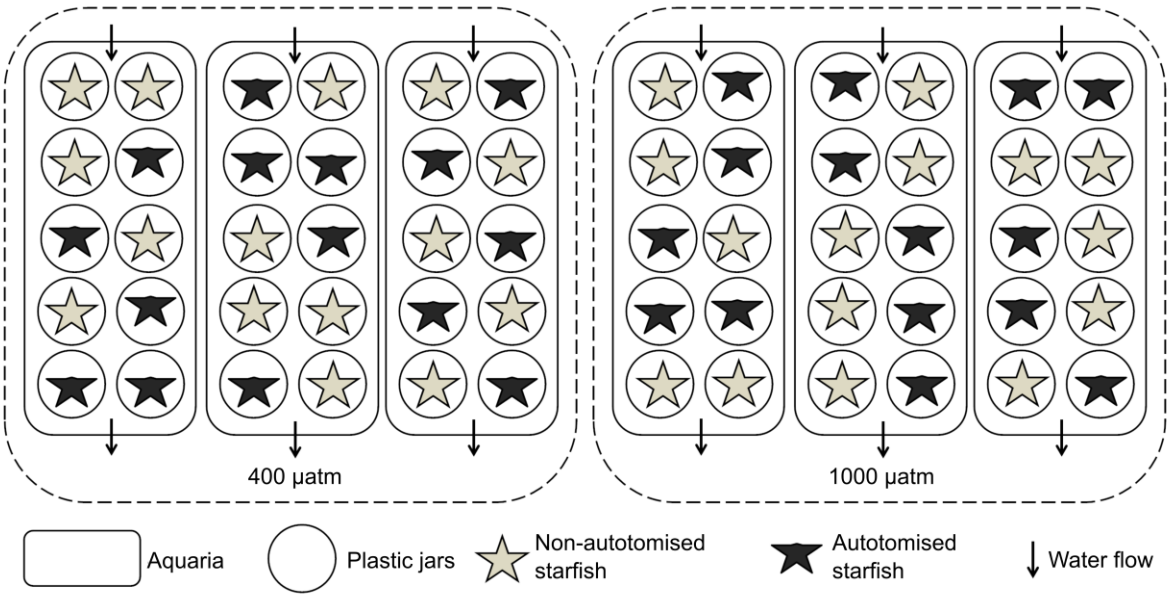
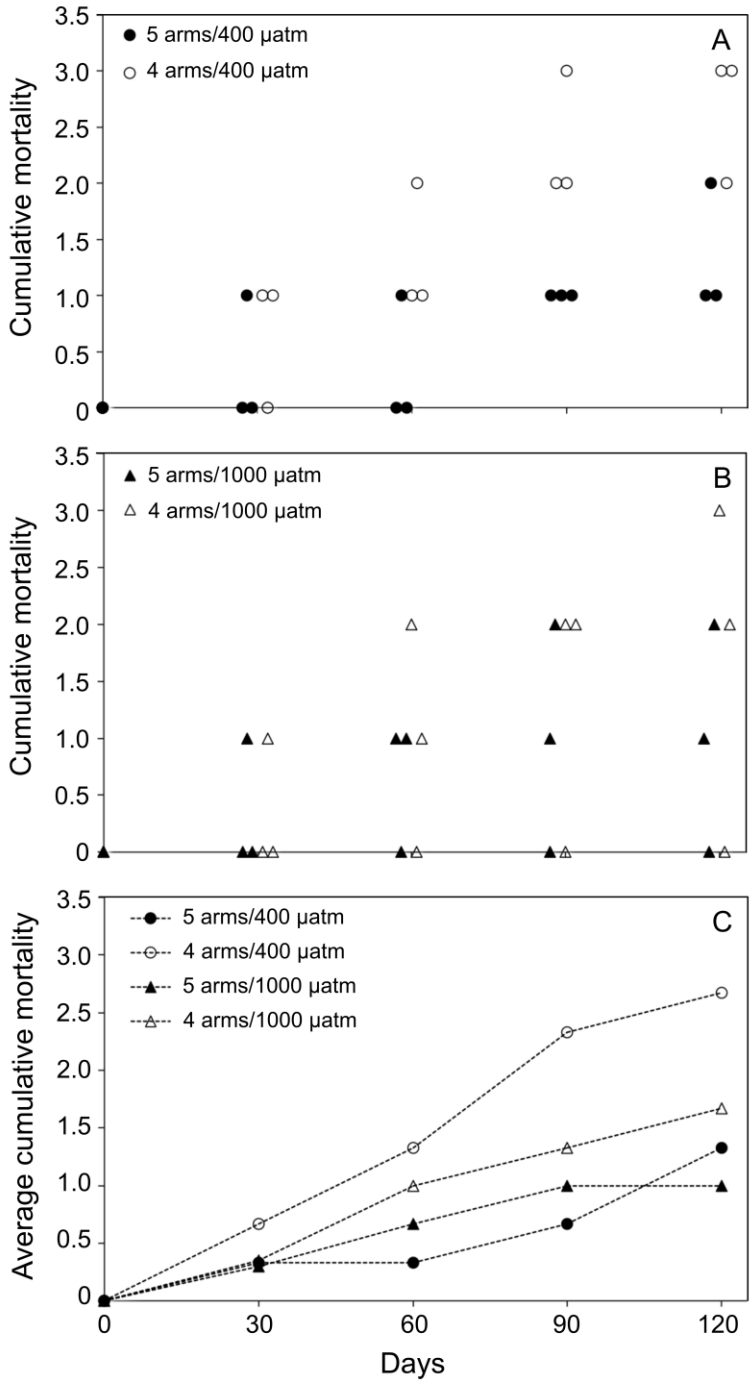
621 **Figure captions**



622

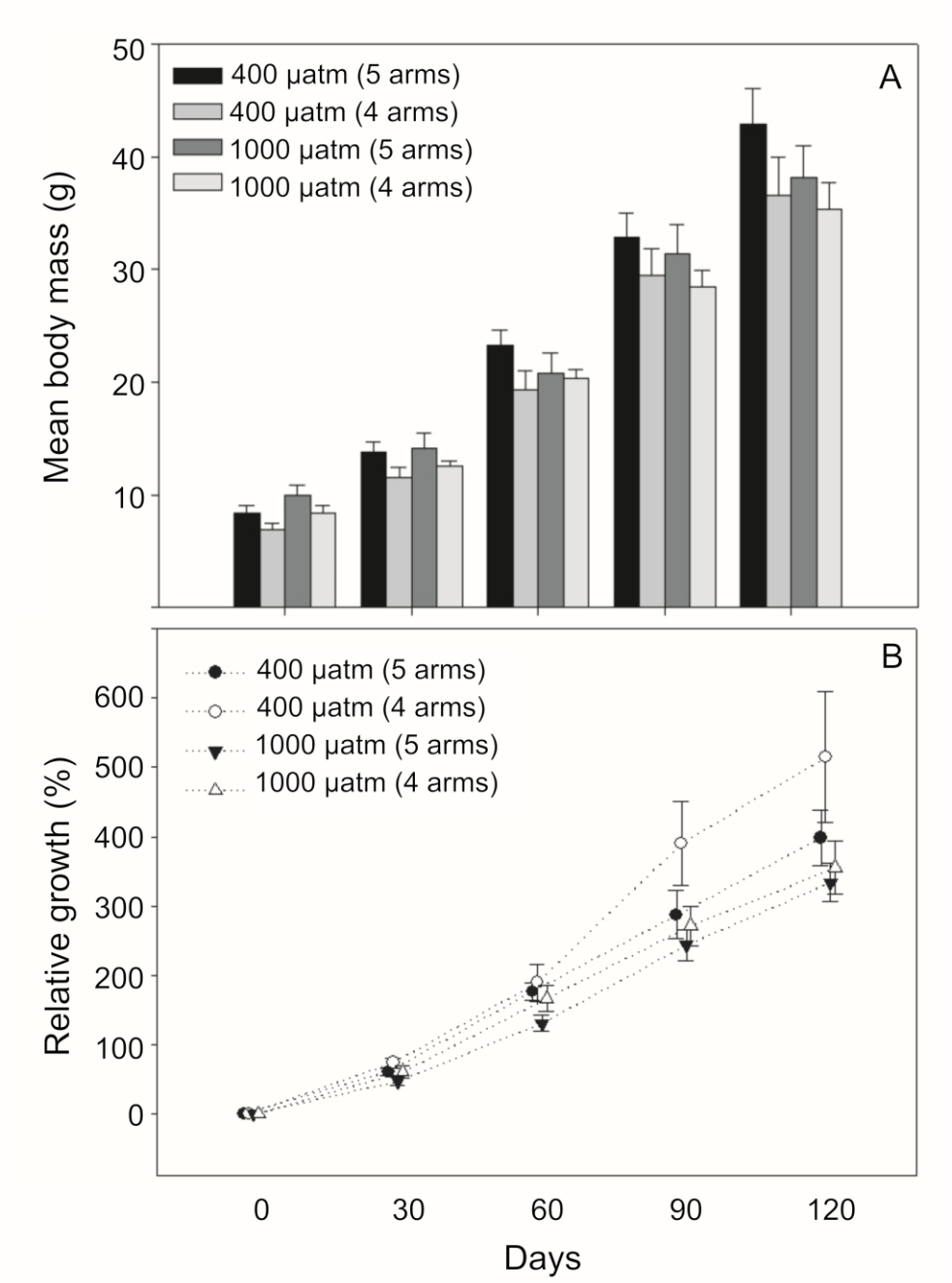
623 Fig. 1. Summary outline of the experimental aquarium design used in present study to determine
624 the impact of pCO_2 control, ~400 µatm; RCP 8.5 'business as usual', ~1000 µatm [IPCC, 2014])
625 and autotomy on *Asterias rubens.*

626



627

628 Fig. 2. Cumulative mortality (number of individuals) of autotomised (4 arms) and non-autotomised
629 (5 arms) *Asterias rubens* reared under different pCO_2 levels: ~400 µatm (control) and ~1000 µatm
630 (RCP 8.5 'business as usual', IPCC, 2014) for 120 days. Data are presented for cumulative
631 mortality in replicate tanks of autotomised (4 arms) and non-autotomised (5 arms) *Asterias rubens*
632 reared at (A) ~400 µatm and (B) ~1000 µatm and (C) average mortality per treatment. Data for
633 each replicate tank in (A) and (B) are slightly offset for clarity.



634

635 Fig. 3. (A) Body mass (g) and (B) percentage relative growth of autotomised (4 arms) and non-
636 autotomised (5 arms) *Asterias rubens* reared under different pCO_2 level (control, ~400 µatm; RCP
637 8.5 'business as usual', ~1000 µatm [IPCC, 2014]) for 120 days. (Data are presented as Mean ±
638 1SE).

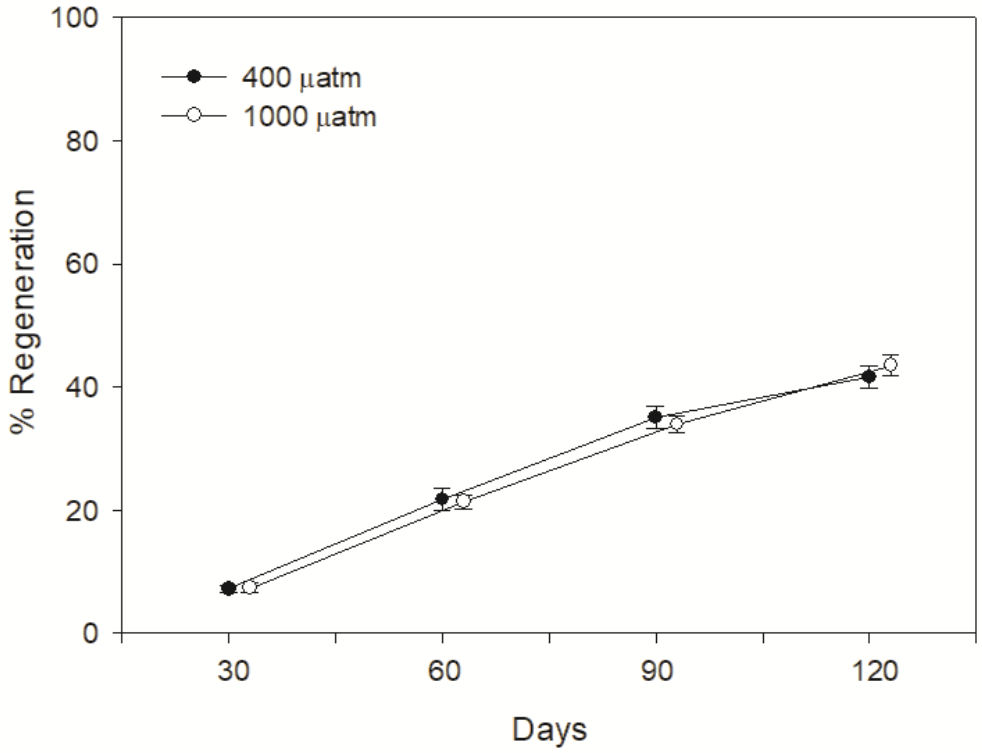


Fig. 4. Percentage regeneration of the autotomised arm of *Asterias rubens* reared under different pCO_2 level (control, ~400 µatm; RCP 8.5 'business as usual', ~1000 µatm [IPCC, 2014]) for 120 days. Percentage regeneration is calculated as 100*(length of the regenerating arm/ length of the longest arm) at each measurement. (Data are presented as Mean ± 1SE).

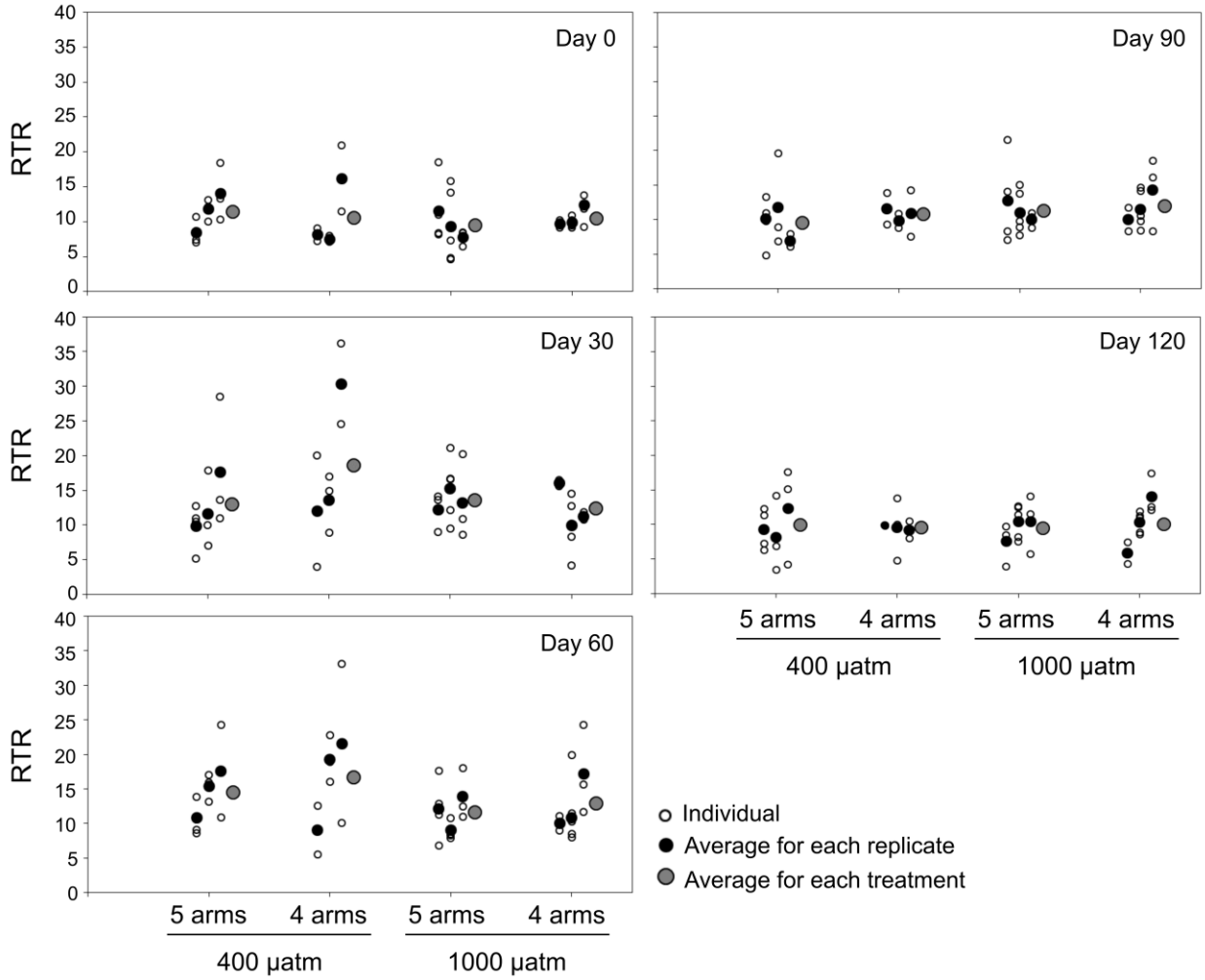


Fig. 5. Righting time responses, expressed by the RTR value, of autotomised (4 arms) and non-autotomised (5 arms) individuals of *Asterias rubens* reared under different pCO_2 level (control, ~400 µatm; RCP 8.5 'business as usual', ~1000 µatm [IPCC, 2014]) for 120 days. Data presented are raw non-transformed data with statistical analyses conducted on log-transformed data (see methods). For each treatment, open circles represent individual RTR values, filled black circles represent the average RTR value for each replicate and the large grey filled circles represent the overall average value for each treatment. Data for each replicate tank are slightly offset for clarity.

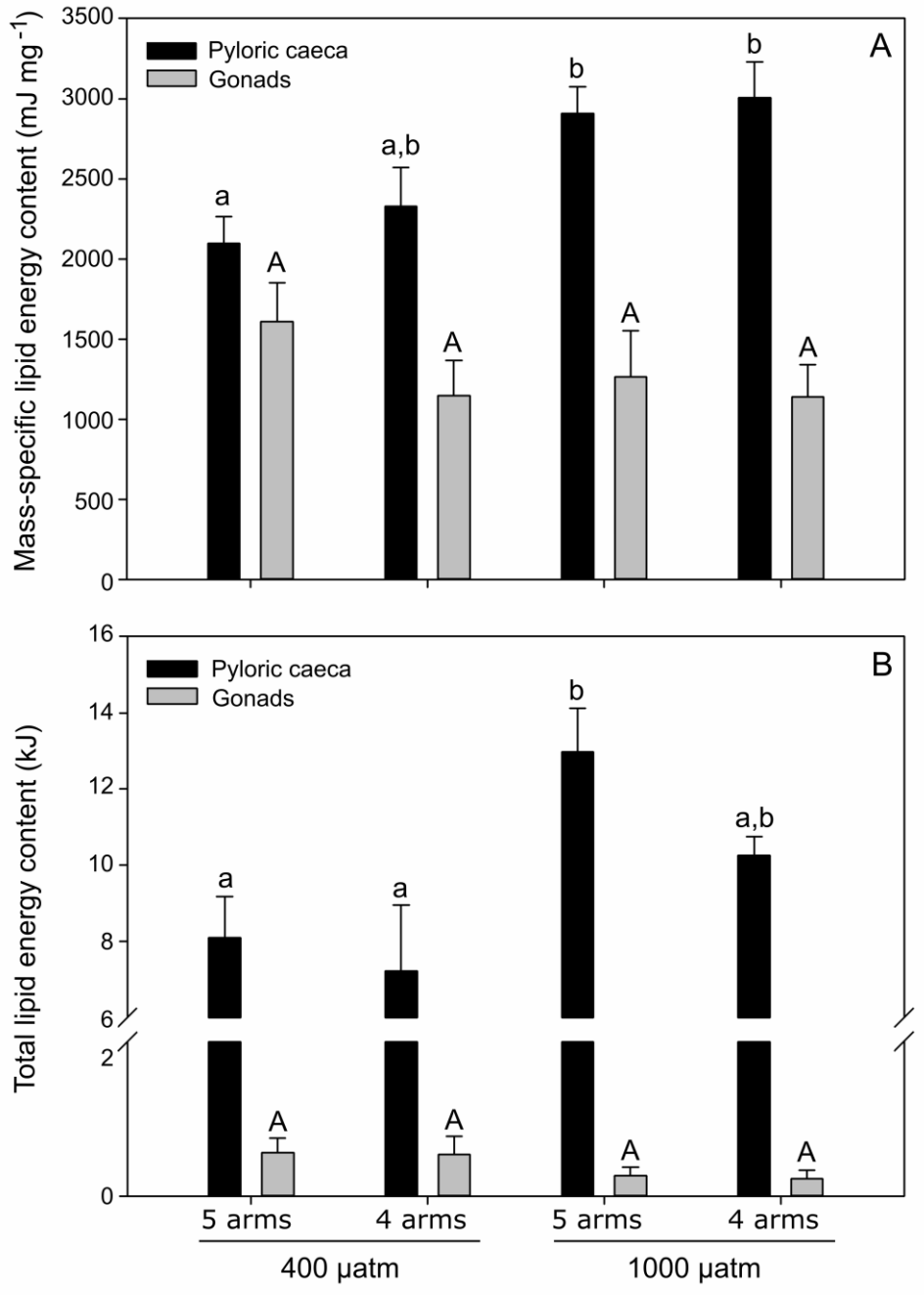


Fig. 6. (A) Mass-specific lipid energy content in the tissues (mJ mg^{-1} wet mass), and (B) total lipid energy content (kJ) in the tissues of autotomised (4 arms) and non-autotomised (5 arms) of *Asterias rubens* reared under different pCO_2 level (control, ~400 µatm; RCP 8.5 'business as usual', ~1000 µatm [IPCC, 2014]) for 120 days. In (A) and (B), different letters indicate significant differences (Tukey *post hoc* test) between treatments for gonads (upper case letters) and pyloric caeca (lower case letters), respectively. (Data are presented as Mean ± 1SE)

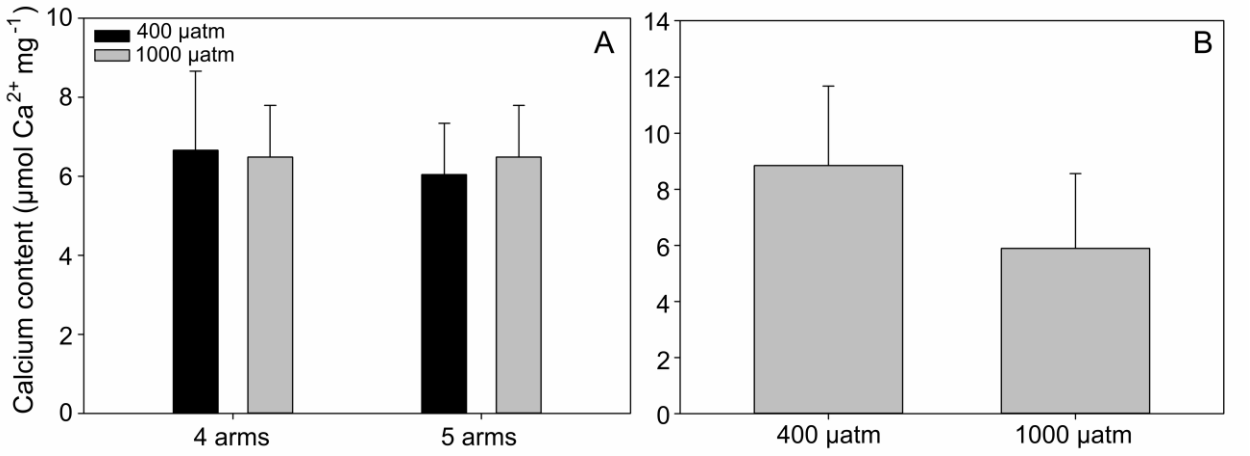


Fig 7. Calcium content, expressed in µmol Ca^{2+} mg^{-1} dry mass in the arms of *Asterias rubens* reared under different pCO_2 level (control, ~400 µatm; RCP 8.5 'business as usual', ~1000 µatm [IPCC, 2014]) for 120 days. Data are presented for (A) the left arm opposite to the position of the madreporite (i.e. the 'control' arm) of individuals subjected to arm autotomy (4 arms) or not (5 arms) and (B) in the regenerating arm of the individuals subjected to autotomy. (Data are presented as Mean ± 1SE).

670 **Tables**

671 Table 1. Average seawater parameters for *Asterias rubens* reared under two different pCO_2 levels
672 (control, ~400 µatm; RCP 8.5 'business as usual', ~1000 µatm [IPCC, 2014]) for 120 days. Data
673 are presented as mean values ± SE with associated 95% confidence interval (CI) range.

674

Parameters	Control (400 µatm)		Experimental (1000 µatm)	
	Mean ± SE	**95% CI**	**Mean ± SE**	**95% CI**
pH	8.03 ± 0.01	8.02 - 8.05	7.69 ± 0.01	7.67 - 7.72
Salinity	32.86 ± 0.07	32.72 - 33.00	32.97 ± 0.06	32.86 - 33.08
Temperature (°C)	12.4 ± 0.04	12.40 - 12.57	12.38 ± 0.04	12.31 - 12.46
TCO_2 (mmol L^{-1})	1808 ± 40	1730 - 1886	1867 ± 40	1789 -1944
pCO_2 (µatm)	519 ± 15	490 – 550	1070 ± 30	1011 - 1130
Ω_{CALC}	2.1 ± 0.10	1.90 – 2.24	1.1 ± 0.10	0.97 – 1.23
Ω_{ARAG}	1.3 ± 0.10	1.21 – 1.43	0.7 ± 0.10	0.62 – 0.79

675

Table 2. Results of Linear Mixed Model used to test the fixed effects of time, autotomy and pCO_2 and random effects of individual and tank on (A) mortality, (B) growth (body mass, g), (C) arm regeneration (percentage regenerated), and (D) the righting time response (RTR) of *Asterias rubens* reared under two different pCO_2 levels (control, ~400 µatm; RCP 8.5 'business as usual', ~1000 µatm [IPCC, 2014]) for 120 days.

Model component	Estimate/ *Variance	Std Error/ Std Dev*	t	Pr(>|t|)
A) Mortality				
Intercept	-0.3000	0.2812	-1.067	0.296
Time	0.3000	0.0688	4.363	**<0.001**
Autotomy	-0.2967	0.3225	-0.827	0.412
Time*Autotomy	0.2667	0.0972	2.742	**0.009**
Tank	0.1861	0.4314*	-	-
B) Growth				
Intercept	1.7888	0.0664	26.932	**<0.001**
Time	0.3962	0.0087	45.373	**<0.001**
Autotomy	-0.1218	0.0668	-1.822	0.078
id	0.0357	0.1891*	-	-
Tank	0.0103	0.1016*	-	-
C) Arm Regeneration				
Intercept	0.1559	0.0160	9.747	**<0.001**
Time	0.1466	0.0053	27.760	**<0.001**
id	0.0008	0.0282*	-	-
Tank	0.0000	0.0000	-	-
D) RTR				
Intercept	1.1321	0.0417	27.155	**<0.001**
Time	-0.0122	0.0076	-1.609	0.110
Autotomy	-0.0248	0.0258	-0.959	0.345
pCO_2	-0.0119	0.0437	-0.272	0.431
id	0.0018	0.0428*	-	-
Tank	0.0019	0.0431*	-	-