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Feeding plasticity more than metabolic rate drives the productivity of economically important filter feeders in response to elevated CO$_2$ and reduced salinity

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Running Head: Effects of CO$_2$ and salinity on feeding and metabolism

Key words: tunicates, bivalves, ocean acidification, scope for growth, metabolism, clearance rate, absorption efficiency.
Abstract

Climate Change driven alterations in salinity and carbonate chemistry are predicted to have significant implications particularly for northern coastal organisms, including the economically important filter feeders *Mytilus edulis* and *Ciona intestinalis*. However, despite a growing number of studies investigating the biological effects of multiple environmental stressors, the combined effects of elevated $pCO_2$ and reduced salinity remain comparatively understudied. Changes in metabolic costs associated with homeostasis and feeding/digestion in response to environmental stressors may reallocate energy from growth and reproduction, affecting performance. Although these energetic trade-offs in response to changes in routine metabolic rates have been well demonstrated fewer studies have investigated how these are affected by changes in feeding plasticity. Consequently, the present study investigated the combined effects of 26 days’ exposure to elevated $pCO_2$ (500 µatm and 1000 µatm) and reduced salinity (30, 23 and 16) on the energy available for growth and performance (Scope for Growth) in *M. edulis* and *C. intestinalis*, and the role of metabolic rate (oxygen uptake) and feeding plasticity (clearance rate and absorption efficiency) in this process. In *M. edulis* exposure to elevated $pCO_2$ resulted in a 50% reduction in Scope for Growth. However, elevated $pCO_2$ had a much greater effect on *C. intestinalis*, with more than a 70% reduction in Scope for Growth. In *M. edulis* negative responses to elevated $pCO_2$ are also unlikely be further affected by changes in salinity between 16 and 30. Whereas, under future predicted levels of $pCO_2$ *C. intestinalis* showed 100% mortality at a salinity of 16, and a >90% decrease in Scope for Growth with reduced biomass at a salinity of 23. Importantly, this work demonstrates energy available for production is more dependent on feeding plasticity, i.e. the ability to regulate clearance rate and absorption efficiency, in response to multiple stressors than on more commonly studied changes in metabolic rates.
Introduction

Climate change is leading to simultaneous alterations in several environmental factors including ocean temperature, pH and salinity (e.g. Doney et al. 2009). Rising levels of CO₂ in the atmosphere are causing increases in sea surface temperature, and worldwide modification of ocean carbonate chemistry, with gradual reductions in pH and carbonate ion (CO₃²⁻) availability in a process known as ocean acidification (e.g. Sabine and Feely 2007; Doney et al. 2009). Elevated atmospheric CO₂ and associated temperature changes are also affecting weather patterns and are altering the Earth’s hydrological cycle, which in turn, affects ocean salinity (Pierce et al. 2012). Freshening of surface salinity has been occurring over past decades with some of the largest reductions in salinity taking place at higher latitudes because of increased precipitation, freshwater runoff, melting freshwater ice and alterations in the meridional overturning circulation (Callaghan et al. 2011). Most studies to date on the effects of elevated $pCO_2$ and reduced salinity have focused on their individual effects. Recently, however, the combined effects of various factors have received some attention, as these may differ from the examination of each factor individually (e.g. Harvey et al. 2013; Sokolova et al. 2016).

Reduced salinity is a prominent stress factor in Arctic and Subarctic coastal areas with future changes predicted to have significant implications for northern estuarine and fjord ecosystems (e.g. Biggs and Cronin 1981; Callaghan et al. 2011). Such species include economically important filter feeders such as the blue mussel Mytilus edulis and the invasive ascidian Ciona intestinalis (Locke and Carman 2009). Colder higher latitude waters also absorb more CO₂ than warmer waters resulting in a greater pH change and lower levels of calcium carbonate saturation at a given $pCO_2$ level (Takahashi et al. 2014). The effect of elevated $pCO_2$ on seawater pH may also be increased in these areas as reduced salinity will reduce the total alkalinity and buffering capacity of seawater (Lee et al., 2006). Tolerances to elevated $pCO_2$, vary among marine invertebrate species, as do tolerances to changes in salinity (e.g. Sokolova et al. 2016; Wood et al. 2016). Little, however, is known about their combined effects and our current understanding in filter feeders is limited to just a few studies where it has been shown that these factors influence the survival, energy metabolism and osmoregulatory capacity as well as weaken shells (e.g. Dickinson et al. 2013; Velez et al. 2016). It is possible that the tolerance of M. edulis and C. intestinalis to the combined effects of elevated $pCO_2$ and reduced salinity may differ. If so, this could potentially
affect community structure via alterations in competitive interactions between the two species, which are known to have an economic impact on *M. edulis* aquaculture (e.g. Locke and Carman 2009).

Although *M. edulis* and *C. intestinalis* have long been considered osmoconformers with little extracellular ionic control (Shumway 1977; 1978) both species have adapted/acclimatised to wide natural gradients in salinity. For example, in the Baltic Sea the lowest salinity limit for development in *C. intestinalis* is as low as 11 (Dybern 1967), and the natural distribution of *M. edulis* is only limited by salinities lower than 4.5 (Segerstråle 1944). However, laboratory studies suggest that optimum fertilisation and early development of *C. intestinalis* occurs above 34 salinity, with much wider tolerance ranges for pH, between 7.4 and 8.8 (Ballas *et al*. 2003). In *M. edulis* adaptation/acclimatisation appears to come at an energetic cost with low salinity populations exhibiting reductions in growth, longevity and reproductive fitness (e.g. Westerbom *et al*. 2002), with metabolic rates (i.e. the cost of living) increase linearly between 30 to 10 salinity (Stickle and Sabourin 1979). However, metabolic responses to reduced salinity are dependent on ion-regulatory capacity, with euryhaline invertebrates demonstrating increased metabolic rates when exposed to reduced salinity and stenohaline invertebrates, such as *M. edulis* and *C. intestinalis*, demonstrating decreased metabolic rates (Shumway 1978). Calcification in *M. edulis* is limited by low salinity (lower salinity threshold for calcification between 14.7 and 20; Malone & Dodd 1967), as well as elevated pCO$_2$ (e.g. Fitzer *et al*. 2016) possibly affecting the overall cost of calcification and so growth. Under elevated pCO$_2$, increased cellular energy demands limit energy available for growth and productivity (Thomsen and Melzner 2010), although food availability and feeding rate are determining factors (Thomsen *et al*. 2013). Both elevated pCO$_2$ and reduced salinity can affect energetic demand and resource allocation affecting performance, productivity and survival. However, changes (both positive or negative) in energy absorption via feeding, which in filter feeders is dependent on clearance rate and absorption efficiency, are likely as important in determining energy budgets as changes in metabolic rate. Metabolic costs changing directly with feeding due to specific dynamic action (Gaffney and Diehl 1986; Sigsgaard *et al*. 2003). Despite the importance of feeding plasticity in determining energy availability for growth and performance, little is known of how responses, such as clearance rate and absorption efficiency, interact to affect overall energy absorption in filter feeders when challenged by elevated pCO$_2$ and/or reduced salinity. In general,
filter feeders reduce pumping rates in response to reduced salinity, linked to decreased clearance rates (e.g. Anderson and Prosser 1953; Shumway 1977; Shumway 1978). However, clearance rates and particle retention are much more plastic that previously supposed (e.g. Denis et al. 1999; Strohmeier et al. 2009; Strohmeier et al. 2012; Cranford et al. 2016), with some bivalves up regulating clearance rates at times of energy limitation (Denis et al. 1999). In addition to clearance rate, absorption efficiency of digestion is also an important determinant of overall energy absorption through feeding, which also shows plasticity. In the Atlantic Deep Sea Scallop (Pectopecten magellanicus), for example, mean absorption efficiency has been shown to increase as filtration rates decreased in an attempt to maintain total energy absorption through feeding (e.g. Cranford and Hargrave 1994). To date, the effects of elevated pCO$_2$ on the absorption efficiency of marine organisms is not well understood (Navarro et al. 2013). Some species, for example Mytilus chilensis, reduce absorption efficiency (Navarro et al., 2013) and others such as the Mediterranean Mussel (Mytilus galloprovincialis) increase absorption efficiency in response to elevated pCO$_2$ (Fernandez-Reiriz et al. 2012). Four-week exposure to reduced salinities in the mussel, Perna viridis, resulted in reduced absorption efficiency (Wang et al. 2011).

The role of feeding plasticity in determining energy budgets is poorly understood. This study investigates the combined effects of elevated pCO$_2$ and reduced salinity on the energy available for growth and performance in M. edulis and C. intestinalis, and the role of feeding plasticity in this process. Both M. edulis and C. intestinalis were exposed to chronic mid-term (26 days) elevated pCO$_2$ and reduced salinity. At the end of the exposure period, oxygen uptake rates were determined as a proxy for routine metabolic rates, and clearance rate and absorption efficiency were determined to assess the ability to exploit feeding plasticity. Fitness/performance was examined by measuring growth and mortality rates. Experiments were used to assess which species would be more likely to survive near future conditions of increasing pCO$_2$ and declining salinity due to occur along northern costs.

Materials and Methods

Animal Collection and acclimation

Adult C. intestinalis (3.8±0.1 g FW, 5.0±0.5 cm length) and M. edulis (16.3±0.6 g FW,
5.0±0.06 cm length) were collected from the shallow subtidal zone at the Institute of Marine Research, Austevoll, Norway (60°05'08.9"N, 05°15'42.5"E) in November 2015. Ninety *C. intestinalis* and forty *M. edulis* were weighed as a baseline to monitor growth. The animals were then glued to pieces of velcro in preparation for attachment to the sides of the experimental tanks, mimicking their natural hanging position. The animals were left to recover for 48 h in aerated ambient seawater prior to acclimation to experimental conditions. Five *C. intestinalis* and three *M. edulis* were assigned to each treatment tank (4 L) and the tanks were triplicated per experimental treatment (N = 15 *C. intestinalis*; N= 9 *M. edulis* per treatment). After being assigned to treatment tanks, salinity and *p*CO$_2$ levels were changed from ambient to the final treatment conditions over approximately 6 h.

The treatments consisted of three salinity levels (30, 23 and 16) and two *p*CO$_2$ levels (500 and 1000 μatm) in a fully crossed design. Treatments were maintained using a flow-through system, using unfiltered seawater pumped (7m depth), directly from the site of animal collection and supplied to each treatment tank at a flow ≈ 50 L h$^{-1}$. This insured that control treatments corresponded to natural *p*CO$_2$ and salinity levels. Seawater salinity levels for each experimental treatment were maintained by mixing with un-chlorinated freshwater (source, Vannområde Vest Austevoll), before being supplied to 6 header tanks (1 per treatment) where *p*CO$_2$ levels were controlled. A nominal control *p*CO$_2$ value of 500 μatm was selected as this corresponded to the natural habitat *p*CO$_2$ level that the organisms were acclimatised to at the time of collection. Carbonate chemistry in Norwegian fjords is extremely dynamic and elevated *p*CO$_2$ levels compared to the open ocean can be associated with seasonal decreases in primary productivity (e.g. Fransson et al 2016). To achieve a predicted future elevated *p*CO$_2$ level of 1000 μatm (e.g. Caldeira and Wickett 2003) the pH was individually controlled for each salinity treatment taking into account the effect of temperature, salinity and total alkalinity (30 = pH 7.676; 23 = pH 7.598; 16 = pH 7.489) calculated using free-access CO$_2$SYS (Lewis and Wallace 1998). CO$_2$ levels were achieved by the addition of elevated $P_{CO_2}$ seawater (pH 5.5) to each header tank via peristaltic pumps controlled according to seawater pH levels via pH electrodes connected a controller (Endress and Hauser, Liquiline CM448; after, Andersen et al., 2013). The flow through system was placed within a temperature controlled room to maintain at 10°C throughout the experiment. Salinity, pH and temperature in each individual tank were
recorded three times a day using a handheld multimeter (labquest 2, vernier). Total 
alcalinity (TA) was measured twice a week by titration (TIM840 titration manager, 
TitraLab). Values for the physicochemical parameters and the associated carbonate 
chemistry values for this system are presented in Table 1. Following 26 days of 
acclimation a number of responses were determined in order to assess energy allocation 
in *C. intestinalis* and *M. edulis* as a result of combined exposure to elevated $pCO_2$ and 
reduced salinity.

*Determination of feeding rate and energy absorption*

Particle clearance rate (CR) of 9 *C. intestinalis* and 9 *M. edulis* was determined as an 
estimate of feeding rate and for the calculation of energy ingestion using the flow 
through feeding chambers developed by Strohmeier *et al.* (2009). These chambers were 
supplied with the same unfiltered seawater as the animals in the respective treatment 
tanks. Three chambers were left empty as controls. Internal dimensions of the *C. intestinalis* feeding chambers were: width 5 x length 22 x height 10 (cm) and the *M. edulis* chambers were: width 3.8 x length 19.5 x height 8 (cm). These chambers have 
been demonstrated to restrict recirculation and therefore inhibit the animals from re-
filtering the water (Strohmeier *et al.* 2009; Cranford *et al.* 2016). The rate of water flow 
was maintained to a level that would also ensure no re-filtration (nominal set values; 
*M. edulis* = 10 l h$^{-1}$; *C. intestinalis* = 6 l h$^{-1}$). The animals were placed in the chambers 
and allowed to rest for 1h undisturbed to resume feeding behaviour prior to sampling 
before the concentration of suspended particles (within 30 size-interval between 1 and 
60μm in diameter) in the out-flow seawater from each chamber was measured using a 
laser particle counter (PAMAS GmbH, Model S4031GO). This protocol was repeated 
3 times on the same 9 individuals and 3 controls from each respective species and 
treatment. As each of the 6 treatments were repeated 3 times, 6 hours apart, and each 
feeding trial took just over 1 h (1h of resting time, a few minutes to collect the water 
and then change the treatment) clearance rate and POM data was collected over a 12 
hour period before the faecal collection as describe below. Therefore, the food in the 
gut that was defecated during faecal collection was cleared by the animal during the 
feeding trials. CR (l h$^{-1}$) was then calculated using the equation:

$$CR = \frac{F(C_{in} - C_{out})}{C_{in}}$$ (1)
Where \( F (1 \text{ h}^{-1}) \) is the measured flow rate of water though each individual chamber. \( C_{\text{in}} \) is the inflow concentration of food represented by the particle concentration from the control chambers, and \( C_{\text{out}} \) is the partial concentration from each experimental chamber.

To calculate energy ingestion from CR particulate organic matter (POM) was determined for each feeding experiment. POM was determined by collecting 4 L of seawater from each of the 3 control chambers and filtering through pre-combusted (450 °C for 5 hours to remove carbon) and pre-weighed 1.5 μm glass microfiber filters (VWR) using 1 ml of ammonium formate to remove salt crystals. Filters were dried to determine dry weight (DW; 60°C for 24 hours) and ash free-dry weight (AFDW; 450°C for 5 hours) to establish organic content of the POM. This protocol was repeated three times for each control chamber. Energy ingested through feeding was then estimated by multiplying CR by the concentration of POM (mg AFDW L\(^{-1}\)) and by the energetic content of POM (23 J mg AFDW \(^{-1}\); Widdows et al. 1979).

Following feeding experiments the animals were placed in individual chambers constructed from sections of PVC pipe (length 12 cm, diameter 8cm) with mesh attached to each end (diameter 375 μm). After 24 h any faecal pellets in the chambers were filtered onto pre-weighed and burned filters (described above) using distilled water. Following this the filters were dried to determine DW (60°C for 24 hours) and AFDW (450°C for 5 hours) to establish organic content of the faecal pellets. Absorption efficiency was then estimated from the ratio of the organic content of the seston (POM) averaged over the 12h feeding period prior to faecal collection and the organic content of the faeces, using the equation (after, Conover 1966):

\[
\text{Absorption Efficiency} = \frac{(F-E)}{((1-E)F)}
\]  

Where \( F \) is the ash-free dry weight: dry weight ratio of the seston during feeding and \( E \) is the ash-free dry weight: dry weight ratio of the faeces. Energy absorption though feeding was then estimated by multiplying the energy ingested by the absorption efficiency.

Rates of oxygen uptake

Oxygen uptake rate was measured as a proxy for metabolic rate (\( \dot{\text{MO}}_2 \)). \( \dot{\text{MO}}_2 \) was measured using stop-flow respirometry after Garilli et al. (2015) and Harvey et al.
In brief, individual *C. intestinalis* and *M. edulis* from each treatment were placed in individual chambers (volume 160 ml) supplied with the same seawater as the respective treatments tanks (flow rate ≈ 10 L h
\(^{-1}\)). Animals were allowed 1 h to recover from handling and regain natural ventilatory behaviour before the flow to each chamber was closed and the decreases in % oxygen saturation continuously measured using a non-invasive optical oxygen system (Oxy-10 mini, PreSense; labquest 2, Vernier) modified from Rastrick and Whiteley (2011) and Calosi et al., (2013). The incubation period was 5 h for *C. intestinalis* and 3 h for *M. edulis*, during which time, % oxygen saturation levels of the seawater did not fall below 70% to avoid hypoxic conditions. A blank chamber with no animal was monitored in parallel to each treatment to account for background respiration in the seawater. Percentage oxygen saturation was converted to oxygen partial pressure \((P_{O_2})\) adjusted for atmospheric pressure and vapour pressure adjusted for relative humidity (continuously monitored using a multimeter; Labquest 2, Vernier). \(M.O_2\) was calculated from the decrease in \(P_{O_2}\) within each chamber multiplied by the oxygen solubility of seawater using coefficients adjusted for the effect of temperature and salinity (Benson and Krause, 1984), and expressed as \(\mu\text{mol O}_2\ h^{-1}\). \(M.O_2\) was then used to estimate the amount of absorbed energy lost via metabolism (routine metabolic maintenance of homeostasis, feeding and digestion) assuming a heat equivalent of oxygen uptake of 0.456 \(\mu\text{mol} \ O_2\) (Gnaiger, 1983).

**Growth**

Estimates of energy availability for growth and reproduction (Scope for Growth; SfG) for each treatment were calculated from estimates of rates of energy absorption though feeding (EA; \(j\ h^{-1}\)) and energy loss via metabolism (EL; \(j\ h^{-1}\); modified from Widdows and Johnson 1988):

\[
\text{SfG (j} h^{-1}) = \text{EA} – \text{EL}
\]

(3)

During the incubation period the wet weight (g) of each animal was recorded twice a week to determine growth rates. At the end of the acclimation the soft tissue of *C. intestinalis* and *M. edulis* individuals was dried to determine dry weight (60°C for 48 hours) and ash free-dry weight (450°C for 5 hours) to establish any treatment effects on carbon richness (energy density) of the tissue.

**Statistical Analysis**
The effects of elevated $pCO_2$ and/or reduced salinity (fixed factors) on all of the measured parameters (dependent factors) were tested using a nested general linear mixed model (GLMM) with body mass as a covariate (to adjusted for the effect of variation in body size between individuals) and tank as a random factor nested within the fixed factors. This considers that replicate tanks were supplied by a single header tank per treatment and therefore, despite being a flow through system, tanks may not be considered true replicates (e.g. Collard et al., 2015; Small et al., 2015). Any observed significant differences were further analysed by F-tests based on pairwise comparisons generated from the estimated marginal means of the GLMM. Proportional data was arc sign square root transformed before statistical analysis. All values are expressed as means ± SEM. All statistical analyses were performed using SPS software (v 20 SPS Chicago, Ill, USA).

Results

Mortality
After 20 days of the 26-day exposure period $C.\ intestinalis$ showed 0% survivorship in the lowest salinity of 16. Consequently, further energetic parameters could not be determined in this treatment. After 26-days the lowest survivorship of 53% was recorded in the 23 salinity and elevated $pCO_2$ treatment, followed by 80% in the ambient salinity of 30 and elevated $pCO_2$ treatment. At ambient $PCO_2$, 87% and 90% survivorship was reported for salinities of 23 and 30, respectively. Conversely over the 26-day exposure period only one mortality was reported for $M.\ edulis$ across all treatments.

Feeding - clearance rate and energy ingestion
In $C.\ intestinalis$ elevated $pCO_2$ significantly influenced the effect of salinity on CR ($F_{1,29}=10.291$, $P=0.003$) and energy ingestion ($F_{1,29}=8.938$, $P<0.01$). In the ambient $pCO_2$ treatments, CR and energy ingestion were maintained across the salinity treatments. However, in the elevated $pCO_2$ treatments a reduction in salinity from 30 to 23 resulted in a significant reduction in CR in $C.\ intestinalis$ from $0.9±0.1$ L h$^{-1}$ to $0.3±0.1$ L h$^{-1}$, respectively ($F_{1,29}=13.829$, $P<0.001$; Figure 1C). This was associated with a significant decrease in energy ingestion between the same treatments ($F_{1,29}=11.940$, $P<0.01$; Table 2). Energy ingestion was also significantly lower in
In *M. edulis*, elevated pCO$_2$ significantly influenced the effect of salinity on CR (F$_{2,45}$ = 11.421, P<0.001; Figure 2, A) and energy ingestion (F$_{1,45}$ = 7.075, P<0.01; Table 3). In the ambient pCO$_2$ treatments, a reduction in salinity from 30 to 16 resulted in a significant reduction in CR from 2.5±0.2 L h$^{-1}$ to 1.0±0.2 L h$^{-1}$, (F$_{2,45}$= 13.748, P<0.001; Figure 1D) and energy ingestion (F$_{2,45}$=31.167, P<0.001; Table 3), respectively. However, in the elevated pCO$_2$ treatments, CR was maintained across the salinity treatments, driving the interaction. Overall in *M. edulis*, elevated pCO$_2$ resulted in an increase in CR (F$_{1,45}$ = 62.555, P<0.001) and energy ingestion (F$_{1,45}$ = 5.640, P<0.05). This was driven by significantly higher CR in the combined elevated pCO$_2$ and reduced salinity treatments (23 salinity, F$_{1,45}$ = 13.345, P<0.001; 16 salinity, F$_{1,45}$ = 70.185, P<0.001).

**Feeding - Absorption Efficiency**

The absorption efficiency of surviving *C. intestinalis* showed no significant variation between salinity treatments of 30 and 23 (F$_{1,26}$=0.065, P=0.801) or pCO$_2$ combinations (F$_{1,4}$=4.730, P=0.099; Table 2). *M. edulis* showed an increase in absorption efficiency at reduced salinity, although this pattern was significantly influenced by pCO$_2$ (F$_{2,39}$ = 7.296, P<0.05; Table 3). In the ambient pCO$_2$ treatments, salinity had a greater effect on absorption efficiency with significantly higher absorption efficiencies at salinities of both 23 and 16 compared with ambient salinity (F$_{2,12}$ = 20.443, P<0.001; Table 3). At elevated pCO$_2$, the effects of salinity were weaker and like *C. intestinalis* there was no significant difference in absorption efficiency between the ambient and 23 salinity treatments. Although, absorption efficiency did significantly increase at the lowest salinity of 16 compared with ambient salinity (F$_{2,12}$ = 4.304, P<0.05; Table 3). This interaction was, in part, driven by a significant reduction in absorption efficiency at elevated PCO$_2$ across all salinity treatments (F$_{2,39}$ = 7.296, P<0.05).

**Feeding - Energy Absorption**

The energy absorption of *C. intestinalis* estimated from energy ingested through feeding and absorption efficiency showed no significant variation between those surviving salinity treatments at ambient or elevated pCO$_2$ levels (F$_{1,29}$ = 0.508, P=0.482). However, energy absorption was lower in *C. intestinalis* at elevated...
compared with ambient $p$CO$_2$ levels, with significant reductions of 66$\%$ ($F_{1,29}=8.378$, $P>0.01$) and 93$\%$ ($F_{1,29}=19.287$, $P>0.001$) in the 30 and 23 salinity treatments, respectively.

In *M. edulis* the effect of salinity on energy absorption was significantly influenced by $p$CO$_2$ levels ($F_{2,41}=18.930$, $P<0.001$). At ambient $p$CO$_2$, energy absorption showed a slight but significant increase between ambient salinity and a salinity of the 23 (mean difference $2.41=4.952\pm2.398$, $P<0.05$). However, at the lower salinity of 16, energy absorption significantly decreased compared to ambient salinity, to levels similar to those reported across the elevated $p$CO$_2$ treatments (mean difference $2.41=-12.465\pm2.393$, $P<0.001$).

**Metabolic Rate**

In *C. intestinalis*, rates of oxygen uptake ($M.O_2$) were significantly lower at a salinity of 23 compared to the ambient salinity at both $p$CO$_2$ levels ($F_{1,4}=146.901$, $P<0.001$; Figure 1A). However, in the same species $M.O_2$ were significantly higher in the elevated compared to the ambient $p$CO$_2$ treatments at both the 23 and the ambient salinity treatments ($F_{1,25}=35.701$, $P<0.001$; Figure 1A).

In *M. edulis* $M.O_2$ was also significantly lower at reduced salinity compared with ambient treatments, but only at the elevated $p$CO$_2$ levels. (23 salinity, $F_{7,51}=5.154$, $P<0.05$; 16 salinity, $F_{7,51}=4.980$, $P<0.05$; Figure 1B). In contrast and similar to *C. intestinalis*, *M. edulis* exhibited a significant increase in $M.O_2$ at elevated $p$CO$_2$ across all salinity treatments ($F_{1,3}=38.089$, $P<0.01$; Figure 1B). In *M. edulis*, $M.O_2$ also decreased significantly in association with a decrease in CR (Spearman Rank, correlation coefficient $r_{58}=0.426$, $P<0.01$).

**Growth**

In *C. intestinalis* estimated energy available for growth and reproduction ($S_fG$) showed no variation among salinity treatments at the ambient ($F_{1,29}=0.022$, $P=0.884$) or elevated $p$CO$_2$ treatments ($F_{1,29}=1.438$, $P=0.240$). Despite conservation of $S_fG$ across the 23 and ambient salinity treatments at ambient $p$CO$_2$ levels, growth rate significantly decreased from $0.035\pm0.013$ g day$^{-1}$ at ambient salinity to $-0.007$ g day$^{-1}$ and $-0.011$ g.
day\(^{-1}\) at salinities of 23 and 16, respectively (F\(_{2,68}=3.521, P<0.05\)). Negative growth at a salinity of 23 was accompanied by a significant increase in AFDW: DW ratio at ambient pCO\(_2\) (F\(_{1,8}=18.396, P<0.01\); Table 2). However, at elevated pCO\(_2\) levels, growth rate was unaffected by a change in salinity from 30 to 23 (F\(_{2,68}=0.692, P=0.504\)).

Despite no changes in SfG between a salinity of 30 and 23 at either pCO\(_2\) level, there were significant decrease in SfG in the elevated compared to the ambient pCO\(_2\) treatments at both salinities (F\(_{1,29}=226.690, P<0.001\)). In the ambient salinity treatment, SfG was reduced by more than 70% at elevated compared with ambient pCO\(_2\) levels (F\(_{1,29}=8.468, P<0.01\)). This was associated with a significant reduction in AFDW:DW ratio of the tissues (F\(_{1,8}=7.414, P<0.05\)). However, due to large variations between individuals, this was not associated with a significant reduction in growth rate (F\(_{1,68}=0.605, P=0.439\)). At a reduced salinity of 23, elevated pCO\(_2\) had a greater effect on SfG than at ambient salinities, with more than a 90% decrease between ambient and elevated pCO\(_2\) treatments (F\(_{1,29}=19.360, P<0.001\)).

Overall, in *M. edulis*, SfG was significantly lower in the elevated pCO\(_2\) treatments compared with ambient pCO\(_2\) levels (F\(_{1,4}=19.162, P<0.05\)). In addition, the effect of salinity on SfG was significantly influenced by elevated pCO\(_2\) (F\(_{2,40}=18.367, P<0.001\)). At ambient pCO\(_2\) *M. edulis* showed a small but significant increase in SfG between ambient and the 23 salinity treatments (mean difference \(2,40= 5.096\pm2.422, P<0.05\); Table 3), but a significant decrease at the lowest salinity of 16 (mean difference \(2,4= -12.352\pm2.418, P<0.001\)). However, there was no significant variation in SfG between salinity treatments at elevated pCO\(_2\) (F\(_{2,41}=1.641, P=0.206\)). Patterns in SfG were reflected in observed growth rate. At ambient pCO\(_2\), growth rate was maintained unchanged between ambient and the 23 salinity treatment (mean difference \(2,39= 0.01\pm0.008, P=0.227\)), but significantly decreased from 0.035±0.006 g day\(^{-1}\) in the ambient salinity treatment to 0.014 g day\(^{-1}\) in the 16 salinity treatment (mean difference \(2,39= -0.021\pm0.008, P<0.05\)). Growth rates did not vary significantly between salinity treatments at elevated pCO\(_2\) levels (F\(_{2,39}=0.754, P=0.477\)).

**Discussion**

*Feeding responses to combined elevated pCO\(_2\) and reduced salinity*

Following 26 days exposure to the combined treatment, surviving tunicates maintained
CR and energy absorption between salinities of 30 and 23 at present ambient levels of 
$pCO_2$. However, $pCO_2$ levels associated with predicted OA had a synergistic effect with 
the lowest CR recorded in the elevated $pCO_2$ and reduced salinity treatment. As there 
was no significant difference in POM between treatments, energy ingestion was also 
lowest under elevated $pCO_2$ and reduced salinity. Reductions in CR may result from 
reduced pumping activity and siphon retraction. *C. intestinalis* have a single inhalant 
siphon which they utilise for feeding and respiration. During repeated short-term 
exposure to a reduced salinity of 19, *C. intestinalis* close their siphons to avoid internal 
exposure to low salinity seawater, thereby avoiding osmotic imbalance (Shumway, 
1978). Pumping rates remained reduced until external salinity levels were restored to 
normal (Shumway, 1978). As *C. intestinalis* exhibited no significant variation in 
absorption efficiency across experimental treatments, reduced energy ingestion resulted 
in an uncompensated decrease in total energy absorption. 

In the ambient $pCO_2$ treatments, *M. edulis* demonstrated a reduction in CR in 
the lowest salinity treatment (16). *M. edulis* has also been shown to exhibit reduced 
pumping activity in order to limit internal exposure to low salinity water (Shumway 
1977). Both species show little ionic- or osmo-regulatory capacity, they have developed 
this response to isolate the tissues from exposure to reduced salinity conditions and 
associated ionic stress. Valve closure in response to decreases (50%) in sea water 
concentration has previously been reported in hard clam (*Mercenaria mercenaria*; 
Anderson & Prosser 1953) and the pacific oyster (*Crassostrea gigas*; Shumway 1977). 
Longer-term (4 week) exposure to reduced salinity levels comparable with the present 
study also led to reduced CR in the mussel, *Perna viridis* (Wang et al. 2011). Here 
reduced salinity did not result in a complete loss of pumping activity, that would restrict 
gas exchange, but reduced CR are likely to be involved with this general strategy to 
limited internal exposure to reduced salinities. 

In contrast to *C. intestinalis*, *M. edulis* do partially compensate for reduced CR 
by up regulating absorption efficiency at lower salinities. The Atlantic Deep Sea 
Scallop (*Placopecten magellanicus*) also increases absorption efficiency as filtration 
rates decreased (e.g. Cranford & Hargrave 1994; cf, Wang et al. 2011). At ambient 
$pCO_2$ this up regulation in absorption efficiency in the 23 salinity treatment leads to a 
slight but significant increase in overall energy absorption. However, at a salinity of 16 
this compensation is incomplete resulting in lower overall energy absorption.
Compensatory changes in absorption efficiency may be limited due to an overall reduction in absorption efficiency at elevated $pCO_2$, as also shown for Juvenile *Mytilus chilensis* (Navarro *et al.* 2013). Sea urchin larvae (*Strongylocentrotus droebachiensis*) exposed to elevated $pCO_2$ also showed reduced digestion rates and a 0.3-0.5 pH unit decrease in gut alkalinity, which was associated with decreased *in vitro* protease activity. Interestingly this $pCO_2$ induced reduction in digestive activity was partly compensated by increased feeding rates (Stumpp *et al.* 2013) as seen here.

In the present study, reduced absorption efficiency reported in the elevated $pCO_2$ treatments may also change the acclimatory strategy of *M. edulis* to reduced salinity. In contrast to ambient $pCO_2$ treatments where CR are reduced at low salinities, at elevated $pCO_2$ CR are maintained across all salinities, possibly to compensate for the reduction in absorption efficiency. CR and particle retention efficiency in bivalves may be much more plastic than previously thought (e.g. Denis *et al.* 1999; Strohmeier *et al.* 2009, Strohmeier *et al.* 2012; Cranford *et al.* 2016). For example, *M. galloprovincialis* can increase CR maintaining energy absorption during food limitation (Denis *et al.* 1999). However, the maintenance of CR at lower salinity and elevated $pCO_2$ here is not sufficient to fully compensate for $pCO_2$-associated reductions in absorption efficiency, resulting in lower overall energy absorption. Relative increases in CR/pumping represents a trade-off between exposure of internal tissues to unfavourable conditions and the attempted maintenance of total energy absorbance though feeding, which is likely sensitive to the length of exposure and size of energy reserves. When exposed to elevated $pCO_2$, the maintenance of ventilation rates, associated with pumping activity, may also facilitate greater CO$_2$ excretion (e.g. Donohue *et al.*, 2012).

**Metabolic responses to combined elevated PCO$_2$ and reduced salinity**

In general *M. edulis* and *C. intestinalis* exhibited similar metabolic responses to combined $pCO_2$ and salinity conditions, with MO$_2$ decreasing in response to reduced salinity and increasing in response to elevated $pCO_2$. Although in *M. edulis* significant decreases in MO$_2$ at the 23 and 16 salinity treatments were limited to the elevated $pCO_2$ treatment. Several studies have reported increased metabolic rates in response to elevated $pCO_2$ in marine invertebrates (e.g. Wood *et al.* 2008; Beniash *et al.* 2010; Calosi *et al.* 2013) including *M. edulis* (Thomsen and Melzner 2010). Elevated metabolic rates may be associated with increased energetic costs of maintaining
physiological homeostasis (Wood et al. 2008; Beniash et al. 2010). Conversely, reduced metabolic rate may help conserve energy at more extreme pCO$_2$/pH levels outside current predictions for ocean acidification (Langenbuch and Portner 2004); as shown by $M$. galloprovinicali following 3 months’ exposure to pH 7.3 (Michaelidis et al. 2005) and $M$. edulis following 2 months’ exposure to pH 7.14 (Thomsen and Melzner 2010). However, metabolic depression may not be sustainable in the longer-term with molluscs adapted/acclimatised to naturally elevated pCO$_2$ ecosystems requiring elevated metabolic rates per gram of tissue to maintain performance (Harvey et al. 2016; Garilli et al. 2015).

Metabolic responses to reduced salinity vary greatly in marine invertebrates with both increases (e.g. Navarro 1988) and decreases (e.g. Shumway 1978) in MO$_2$ reported. In general, this response is dependent on ion-regulatory capacity, with stenohaline invertebrates, such as $M$. edulis and $C$. intestinalis, demonstrating a decrease in MO$_2$ (Shumway 1978), as reported here. $C$. intestinalis also reduces MO$_2$ in response to decreased salinity, possibly associated with a reduction in ventilation and pumping activity (Shumway 1978). $M$. edulis and other bivalves also demonstrate reduced pumping to protect their internal structures from reduced salinity seawater (Anderson and Prosser 1953; Shumway 1977). Reduced pumping has been shown to reduce oxygen tension in the mantle cavity of $M$. edulis (Tang and Riisgård 2016) and the clam Arctica islandica (Taylor 1976). Valve control in $M$. edulis is postulated to reduce metabolic rate via reduction of oxygen partial pressure in the mantle cavity conserving energy during starvation (Tang and Riisgård 2016). However, as oxygen uptake is the result of metabolic demand for ATP and not an adaptive/acclimatory response, there is no known mechanism to explain how lowering oxygen availability via valve control could reduce oxygen demand without enzymatic feedback associated with harmful anaerobic pathways. This assumption also presumes that food limitation is ubiquitous with restricted valve opening and reduced CR, but this has been repeatedly questioned (e.g. Denis et al. 1999; Strohmeier et al. 2009; Strohmeier et al. 2012). Reduced activity due to restricted valve/siphon opening could reduce metabolic demand in response to low salinity (Shumway, 1978). Although, direct costs of pumping are estimated to be inconsequential in filter feeders (Jørgensen et al. 1986), reductions in metabolic rate due to reduced feeding and associated specific dynamic action (SDA) could be more significant. SDA accounts for approximately 20% of oxygen uptake rates in both $M$. edulis and $C$. intestinalis, depending on food quality.
In a wide variety of filter feeders, including *M. edulis*, reduced feeding is associated with reduced metabolism (Thompson and Bayne 1972). Observed decreases in routine metabolic rate with decreased salinity here do not exclude the theoretical possibility that costs of maintaining homeostasis could increase, and that this ATP is reallocated from other energetically demanding processes such as feeding and digestion. As routine metabolic rates were determined in naturally fed animals no attempt is made to separate costs associated to maintaining homeostasis and costs associated with energy assimilation via feeding. This study instead focuses on the ecologically relevant overall energy requirement of the animal that, unlike some studies on starved animals, considers that natural levels of feeding and digestion have an energetic cost that may also lead to important trade-offs with growth and should therefore be included in a general assessment of energetic costs.

**Resource allocation to growth**

After 26 days incubation surviving tunicates showed a significant reduction in energy available for growth and reproduction (SfG) in the elevated pCO₂ treatments. This was a result of decreased energy absorption through feeding and to a lesser extent increased routine metabolic costs (including the costs of maintaining homeostasis and energy assimilation via feeding). In sea urchin larvae (*Strongylocentrotus purpuratus*) elevated pCO₂ (1271 μatm); also reduced SfG, attributed to increased allocation of absorbed energy to metabolism. (Stumpp *et al.* 2011). Larvae in ambient pCO₂ conditions allocated between 78 and 80% of available energy to growth, whereas, larvae incubated at elevated pCO₂ invested only 39-45% (Stumpp *et al.*, 2011). Increased costs of maintaining physiological homeostasis have also been postulated to reduce energy available for growth in the brittle star, *Amphiura filiformis*, exposed to simulated OA (Wood *et al*. 2008) and in gastropods inhabiting naturally elevated pCO₂ environments (Harvey *et al*. 2015; Garilli *et al.*, 2015). However, in the present study increased routine metabolic rates in *C. intestinalis* at elevated pCO₂ only accounted for a 1.2 j/day and 0.6 j/day decrease in SfG, at salinities of 30 and 23 respectively (calculated from differences in MO₂ between pCO₂ treatments, Fig 1A, assuming a heat equivalent of oxygen uptake of 0.456 J μmol⁻¹ O₂; Gnaiger 1983). Whereas, reductions in energy absorbed though feeding at elevated pCO had a much greater effect on SfG, reducing energy availability by 328.8 J day⁻¹ at a salinity of 30 and 244.8 j day⁻¹ at a salinity of 23 (calculated from differences in energy absorption between pCO₂
treatments, Table 2). Despite reductions in SfG, no significant reduction in growth rate could be attributed to elevated $pCO_2$ at the ambient salinity, probably due to large individual variability. However, patterns in SfG were consistent with patterns in mortality among treatments. In treatments with surviving *C. intestinalis* after 26 days the lowest SfG and highest mortality was observed at a salinity of 23 and elevated $pCO_2$ with the lowest mortalities observed in ambient $pCO_2$ treatments where SfG was conserved between the ambient (30) and 23 salinity treatments.

Salinity caused mortality of 100% after 20 days in *C. intestinalis* similar to other studies (e.g. Vercaemer et al 2011). In the 23 salinity and elevated $pCO_2$ treatment *C. intestinalis* showed 53% mortality, here a selection may be possible as the survivors that are examined are the most tolerant individuals within the population. Since survivorship was above 80% in all other treatments, a selection effect is unlikely. Growth rates also significantly decreased with salinity with a reduction in body mass (negative growth) observed in all reduced salinity treatments. In the ambient $pCO_2$ treatment this reduction in body mass at reduced salinity occurred despite the maintenance of SfG and survivorship, possibly attributable to a significant increase in tissue AFDW:DW ratio. Consequently, in the 23 salinity treatment, available energy (SfG) may be diverted toward storage and increased carbon richness of tissues at the expense of overall growth. Although this is likely to increase the density of energy stores the benefits of a reduction in body size are difficult to explain. Paleontological and present reductions in body size, known as the Lilliput effect, associated with adaptation to natural elevations in $pCO_2$ may, in part, help to maintaining metabolic efficiency (Garilli *et al*. 2015). Although beyond the scope here, reduced body mass of *C. intestinalis* in the ambient $pCO_2$ and reduced salinity treatment is associated with an increase in mass specific metabolic rates while conserving whole animal energetic demand compared to controls, possibly facilitating metabolic efficiency.

Across all treatments only one individual *M. edulis* died during the 26 day incubation, in the ambient $pCO_2$ and salinity treatment. Sessional variation in body mass in Norwegian populations of *M. edulis* is highly dependent on reproduction, with body mass increasing in early summer before decreasing with spawning between June and August and then increasing again between September and December (e.g. Strohmeier *et al*. 2015). Making late Autumn growth, as documented in the present study, important to winter survival. The 8% increase recorded over 26 days under ambient conditions at
the same time of year is similar to previous studies (15-30% Strohmeier et al 2015).

As in *C. intestinalis*, *M. edulis* showed a decrease in SfG with an elevation in
$pCO_2$ in the ambient and 23 salinity treatments, attributable to reduced energy available
through feeding and to a lesser extent an increase in metabolism. Despite less energy
available for growth at elevated $pCO_2$, there was no significant effect of $pCO_2$ on
growth rate within the 26 day period of exposure. Elevated $pCO_2$ can negatively affect
the growth of *M. edulis* both under natural conditions in the Baltic Sea that resemble
predicted OA (Thomsen and Melzner 2010; Thomsen *et al*., 2013), and in the laboratory
(e.g. Fitzner *et al*. 2015), although $pCO_2$ levels and length of exposure varied.

At ambient $pCO_2$ levels SfG showed a slight but significant increase in the 23
salinity treatment compared to the controls, attributable to an elevation in absorption
efficiency (discussed above), and results in the conservation of growth rates in this
treatment. However, at the lowest salinity increased absorption efficiency cannot
compensate for reduced CR and despite significant reduction in metabolic rates, SfG is
reduced leading to a significant reduction in growth rate. Comparisons between
populations of *M. edulis* from the North Sea and in the Baltic Sea where Baltic mussels
living at comparatively lower salinities are frequently smaller than North Sea mussels,
also attributed decreased growth to increased metabolic costs at lower salinities
(Tedengren and Kautsky 1986) whereas in the present study decreases in SfG and
associated growth rate at a salinity of 16 is due to reduced CR and not changes in
metabolic rate. Interestingly salinity had no further effect on SfG or growth rates at
elevated $pCO_2$ levels. Ammonia excretion only amounts to 1-2% of energy loss via
metabolism during autumn (Bayne and Widdows 1978) and was therefore not
considered here. In the high $pCO_2$ ambient salinity treatment where metabolic rates
were highest ammonia excretion would amount to an estimated loss of 0.014-0.028 J h$^{-1}$
which only represents 0.11-0.23% of the energy absorbed though feeding in this
treatment and so any overestimation of SfG is considered inconsequential.

**Conclusion and implications** Under ambient salinities of 30 energy for mussel growth
and reproduction could be reduced by up to 50% after mid-term exposure to elevated
$pCO_2$ levels predicted for the end of the century, leading to possible losses for the
aquaculture industry. However, growth rate of *C. intestinalis*, was reduced by 70% in
energy for growth and reproduction under the same conditions possibly relieving
pressure on the industry from this invasive tunicate. The reduction in SfG and growth
rate in mussels as a result of elevated $p\text{CO}_2$ is unlikely to be further affected by changes in salinity between 16 and 30. Whereas, under future predicted levels of $p\text{CO}_2$, *C. intestinalis* showed 100% mortality at a reduced salinity of 16 and showed more than 90% decrease in SfG with an associated mean reduction in biomass (negative growth) at a salinity of 23. Although future levels of ocean acidification may reduce mussel productivity, the effect on the industry may be, in part, compensated by the reduced productivity of invasive tunicates particularly during times of low salinity (e.g. seasonal precipitation or melt-water). Consequently, an elevated $p\text{CO}_2$ in future mussel aquaculture could also benefit from lower salinity sites. Although mid-term exposures, as in the present study, give an indication of acclimatisation capacity and are ecologically relevant to seasonal changes in salinity and carbonate chemistry, caution should be applied when extrapolating theses result to naturally assembled ecosystems. Lifelong and multigenerational responses to chronic changes in $p\text{CO}_2$ and salinity need further investigation. For example, reductions in feeding by the grazing mollusc *Littorina littorea* in response to elevated $p\text{CO}_2$ and temperature are no longer observed after 5 months of acclimation (Russell *et al* 2013). The relationship between energy available for growth and growth rate is complex. For example, *C. intestinalis* showed a loss in biomass in the ambient $p\text{CO}_2$ reduced salinity treatment despite the maintenance of SfG. This disconnect between energy available for growth and actual growth is likely to be due to changes in the carbon richness (i.e. energetic density/storage) of the tissues, the length of exposure to adverse conditions, and possibly changes in metabolic efficiency associated with body size.

Changes in carbonate chemistry and salinity may interact resulting in a variety of feeding and metabolic responses, effecting energy acquisition and utilisation that in-turn determines productivity. Interestingly, under natural feeding conditions, energy available for production is more dependent on feeding plasticity (i.e. the ability to regulate clearance rate and absorption efficiency) in response to elevated $p\text{CO}_2$ and reduced salinity than on changes in routine metabolic rates. This dependence on feeding plasticity shows the importance of understanding feeding plasticity, in addition to more commonly studied metabolic rates, in determining the comparative acclimatisation capacity of competing species to future climate change.
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### Table 1. Physico-chemical seawater measurements from each of the six nominal $p$CO$_2$ and salinity treatments over the 26 day exposure period

<table>
<thead>
<tr>
<th>Nominal $p$CO$_2$ treatment (atm)</th>
<th>500</th>
<th>500</th>
<th>500</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal salinity treatment</td>
<td>30</td>
<td>23</td>
<td>16</td>
<td>30</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>$p$CO$_2$ treatment (atm)</td>
<td>602±17.9$^a$</td>
<td>548±21.8$^a$</td>
<td>611±14.1$^a$</td>
<td>1045±54.0$^b$</td>
<td>964±19.2$^b$</td>
<td>1054±24.4$^b$</td>
</tr>
<tr>
<td>Salinity</td>
<td>30.5±0.11$^a$</td>
<td>22.9±0.22$^b$</td>
<td>16.2±0.15$^c$</td>
<td>30.3±0.17$^a$</td>
<td>22.9±0.39$^a$</td>
<td>15.6±0.16$^a$</td>
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<tr>
<td>Temperature (°C)</td>
<td>10.5±0.20$^a$</td>
<td>10.14±0.23$^a$</td>
<td>9.74±0.26$^a$</td>
<td>11.0±0.47$^a$</td>
<td>10.6±0.33$^a$</td>
<td>10.3±0.41$^a$</td>
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<tr>
<td>TA (μmol kg$^{-1}$)</td>
<td>2206±7$^a$</td>
<td>1678±8$^a$</td>
<td>1227±12$^a$</td>
<td>2201±9$^a$</td>
<td>1625±24$^b$</td>
<td>1161±26$^c$</td>
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<tr>
<td>pH</td>
<td>7.88±0.01$^a$</td>
<td>7.84±0.02$^a$</td>
<td>7.67±0.01$^b$</td>
<td>7.67±0.01$^b$</td>
<td>7.60±0.02$^c$</td>
<td>7.43±0.01$^b$</td>
</tr>
<tr>
<td>DIC (μmol kg$^{-1}$)</td>
<td>2105±5.10$^a$</td>
<td>1624±5.10$^b$</td>
<td>1226±4.31$^c$</td>
<td>2162±3.93$^d$</td>
<td>1618±29.7$^b$</td>
<td>1194±1.42$^c$</td>
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<tr>
<td>HCO$_3$ (μmol kg$^{-1}$)</td>
<td>1989±6.24$^a$</td>
<td>1546±6.81$^b$</td>
<td>1172±0.93$^c$</td>
<td>2060±3.55$^d$</td>
<td>1553±1.94$^a$</td>
<td>1131±0.75$^b$</td>
</tr>
<tr>
<td>CO$_2$ (μmol kg$^{-1}$)</td>
<td>87.8±2.53$^a$</td>
<td>52.5±2.69$^b$</td>
<td>21.6±0.37$^c$</td>
<td>56.9±1.48$^b$</td>
<td>28.8±0.81$^b$</td>
<td>11.7±0.29$^c$</td>
</tr>
<tr>
<td>$\alpha_{\text{aq}}$</td>
<td>2.15±0.01$^a$</td>
<td>1.33±0.07$^b$</td>
<td>0.57±0.01$^c$</td>
<td>1.40±0.04$^b$</td>
<td>0.74±0.02$^c$</td>
<td>0.31±0.01$^d$</td>
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<tr>
<td>$\alpha_{\text{eq}}$</td>
<td>1.35±0.04$^a$</td>
<td>0.82±0.04$^b$</td>
<td>0.33±0.01$^c$</td>
<td>0.88±0.03$^b$</td>
<td>0.45±0.01$^c$</td>
<td>0.18±0.01$^d$</td>
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<td>POM (mg L$^{-1}$)</td>
<td>1.29±0.05$^a$</td>
<td>1.25±0.05$^a$</td>
<td>0.97±0.14$^a$</td>
<td>1.04±0.05$^a$</td>
<td>0.87±0.05$^a$</td>
<td>0.74±0.15$^a$</td>
</tr>
<tr>
<td>Suspended Particles 10mL$^{-1}$ 1-1.5μm</td>
<td>26890±3230$^a$</td>
<td>21189±2327$^a$</td>
<td>25700±3926$^a$</td>
<td>28039±2465$^a$</td>
<td>25069±3026$^a$</td>
<td>26594±3257$^a$</td>
</tr>
<tr>
<td>Suspended Particles 10mL$^{-1}$ 1.5-2μm</td>
<td>4944±163$^a$</td>
<td>4544±167$^a$</td>
<td>3683±131$^a$</td>
<td>6051±372$^a$</td>
<td>5430±345$^a$</td>
<td>4439±241$^a$</td>
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<td>Suspended Particles 10mL$^{-1}$ 2-2.5μm</td>
<td>2064±84$^a$</td>
<td>1925±106$^a$</td>
<td>1524±69$^a$</td>
<td>2468±165$^a$</td>
<td>2181±145$^a$</td>
<td>1748±97$^a$</td>
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<tr>
<td>Suspended Particles 10mL$^{-1}$ 2.5-3μm</td>
<td>1033±42$^a$</td>
<td>982±57$^a$</td>
<td>796±53$^a$</td>
<td>1209±87$^a$</td>
<td>1072±60$^a$</td>
<td>865±57$^a$</td>
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<tr>
<td>Suspended Particles 10mL$^{-1}$ 3-4μm</td>
<td>134±59$^a$</td>
<td>1279±88$^a$</td>
<td>1087±114$^a$</td>
<td>1507±119$^a$</td>
<td>1332±82$^a$</td>
<td>1116±80$^a$</td>
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<tr>
<td>Suspended Particles 10mL$^{-1}$ 4-5μm</td>
<td>1365±40$^a$</td>
<td>1301±84$^a$</td>
<td>1102±74$^a$</td>
<td>1560±142$^a$</td>
<td>1388±88$^a$</td>
<td>1183±111$^a$</td>
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<tr>
<td>Suspended Particles 10mL$^{-1}$ 5-6μm</td>
<td>630±23$^a$</td>
<td>588±23$^a$</td>
<td>466±26$^a$</td>
<td>730±82$^a$</td>
<td>646±52$^a$</td>
<td>555±67$^a$</td>
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<td>Suspended Particles 10mL$^{-1}$ 6-7μm</td>
<td>371±13$^a$</td>
<td>350±15$^a$</td>
<td>266±15$^a$</td>
<td>425±51$^a$</td>
<td>364±28$^a$</td>
<td>302±32$^a$</td>
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<tr>
<td>Suspended Particles 10mL$^{-1}$ 7-8μm</td>
<td>263±12$^a$</td>
<td>244±8$^a$</td>
<td>196±13$^a$</td>
<td>313±40$^a$</td>
<td>269±22$^a$</td>
<td>210±21$^a$</td>
</tr>
<tr>
<td>Suspended Particles 10mL$^{-1}$ 8-9μm</td>
<td>178±7$^a$</td>
<td>164±6$^a$</td>
<td>125±7$^a$</td>
<td>209±25$^a$</td>
<td>181±16$^a$</td>
<td>129±11$^a$</td>
</tr>
<tr>
<td>Suspended Particles 10mL$^{-1}$ 9-10μm</td>
<td>123±5$^a$</td>
<td>109±4$^a$</td>
<td>86±6$^a$</td>
<td>143±15$^a$</td>
<td>126±12$^a$</td>
<td>88±7$^a$</td>
</tr>
</tbody>
</table>
Temperature, salinity and pH (NBS scale) were measured 3 times daily. Total alkalinity (TA) was measured twice weekly. All other parameters [pCO₂; DIC (total dissolved inorganic carbon); calcite and aragonite saturation state (Ω_{calc} and Ω_{arag}, respectively); HCO₃⁻; and CO₃²⁻] were calculated from pH and AT with CO2SYS (Lewis and Wallace, 1998) using the dissociation after Dickson and Millero (1987). Particulate organic matter (POM) is a mean across species at the time of CR determination. Concentration of suspended particles, within 10 size-intervals between 1 and 10μm in diameter, were determined every 48 h during the 26-day incubation using a laser particle counter (PAMAS GmbH, Model S4031GO), values are presented as the mean number of particles 10ml⁻¹ of seawater. Values are means ± s.e.m. Different superscript letters indicate significant variation between treatments (ANOVA, Tukey HSD post hoc, P<0.05).

**Table 2.** Estimated energetic parameters for surviving *C. intestinalis* after 26 days exposure to combined elevated pCO₂ and reduced salinity treatments.

<table>
<thead>
<tr>
<th>Nominal pCO₂ treatment (µatm)</th>
<th>500</th>
<th>500</th>
<th>1000</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal salinity treatment</td>
<td>30</td>
<td>23</td>
<td>30</td>
<td>23</td>
</tr>
<tr>
<td>Energy Ingested (J h⁻¹)</td>
<td>34.9±3.41⁵²A</td>
<td>40.0±3.05⁵³A</td>
<td>19.4±2.97¹B</td>
<td>5.7±3.21²B</td>
</tr>
<tr>
<td>Absorption Efficiency (%)</td>
<td>34.8±7.51</td>
<td>39.2±3.78</td>
<td>23.4±1.49</td>
<td>27.5±1.62</td>
</tr>
<tr>
<td>Energy Absorbed (J h⁻¹)</td>
<td>14.8±2.39⁵²A</td>
<td>15.3±2.28⁵³A</td>
<td>1.1±2.39⁵³B</td>
<td>5.1±2.22²B</td>
</tr>
<tr>
<td>Scope for Growth (J h⁻¹)</td>
<td>14.7±2.64⁵²A</td>
<td>15.2±2.28⁵³A</td>
<td>4.9±2.22²B</td>
<td>1.0±2.39²B</td>
</tr>
</tbody>
</table>

Values are estimated means ± s.e.m generated from the GLMM (pCO₂×Salinity) and adjusted to the mean mass of sampled individuals (120.1 mg DW). Different superscript numbers and letters indicate significant variation (p >0.05) established by F-tests based on linearly independent pairwise comparisons among the estimated marginal means. For Absorption Efficiency values are mean % ± s.e.m with statistical comparisons as above but based on arc sign square root transformed data. Numbers indicate significant effects of salinity within each level of pCO₂. Letters indicate significant effects of pCO₂ within each level of salinity.
Table 3. Estimated energetic parameters for *M. edulis* after 26 days exposure to combined elevated $p$CO$_2$ and reduced salinity treatments.

<table>
<thead>
<tr>
<th>Nominal $p$CO$_2$ treatment (μatm)</th>
<th>500</th>
<th>500</th>
<th>500</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal salinity treatment</td>
<td>30</td>
<td>23</td>
<td>16</td>
<td>30</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>Energy Ingested (J h$^{-1}$)</td>
<td>68.7±4.64$^{1}$</td>
<td>58.3±4.38$^{1}$</td>
<td>21.5±4.37$^{2A}$</td>
<td>62.5±4.37$^{1L2}$</td>
<td>63.8±4.67$^{1}$</td>
<td>48.1±4.36$^{2B}$</td>
</tr>
<tr>
<td>Absorption Efficiency (%)</td>
<td>32.8±2.34$^{1A}$</td>
<td>47.5±1.07$^{2A}$</td>
<td>48.1±0.99$^{2A}$</td>
<td>19.5±0.93$^{1B}$</td>
<td>31.3±0.79$^{1L2B}$</td>
<td>44.6±1.58$^{2B}$</td>
</tr>
<tr>
<td>Energy Absorbed (J h$^{-1}$)</td>
<td>22.8±1.79$^{1A}$</td>
<td>27.7±1.70$^{2A}$</td>
<td>10.3±1.70$^{3A}$</td>
<td>12.1±1.70$^{B}$</td>
<td>14.3±1.80$^{B}$</td>
<td>15.8±1.70$^{B}$</td>
</tr>
<tr>
<td>Scope for Growth (J h$^{-1}$)</td>
<td>22.2±1.82$^{1A}$</td>
<td>27.3±1.72$^{2A}$</td>
<td>9.8±1.72$^{3}$</td>
<td>10.7±1.72$^{B}$</td>
<td>13.4±1.84$^{B}$</td>
<td>15.0±1.82</td>
</tr>
</tbody>
</table>

Values are estimated means ± s.e.m generated from the GLMM ($p$CO$_2$*Salinity) and adjusted to the mean mass of sampled individuals (598.5 mg DW). Different superscript numbers and letters indicate significant variation ($p >0.05$) established by F-tests based on linearly independent pairwise comparisons among the estimated marginal means. For Absorption Efficiency values are mean % ± s.e.m with statistical comparisons as above but based on arc sign square root transformed data. Numbers indicate significant effects of salinity within each level of $p$CO$_2$. Letters indicate significant effects of $p$CO$_2$ within each level of salinity.
Figure legends:

**Figure 1.** Oxygen uptake and Clearance rate in *C. intestinalis* (A and C respectively) after 26 days exposure to 23 or 30 salinity and *M. edulis* (B and D respectively), after 26 days exposure to 16, 23 or 30 salinity at ambient (500 µatm; black bars) or elevated (1000 µatm; white bars) pCO$_2$. No data is shown for *C. intestinalis* at 16 salinity due to 100% mortality in this treatment. Values are estimated means ± s.e.m generated from the GLMM (pCO$_2$*Salinity) and adjusted to the mean mass of sampled individuals (*C. intestinalis* = 120.1 mg DW; *M. edulis* = 598.5 mg DW). Different numbers and letters indicate significant variation (p >0.05) established by F-tests based on linearly independent pairwise comparisons among the estimated marginal means. Numbers indicate significant effects of salinity within each level of pCO$_2$. Letters indicate significant effects of pCO$_2$ within each level of salinity.

**Figure 2.** SfG, AFDW:DW and Growth rate for *C. intestinalis* (A, C and E respectively) after 26 days exposure to 23 or 30 salinity and *M. edulis* (B, D and F respectively) after 26 days exposure to 16, 23 or 30 salinity at ambient (500 µatm; black bars) or elevated (1000 µatm; white bars) pCO$_2$. No data is shown for *C. intestinalis* at 16 salinity due to 100% mortality in this treatment. Values are estimated means ± s.e.m generated from the GLMM (pCO$_2$*Salinity) and adjusted to the mean mass of sampled individuals (*C. intestinalis* = 120.1 mg DW; *M. edulis* = 598.5 mg DW). Different numbers and letters indicate significant variation (p >0.05) established by F-tests based on linearly independent pairwise comparisons among the estimated marginal means. Numbers indicate significant effects of salinity within each level of pCO$_2$. Letters indicate significant effects of pCO$_2$ within each level of salinity.
Figure 1:
Figure 2