Effects of seawater alkalinity on calcium and acid-base regulation in juvenile European lobster (*Homarus gammarus*) during a moult cycle

Karen L. Middlemiss[1]*, Mauricio A. Urbina[1,2]*, Rod W. Wilson[1]*

[1] Biosciences, College of Life and Environmental Sciences, Geoffrey Pope Building, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK.

[2] Departamento de Zoología, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile.

E-mail addresses: kombi@xtra.co.nz (K.L. Middlemiss); mauriciourbina@udec.cl (M.A. Urbina); r.w.wilson@exeter.ac.uk (R.W. Wilson).

* Corresponding authors. Biosciences, College of Life and Environmental Sciences, Geoffrey Pope Building, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK. Tel.: +44 (0)1392 725171; fax: +44 (0)1392 263434. *E-mail addresses*: kombi@xtra.co.nz (K.L. Middlemiss); m.a.urbina-foneron@ex.ac.uk (M.A. Urbina); r.w.wilson@ex.ac.uk (R.W. Wilson).

M.A. Urbina present address: Departamento de Zoología, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile. *E-mail address:* mauriciourbina@udec.cl

Abstract

Fluxes of NH_4^+ (acid) and HCO_3^- (base), and whole body calcium content were measured in European lobster (*Homarus gammarus*) during intermoult (megalopae stage), and during the first 24 h for postmoult juveniles under control (~2000 µeq/L) and low seawater alkalinity (~830 µeq/L). Immediately after moulting, animals lost 45% of the total body calcium via the shed exoskeleton (exuvia), and only 11% was retained in the uncalcified body. At 24 h postmoult, exoskeleton calcium increased to

~46% of the intermoult stage. Ammonia excretion was not affected by seawater alkalinity. After moulting bicarbonate excretion was immediately reversed from excretion to uptake (~4-6 fold higher rates than intermoult) over the whole 24 h postmoult period, peaking at 3-6 h. These data suggest that exoskeleton calcification is not completed by 24 h postmoult. Low seawater alkalinity reduced postmoult bicarbonate uptake by 29 % on average. Net acid-base flux (equivalent to net base uptake) followed the same pattern as HCO_3^- fluxes, and was 22 % lower in low alkalinity seawater over the whole 24 h postmoult period. The common occurrence of low alkalinity in intensive aquaculture systems may slow postmoult calcification in juvenile *H. gammarus*, increasing the risk of mortalities through cannibalism.

Keywords: ammonia excretion, bicarbonate excretion, calcification, carbonate, crustacean, exuvia, moult cycle

1. Introduction

Around 80% of the total body calcium present in calcifying marine organisms is located in the exoskeleton as calcium carbonates (Wheatly et al., 2002) and environmental availability of Ca^{2+} ions and HCO_3^- equivalents (HCO_3^- and/or CO_3^{2-}) is critical to postmoult shell hardening. Internal Ca^{2+} stores are also important during moulting, mainly for terrestrial and freshwater crustaceans (Li and Cheng, 2012; Zanotto and Wheatly, 2003), but only of minor importance during postmoult calcification in marine crustaceans. For example, the amount of reabsorbed and internally stored calcium varies greatly from ~65% in the freshwater/land crab *Holthuisana transversa* (Sparkes and Greenaway, 1984) to <10% in the marine European shore crab, *Carcinus maenas* (Robertson, 1937).

During moulting, a major portion of total body Ca^{2+} is lost via the shed exoskeleton (exuvia) or excreted to the surrounding medium while smaller portions can be stored internally (e.g. in gastroliths, hepatopancreas, and haemolymph) (Ahearn et al., 2004; Greenaway, 1985, 1988; Hecht, 1914; Wheatly and Ayers, 1995; Zanotto and Wheatly, 2003). Postmoult, the external seawater, internal stores, and ingested material (especially shed exuvia) are collectively critical components required to achieve re-calcification of the exoskeleton.

Calcium ions and HCO_3^- equivalents can become limiting factors in the natural environment and in aquaculture facilities (e.g. due to changes in salinity and alkalinity). In freshwater environments the level of the cation Ca^{2+} varies by more than 100-fold from <50 µM to >5 mM (depending on factors such as underlying geology and rainfall), whereas in marine environments Ca^{2+} levels are far higher and more stable, typically being ~10 mM (Greenaway, 1985). Therefore, calcium availability is not normally a limiting factor in either natural marine environments or aquaculture facilities. However, in seawater recirculating aquaculture systems (RAS) the availability of HCO_3^- equivalents is often much lower than in natural environments. In RAS, where calcifying crustaceans are cultured at high densities and moult frequently (e.g. European lobster, *H. gammarus*; Middlemiss et al., 2015a), this occurs partly due to the consumption of alkalinity during the calcification process, described by the net reaction that encompasses all the relevant acid-base changes occurring (Hofmann et al., 2010):

$$Ca^{2+} + 2HCO_3^- \Leftrightarrow CaCO_3 + CO_2 + H_2O$$

However, in RAS the microbially-mediated nitrification (conversion of ammonia to nitrate) that occurs within the biofiltration systems, also consumes significant amounts of HCO_3^- ions (Eshchar et al., 2006). As a result, the alkalinity of seawater in RAS declines over time if seawater replacement rates are insufficient. The alkalinity and Ca^{2+}

in seawater has been shown to directly affect the rate of postmoult calcification in crustacean exoskeletons and subsequent growth and survival rates in adults (Cameron and Wood, 1985; Greenaway, 1983; Whiteley, 2011). Slowing of the postmoult calcification process in marine crustaceans can be detrimental not only to natural populations, but also to aquaculture productivity by a) prolonging the period when the soft-bodied postmoult animal is more vulnerable to cannibalism (Borisov et al., 2007), or b) disease (Cawthorn, 1997; Scolding et al., 2012), and c) extending the sensitive period when they lack hardened mouthparts which allow feeding to resume postmoult (Whiteley, 2011).

As with many intensively harvested marine species, European lobster have experienced a general population decline. A more recent 4-fold increase in UK capture rates in 2013 compared to the 1950's (FAO, 2014), has been attributed to conservation measures. These include the hatchery-based culturing of pelagic larval stages through to early benthic juvenile stages for release into coastal waters as part of stock enhancement programmes (Daniels et al., 2010; Tully, 2004). However, cannibalism and mortalities of the early life stages, exacerbated during moulting and by an elevated moult frequency (e.g. 4 moults in around 30 days at 17-19 °C in European lobster; Middlemiss et al., 2015a), is a major limitation for crustacean aquaculture (Hecht and Appelbaum, 1988; Marshall et al., 2005; Middlemiss et al., 2015b). Currently there is no knowledge of how low alkalinity seawater that commonly occurs in RAS, may influence the moulting success, survival, particularly of the early life stages, and overall productivity of the culture of European lobster.

The aim of the current study was to investigate the effects of low alkalinity seawater on the moulting and exoskeleton hardening in juvenile European lobster (megalopae to juveniles). Specifically, this study assessed the change in body calcium stores, and the

fluxes of acid-base relevant ions (HCO_3^- and NH_4^+) into and out of the body (uptake and excretion), at different times in the moult cycle, between megalopae and juvenile stages. We hypothesized that calcification of the new postmoult exoskeleton will be slowed down under low alkalinity conditions.

2. Methods

2.1 Animals

Animals were reared at the National Lobster Hatchery (NLH; Padstow, North Cornwall, UK) until they reached the 5 day old megalopae (stage IV), at which point 120 individuals were transferred to the University of Exeter (Devon, UK) and held in the Aquatic Resource Centre within the Biosciences department. Larvae were left undisturbed for a 4 day period before experimental work started, to allow recovery from any disturbance during transportation. Animals were fed a diet of NLH formulated pellet feed once daily and housed in individual cells within a closed recirculation seawater system. Water temperature was maintained at 21.5 °C and at a salinity of 35 ppt. Photoperiod (12L:12D) was set at 08:00 hours (dawn) and 20:00 hours (dusk). The moult cycle was approximately 16 days between megalopa (stage IV) and juvenile (stage V). Intermoult megalopae were sampled 8 days post metamorphosis. Postmoult sampling was conducted over a 24 h period commencing immediately after moulting to first juvenile (stage V).

2.2 Experimental setup

For measuring the flux of acid-base relevant ions between lobsters and the ambient seawater, individual animals were transferred into 40 mL aerated chambers that were partially submerged in a water bath maintained at 21.5 °C.

131 Flux measurements were carried out over a 24 h period in seawater with one of two
132 different total alkalinity (TA) treatments; TA = 2007 ± 3 µeq/L, pH 8.153 ± 0.015,
133 pCO_2 371 ± 15 µatm (control; N = 18) or, TA = 833 ± 4 µeq/L, pH 7.794 ± 0.004, pCO_2
134 381 ± 3 µatm (low alkalinity; N = 19). Note that pCO_2 was almost identical between
135 treatments because both were equilibrated with atmospheric air by continuous aeration
136 (see below). All other water chemistry parameters were maintained identical between
137 treatments, as detailed in Table 1. Each treatment was formulated from an artificial sea
138 salt mix (Tropic Marin; TMC, UK) dissolved in deionised water. To achieve the desired
139 total alkalinity and pH values for the two treatments, firstly the alkalinity of freshly
140 made up seawater was measured (see below). Sufficient hydrochloric acid (1 M) was
141 then added to each seawater treatment to achieve the desired alkalinity (either ~2,000 or
142 ~830 µeq/L) following vigorous overnight aeration to ensure equilibration with
143 atmospheric CO_2 for both treatments. Flux measurements in the intermoult animals
144 (mean wet weight ~63 mg ± 1.4 SE) were measured for 24 hours (n=15) in control
145 seawater only (TA ~2000 µeq/L).

146 Table 1 insert here

147 **Table 1** Water chemistry parameters maintained throughout *H. gammarus* moult cycle during
148 measurement of calcium and acid-base regulation in intermoult megalopae and postmoult
149 juvenile stages for both control and low alkalinity seawater treatment groups. Alkalinity and pH
150 of both treatments are stated in the Methods text (Section 2.2)

Parameter	Mean ± SE
Temperature (°C)	21.0 ± 0.5
Salinity (%)	35.0 ± 1.0
Na^+ (mM)	518.1 ± 13.0
Ca^{2+} (mM)	7.56 ± 0.17
K^+ (mM)	10.1 ± 0.4
Mg^{2+} (mM)	49.7 ± 1.4

151

Using a separate group of lobsters from the same batch, and therefore undergoing the same moult cycle, samples were taken for measuring the calcium (as carbonate) content of the whole animal. These included samples for: 1) whole body during intermoult (n=15); 2) whole body at ~0.5 h postmoult (n=8), 3) exuvia at ~0.5 h postmoult (n=8); and 4) whole body at 24 hour postmoult (n=10). Animals were placed in a -80 °C freezer to euthanase. They were blotted dry, weighed, then dried at 40 °C in an oven for ~48 hours in pre-dried and weighed centrifuge tubes. Following drying, all 4 sample groups, as detailed above, were weighed and placed in 1.5 mL of 5% (w/v) sodium hypochlorite (NaOCl), until organic components were digested leaving only the remaining white calcium (as carbonate) stores (primarily exoskeleton and gastroliths if present). This treatment has no discernible effect on carbonate mineralogy and causes no detectable dissolution (Gaffey and Bronnimann, 1993). Samples were then rapidly rinsed in deionised water three times to remove traces of the hypochlorite (centrifuging and decanting the supernatant between rinses). They were then dried (as above), weighed, and digested in a volume of 1 M HCl (40 µL acid per mg of sample; Walther et al., 2011), for later calcium analysis (see below).

To assess the ion flux rates, seawater samples were taken at the beginning and at the end of the 0-3, 3-6, and 6-24 h flux periods, representing the initial and final conditions within each chamber for each flux period. Seawater samples were preserved and stored at 4 °C for titratable alkalinity analysis (see below), a subsample frozen at -20 °C for total ammonia analysis, and another subsample immediately diluted (see below) and stored at -20 °C for ion analysis. Postmoult experiments were carried out on newly moulted animals (n=10) within 30 minutes of moulting. Animals moulted naturally and at different times over a period of 2 days, and they were distributed alternately to chambers containing either control or low alkalinity seawater. This was achieved by checking the holding trays every 30 min during two days, thus ensuring an equal

number of animals exposed to each treatment, and limiting any bias associated with time of moult. At the start of each flux, animals were rinsed (using seawater from their allocated treatment), and transferred to their chamber containing clean seawater of the relevant alkalinity.

2.3 Ion analysis

Seawater samples for measuring titratable alkalinity were preserved by adding 4 µl of 4% (w/v) mercuric chloride per 10 mL of seawater (Dickson et al., 2007), and stored at 4 °C until analysis by double titration using a Metrohm autotitrator (815 Robotic USB Sample Processor XL, Switzerland). Alkalinity was measured by titration of a 20 mL sample to pH 3.89 with 0.02 N HCl whilst gassing with CO_2-free nitrogen, followed by return to the starting pH with 0.02 N NaOH, similar to the method described by Cooper et al. (2010). Ammonia concentration was measured using a modified version of the colourimetric method of Verdouw et al., (1978), using a microplate reader (Infinite® M200 PRO, Tecan, UK). A calibration curve was constructed using NH_4Cl standards made up in the relevant seawater (control or low alkalinity). Calcium concentrations in the seawater and total body calcium were measured by ion chromatography (Dionex ICS-1000), and flame photometry (Corning 410), respectively. However, the Dionex was unable to detect the very small changes over each flux period against the very high background in seawater calcium and therefore no data are included in this study for calcium fluxes. Acid-digested body samples for calcium analysis were diluted 201-fold in ultrapure deionised water prior to analysis and calcium content data were not corrected for individual animal or exuvia weights.

2.4 Calculations

202 Acid-base relevant fluxes (positive values representing uptake and negative
203 values representing excretion) were calculated (μeq kg^{-1} h^{-1}) using the following
204 equation:

205 $$J_X = [([X]_i - [X]_f) \times V] / (M \times t)$$

206 as described in Wilson and Grosell, (2003), where V is the volume of water (L); M is
207 the mass of the lobster (kg); t is the duration of the flux period (h); and $[X]_i$ and $[X]_f$ are
208 the ion concentrations in the seawater (μmol L^{-1}) at the beginning and end of the flux
209 period, respectively. Titratable acid fluxes (J_{TA}) were calculated using the above
210 equation by reversing initial and final values to achieve acid instead of base fluxes. Net
211 flux of acid-base equivalents (J_{H+}^{net}) was then calculated as the sum of J_{TA} (μeq kg^{-1} h^{-1})
212 and ammonia fluxes J_{Amm} (μeq kg^{-1} h^{-1}) as described by McDonald and Wood (1981).
213 Seawater pCO_2 was calculated using the CO2sys programme (using the constants from
214 Mehrbach et al. (1973), refitted by Dickson and Millero (1987), and using the KSO_4
215 dissociation constants from Dickson (1990)) following direct measurements of pH
216 (NBS scale) and total alkalinity. Having data on lobsters held in seawater at different
217 concentrations of bicarbonate gave us the opportunity to roughly estimate some
218 characteristics of the bicarbonate uptake transport system. Specifically, the affinity
219 constant (K_m) for external bicarbonate uptake and the maximum rate of uptake once the
220 transport system was saturated (V_{max}). Assuming that the transport system for
221 bicarbonate in lobsters displays classical Michaelis-Menten kinetics (as it does in other
222 aquatic animals; Goss et al. (1993)), the K_m and V_{max} values were determined by
223 transformation of the data using Lineweaver-Burk and Eadie-Hofstee regression plots
224 (Perry and Rivero-Lopez. 2012). This analysis was carried out for data collected during
225 the three flux periods after moulting (0-3 h, 3-6 h, and 6-24 h) from all individual
226 lobsters for measured bicarbonate flux and measured average bicarbonate concentration

during the relevant flux period. To visualise the transport characteristic graphically, the K_m and V_{max} values were then used to generate kinetics curves for each time period by substitution into the Michaelis-Menten equation:

$$HCO_3^- \text{ flux rate} = (V_{max} \times [HCO_3^-]) \ / \ (K_m + [HCO_3^-])$$

2.5 Statistical analysis

Analysis was carried out using SigmaPlot V.11.0 (Systat Software Inc., USA) and data are presented as the mean ± SE. Total body Ca^{2+} data was $\log_{10}$ transformed to meet parametric assumptions, and then analysed using a one-way ANOVA. Data for HCO_3^-, NH_4^+ and net H^+ fluxes were analysed using a two-ways ANOVA (time and treatment as factors). In order that parametric assumptions of equal variance and normal distribution were met, NH_4^+ data were transformed using square root. When ANOVA tests were significant, data were subjected to post hoc analysis (Tukey). Differences were considered significant with P value ≤ 0.05.

3. Results

3.1 Exoskeleton and whole animal calcium

Mean total whole animal calcium (as carbonate) in intermoult animals was 24.3 µmol/animal, and immediately after moulting this had significantly declined by ~90% (Fig. 1, $P<0.05$). Almost four times as much Ca^{2+} (as carbonate) was present in the discarded exuvia compared to the freshly moulted body. Compared to the intermoult animals, data suggest that 45% of whole animal Ca^{2+} (as carbonate) was lost in the shed exoskeleton (exuvia), 11% was retained in the uncalcified body, with the remaining 44% presumably excreted to the seawater during the pre-moult period or maintained in organic compartments (Fig. 1). After a 24 h period the newly moulted animal had increased whole animal Ca^{2+} (as carbonate) content by 4-fold (compared to the

252 immediate postmoult whole animal), but this was still less than 50% of the previous
253 intermoult animal ($P<0.001$).

254 Fig. 1 insert here

255 3.2 Acid-base fluxes

256 Lobsters exhibited low excretion rates of both NH_4^+ and HCO_3^- during
257 intermoult (megalopae) (Fig. 2A, B). Bicarbonate (base) excretion was ~2.7 fold higher
258 than the NH_4^+ (acid) excretion rate, and the difference between these two variables
259 therefore culminated in a positive net H^+ flux during intermoult (representing net base
260 excretion or acid uptake). In the first 3 hours immediately postmoult, NH_4^+ excretion
261 was ~5-fold higher than the intermoult rate (Fig. 2A). Thereafter, the ammonia
262 excretion rate steadily declined at each subsequent postmoult flux period, but remained
263 elevated in relation to the intermoult (still ~1.5 fold higher 24 h postmoult). Ammonia
264 excretion rate was ~60% lower during the second postmoult flux (3-6 h) compared to
265 the first (0-3 h; Fig. 2A, $P<0.001$) and decreased further to ~30% of the 0-3 h period
266 during the 6-24 h postmoult flux ($P=0.014$). Low alkalinity had no effect on the net
267 NH_4^+ excretion rate in postmoult animals ($P=0.087$) (Fig. 2A).

268 During postmoult periods, HCO_3^- fluxes were reversed in comparison to intermoult (i.e.
269 HCO_3^- uptake instead of excretion), and were of much greater magnitude (~6-fold and
270 4-fold peak increase in control and low alkalinity treatments, respectively, during the 3-
271 6 h postmoult period; Fig. 2B). Under low alkalinity conditions, net bicarbonate uptake
272 rates were substantially smaller than control alkalinity conditions (Fig. 2B; 37, 29 and
273 21% respectively for the 0-3, 3-6 and 6-24 h periods). There was no interactive effect
274 between time and treatment ($P=0.942$) between the control and low alkalinity HCO_3^-
275 treatment groups. In both the control and low alkalinity treatments, HCO_3^- uptake was
276 significantly higher at 3-6 h than at 0-3 h postmoult ($P=0.006$ and $P=0.003$, respectively

277 Fig. 2B). At all time points postmoult there was a significantly higher uptake of HCO_3^-
278 in the control alkalinity treatment group than low alkalinity (P=0.007, P=, P=0.012 and
279 P=0.013 respectively). In both high and low alkalinity treatment groups, HCO_3^- showed
280 an increase between 0-3 and 3-6 h postmoult, and then a decrease at 6-24 h postmoult,
281 however rates were still higher than at the 0-3 h time point.

282 Rates of HCO_3^- uptake were substantially larger than the amount of NH_4^+ excretion,
283 resulting in HCO_3^- having a far greater impact on net H^+ fluxes (Fig. 2C). Subsequently,
284 during postmoult periods, all animals displayed large negative net H^+ fluxes (i.e., net
285 acid excretion rates equivalent to net base uptake). Net base uptake was significantly
286 higher in the controls than in the low alkalinity seawater treatment group at 0-3 and 3-6
287 h postmoult (P=0.032, P=0.002, respectively; Fig. 2C). There was no interaction
288 between factors time and treatment (P=0.436). Within the control treatment, net base
289 uptake during the 6-24 h postmoult period was significantly lower (P=0.05) than the
290 previous 3-6 h period (Fig. 2C). In both alkalinity treatment conditions, net H^+ fluxes
291 were highest from 3-6 h postmoult, and then from 6-24 h they decreased to below the 0-
292 3 h rates.

293 Table 2 shows that the K_m values for bicarbonate uptake were almost identical
294 regardless of the analysis method used (Lineweaver-Burk or Eadie-Hofstee). The K_m
295 values were similar during the first two time points (0-3 and 3-6 h) after the moult
296 (ranging from 889 to 945 µM), but this was reduced by almost 4-fold by the 6-24 h flux
297 period (K_m values of 270 to 298 µM).

298 Table 2 insert here

299 **Table 2**. The kinetics of HCO_3^- uptake in *H. gammarus*, first juvenile (stage V), during
300 the first 24 h of moulting in waters of two different alkalinities. K_m is the HCO_3^-
301 concentration needed to achieve a half-maximum HCO_3^- uptake, and V_{max} is the
302 maximum rate of HCO_3^- uptake. Both K_m and V_{max} were calculated by following both
303 Lineweaver-Burk (1934) and Hanes-Woolf methods.

	Lineweaver-Burk		Hanes-Woolf	
Flux Period (h)	K_m (µeq l^{-1} CO_3^-)	V_{max} (µeq $HCO_3^- h^{-1}$)	K_m (µeq l^{-1} HCO_3^-)	V_{max} (µeq $HCO_3^- h^{-1}$)
0-3	923	12201	945	12322
3-6	889	19419	899	19514
6-12	270	10339	298	10557

Fig. 2 insert here

4. Discussion

The focus of the current study was to examine the effect of low alkalinity seawater on the ability to take up external (seawater) HCO_3^- during the rapid postmoult calcification of their exoskeleton in *H. gammarus*. In comparison to the control treatment, rates of bicarbonate uptake by *H. gammarus* were significantly reduced in low alkalinity seawater. This would presumably result in slower calcification rates and a subsequent increase in time to complete exoskeleton mineralisation. It should be noted that in early life stages of lobster, Zoeal exoskeletons are not calcified, megalopae are partially calcified, and full calcification is present from the juvenile stage onwards (Anger, 2001). Therefore, the differences and effects found in the present study are expected to be even greater from juveniles to adults.

The availability of HCO_3^- ions in seawater is typically ~5 times lower than for Ca^{2+} ions and much less stable than Ca^{2+} in natural marine environments. In the present study pCO_2 was kept constant whilst varying alkalinity to reproduce aquaculture conditions. As a result, the low alkalinity seawater treatment was also lower pH (~7.8 compared to ~8.1 in the controls). However, it is commonly considered that changes to seawater pH per se within this range are unlikely to explain the effects we observed on HCO_3^- uptake in marine animals (e.g. Esbaugh et al., 2012). We have therefore assumed that changes

in calcification under low alkalinity conditions used here would likely be entirely due to the 60% reduction in availability of seawater bicarbonate for uptake via the gills.

Alkalinity, pH and pCO_2 are inextricably linked, and therefore the resulting consequence of a change in one parameter is a simultaneous change in either/both of the others (i.e. changing pH will by default result in subsequent increase/decrease in pCO_2 and/or alkalinity; Orr et al. 2005). In the current study, we avoided the issue of pCO_2 and focused on the effects of low alkalinity, a common problem in intensive aquaculture conditions. The experimental design used allowed evaluation of an acute (24 h) exposure to low alkalinity. Further experiments are needed in order to determine long term effects of low seawater alkalinity on calcification rates and subsequent impact on cultured animals.

4.1 Whole animal $CaCO_3$ stores

Immediately after moulting the juvenile *H. gammarus* had lost around 89% of their total stores of calcium (as carbonates) either via the exuvia or excreted to the surrounding water, leaving only 11% in the newly moulted animal. This is similar to findings by Graf (1978) in *Carcinus maenas*, supporting the knowledge that marine crustaceans readily acquire the required amounts of Ca^{2+} from surrounding water, and not from internal Ca^{2+} stores. Also, the combined sum of calcium (carbonates) in the newly moulted animal and the shed exuvia was ~56% of that present in the intermoult whole animal, indicating that around 44% of this calcium is lost during the demineralisation process to the surrounding medium, or stored in organic compartments (e.g. haemolymph and intracellular stores). It should be noted that the dissolved or organically-bound component of total body Ca^{2+} was not measured. Therefore we cannot distinguish between the amount of Ca^{2+} transferred from the exoskeleton during the pre-moult period into either internal organic stores or the surrounding medium. At

24 h postmoult, the calcium (as carbonate) present in the exoskeleton was still <50% of intermoult animals showing that quantitatively significant calcification of the new exoskeleton must continue beyond 24 h postmoult. This corresponds with the continued high rates of HCO_3^- uptake 24 h postmoult as discussed below.

Increased time for calcification results in higher energetic costs, reducing energy required for other physiological processes such as growth (Keppel et al., 2012). Results from the current study for juvenile *H. gammarus* show a reduced rate of postmoult bicarbonate uptake in low seawater alkalinity (which had simultaneously lower pH at unchanged CO_2 levels). Reduced seawater alkalinity in a recirculating aquaculture environment could therefore result in delayed hardening of the exoskeleton and reduced hatchery success (see below). However, the chemistry of seawater in aquaculture systems can be controlled through management of water quality (i.e. enrichment of rearing water with calcium, or more likely with bicarbonate as the greater limiting factor, as previously discussed). Obviously, upon release of cultured lobster into their natural environment they would then be faced with a number of naturally occurring environmental pressures. Over the coming centuries this will likely include ocean acidification, which will not change Ca^{2+} availability, but will reduce the concentration of CO_3^{2-} ions, whilst HCO_3^- would be largely unchanged (in fact slightly increased; Zeebe, 2012). Although the exoskeleton of early larvae stages in crustaceans are not fully mineralized, it is not yet known to what extent such changes would influence exoskeleton hardening in early life stages of crustaceans in general. Ocean acidification conditions have been shown to reduce calcification in some marine calcifiers, including our study species *H. gammarus* (Arnold et al., 2009), but increase it in others such as brittlestar (*Amphiura filiformis*; Wood et al., 2008), so based on our current understanding it is difficult to predict how crustaceans in general will respond to future conditions.

376 4.2 Acid-base fluxes

377 Calcification in Homarus species is known to take place rapidly during the first
378 day or two postmoult (Horne and Tarsitano, 2007). In the current study, low alkalinity
379 seawater caused a large reduction in the uptake of HCO_3^- equivalents via the gills (21-
380 37% across the three flux periods postmoult) which would subsequently slow down the
381 supply of internal CO_3^{2-} ions for calcification. Net acid-base fluxes (net base uptake in
382 this case, equivalent to net acid excretion) were also much reduced in low alkalinity
383 seawater during all three flux periods, in line with decreases in uptake of basic HCO_3^-
384 (whilst acidic NH_4^+ excretion was unaffected). These findings, confirm previous results
385 in Cameron (1985) on adult blue crab (*Callinectes sapidus*), which showed that reduced
386 HCO_3^- in seawater led to reduced uptake of HCO_3^- (and also net base uptake a.k.a. net
387 acid excretion). This would clearly translate into low alkalinity environments causing a
388 dramatic increase in the time it takes to complete calcification. This is very likely to be
389 detrimental for the successful culture of early developmental stages in lobsters and other
390 crustaceans for two main reasons. Firstly, slow hardening of the mouthparts may delay
391 the initiation of feeding which would result in metabolically active larval/juvenile
392 animals quickly depleting energy reserves. Therefore, a quick return to active feeding
393 will be essential not only for their subsequent survival postmoult, but also to recover
394 optimal growth rates (desired for aquaculture). Secondly, lobsters are highly
395 cannibalistic, and larvae that are slower to harden their exoskeletons will be even more
396 prone to predation by their intermoult peers in intensive aquaculture situations.
397
398 Estimates of the affinity constant (K_m) for bicarbonate uptake, whilst tentative, provide
399 some potential insight into how lobsters may be able to regulate their bicarbonate
400 transport systems after a moult. The almost 4-fold decrease in K_m for the 6-24 h period
401 compared to the first 6 hours suggests two possibilities. Firstly, it could mean that

lobsters are genetically programmed to switch to expressing higher affinity bicarbonate transporters after moulting, for which translation of the relevant genes takes more than 6 hours. This would potentially aid in enhancing bicarbonate uptake at the time of highest demand, i.e. rapid postmoult calcification of the exoskeleton. Alternatively, it could be that the data for lobsters held in low alkalinity seawater are having the major influence on this K_m estimate and that only this group were undergoing any temporal change in bicarbonate transport kinetics. If this second interpretation is correct, then it raises the possibility that lobsters already possess the genetic flexibility to acclimate to reduced alkalinity conditions (e.g. in commercial RAS) in terms of regulating bicarbonate uptake to provide the needs of postmoult calcification. Either way, this would be an interesting area for further investigation, to determine the precise physiological and molecular mechanisms that underlie the postmoult changes in acid-base regulation for the purposes of exoskeleton hardening.

5. Conclusion

Crustaceans require significant levels of calcium and bicarbonate equivalents to harden their new exoskeletons after ecdysis. Prior to and during a moult, most of the calcium present in the exoskeleton and internal carbonate stores during intermoult periods is lost either to the shed exuvia or via excretion to the surrounding medium, and a proportion is moved to internal non-carbonate stores. A 60% reduction in seawater alkalinity correspondingly reduced the rate of bicarbonate uptake (equivalent to acid excretion) by 29-42%, which is a major source for the carbonate component of the exoskeleton. Thus acute exposure (24 h) to low alkalinity seawater is likely to translate into a similarly slower calcification rate, and hence prolonged time to complete mineralisation and hardening of the exoskeleton. This could contribute to reduced feeding and growth rates and enhanced mortality from cannibalism. The current

research highlights the importance of seawater alkalinity with respect to potential economic and conservation gains for the crustacean aquaculture industry through increased survival rates from improved water management techniques. Future research should investigate the potential for longer term exposures to low alkalinity seawater commonly found in aquaculture facilities, to examine whether (and how) lobsters can acclimate in terms of postmoult calcification rates.

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586 Figure Legends

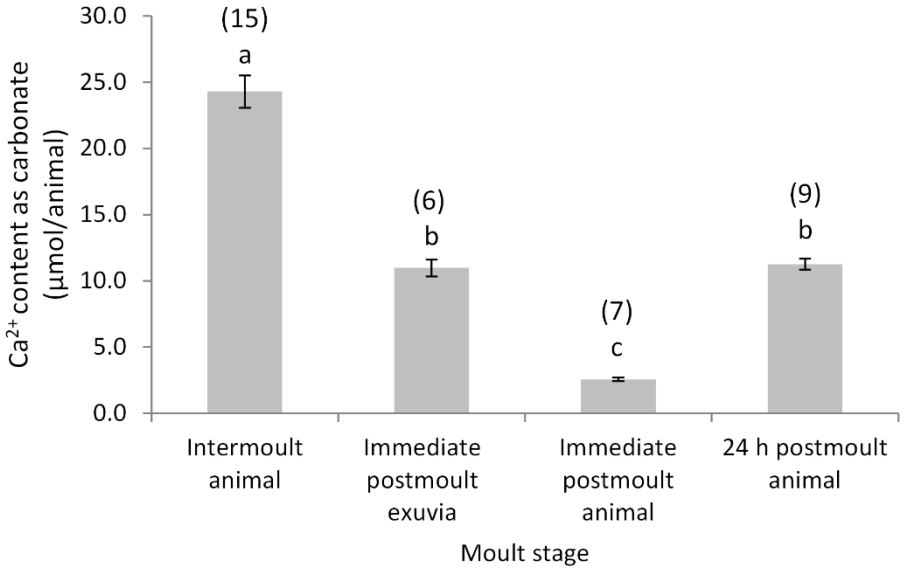
587

588 **Fig. 1** Calcium (bound to carbonate) stores in *H. gammarus* at intermoult (megalopae), exuvia
589 immediately postmoult and whole animal immediately postmoult and 24 h postmoult (juvenile).
590 Numbers in parenthesis are sample sizes. Data presented as the mean ± SE. Significant
591 differences represented by different letters and significance accepted at $P \leq 0.05$

592

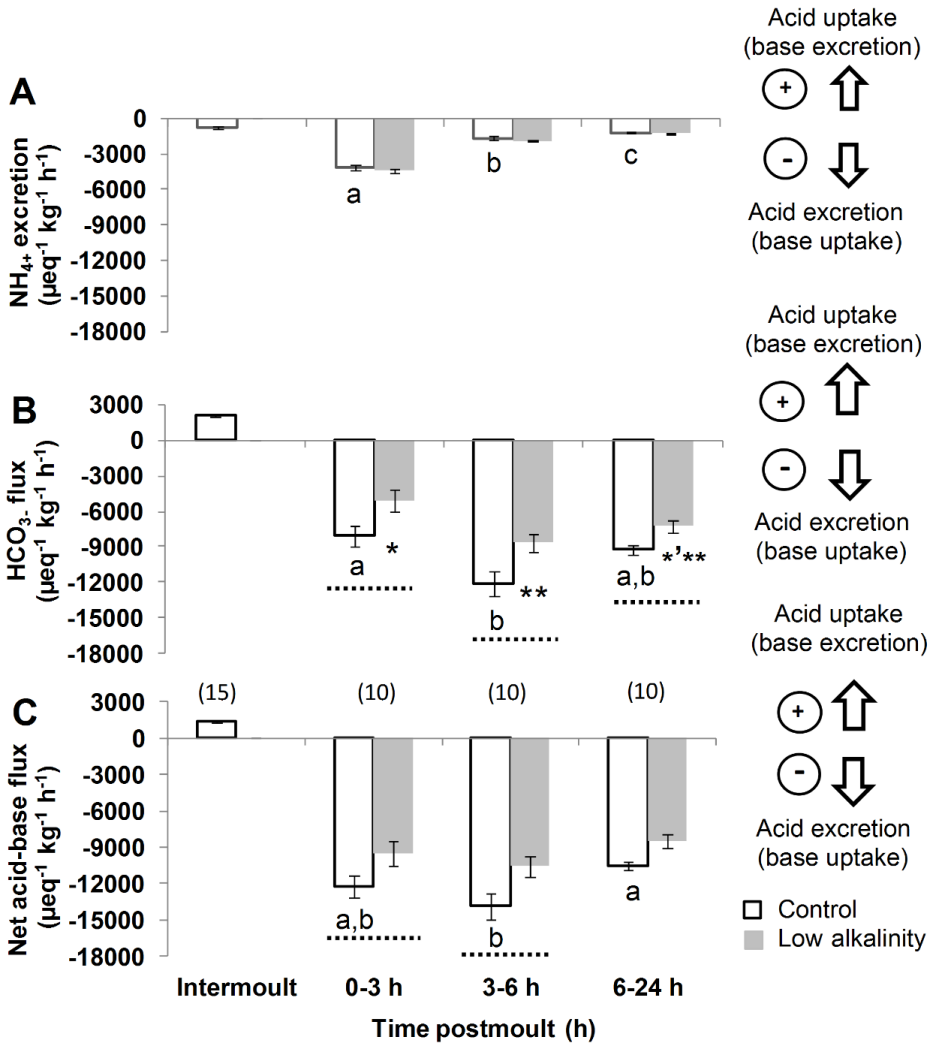
593 **Fig. 2** The NH_4^+ flux (A), HCO_3^- flux (B) and net acid-base flux (C) in intermoult (megalopae),
594 and 0-3, 3-6, and 6-24 h postmoult (juvenile) European lobsters in control and low alkalinity
595 seawater treatments. Numbers in parenthesis represent sample size. Data represent the mean ±
596 SE. Significant differences between time points in the control and low alkalinity treatment
597 groups are indicated by different letters and symbols respectively. Significant differences
598 between treatment groups at each time point are indicated with dotted lines. Significance
599 accepted at $P \leq 0.05$

600 **Figure 1 below**



601

602 **Figure 2 below**


A
B
C
NH_{4+} excretion (µeq^{-1} kg^{-1} h^{-1})
HCO_{3-} flux (µeq^{-1} kg^{-1} h^{-1})
Net acid-base flux (µeq^{-1} kg^{-1} h^{-1})
0
-3000
-6000
-9000
-12000
-15000
-18000
3000
a
b
c
a,b
*
**
*,**
(15)
(10)
(10)
(10)
Intermoult
0-3 h
3-6 h
6-24 h
Time postmoult (h)
Acid uptake (base excretion)
Acid excretion (base uptake)
Control
Low alkalinity

603