Effects of ocean acidification on embryo development: Does encapsulation matter?

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Abstract

As the concentration of CO_2 in surface seawaters increases (ocean acidification, or OA) the saturation of calcium carbonate decreases, preventing marine organisms from creating shells and other calcified structures. These effects of elevated CO_2 on calcification have been previously shown in free-spawning larvae, but are not as well-studied in larvae that spend their early life stages in encapsulation. The focus of our study was to determine what effects CO_2 would have on a diversity of encapsulated embryos, and whether different types of encapsulating structures provided different levels of protection against OA. We found only a moderate larval response to low (600 ppm), medium (1050 ppm), and high (1500 ppm) CO_2 concentrations across all species taken as a whole, but did observe that several species/ populations exhibited a decline in shell length with no corresponding decline in inorganic content. This suggests that while calcification was not significantly decreased by our OA conditions, perhaps the morphology of certain shells changed, becoming wider and shorter. Our hatch times, which increased with elevated CO_2, confirmed that increased CO_2 placed embryos under stress during development.

Introduction

Rapid climate change increasingly tests marine organisms in their ability to adapt and survive, and to so at speed. A key challenge to marine survival is OA due to anthropogenic CO_2, which results in increased carbonic acid in surface seawater and thus decreased pH (Ellis et al. 2009). By the beginning of the next century the average pH of oceanic surface waters may lower by 0.5 units, to a pH between 7.6 and 7.8 (Ellis et al. 2009, Clark et al. 2009). This increase in CO_2 and its resulting decrease in pH are likely

to have a variety of effects, both predictable and unexpected in the present moment; the focus of this study will be their effect on the development of encapsulated larvae.

OA has already been shown to have an effect on the fitness of calcifying marine larvae. For example, when exposed to a lower pH sea urchin larvae exhibit a decrease in both growth and calcification, which would likely impact their ability to protect themselves and survive as adults (Clark et al. 2009). Similarly, under conditions of increased pCO_2 *Nassarius obsoletus* hatchlings experience a decrease in inorganic content, primarily due to a decline in calcification (Fernandes & Podolsky, in review). OA has such an effect because its decrease in pH reduces the saturation state of calcite and aragonite, causing these minerals to dissolve. This undersaturation is happening quickly, and in the case of aragonite could spread throughout the Southern Ocean as soon as 2100 (Orr et al. 2005). Since these two forms of calcium carbonate are the main component of calcified structures in many marine animals, their undersaturation directly decreases the organisms' inorganic content, such as shells (Clark et al 2009).

Inorganic dissolution due to OA may be prevented by encapsulation of marine embryos in egg masses or capsules, a tactic commonly used by polychaetes and gastropods (Pechenik 1979). These structures are already known to buffer a variety of aquatic chemical changes, preventing rapid shock from reaching the embryos. For example, the gel of an egg mass, such as that of *Melanochlamys diomedea*, may either slow internal salinity change or reduce a period of low salinity within the mass (Pechenik 1983, Wood & DeSilets 1997). The intercapsular fluid of a mass or capsule may prevent desiccation through the maintenance of liquid around the embryos (Pechenick 1979). These extracapsular and intercapsular buffers can be critical in an intertidal habitat,

which is prone to highly variable conditions (Fernandes & Podolsky, in review). In the context of OA, the ability to handle such a range of situations could make encapsulated embryos more resilient to rapid changes in different variables: CO_2 and pH.

The effects of CO_2 and pH on encapsulated embryos are not as well-studied as the effects of O_2, but O_2 studies can still provide a useful foundation for the potential effects of OA. The focus of many O_2 studies is the risk of hypoxia in egg masses, for in a dense cluster of eggs anchored to an immobile surface it can be difficult for O_2 to reach central embryos. Photosynthetic substrates, such as *Zostera marina*, can increase O_2 exchange to the center of a mass, decreasing hypoxia (Woods & Podolsky 2007, Fernandes & Podolsky 2011). This exchange increase in the presence of heightened O_2 suggests that with raised CO_2 levels in the ocean, even the central eggs in a mass will likely be affected. The natural hypoxia in egg masses also suggests, however, that CO_2 from respiring larvae does not escape the egg mass quickly, potentially lowering internal pH of the mass itself. This naturally low pH may mean that species with encapsulation fair better under OA conditions than those without a naturally low pH environment in early life stages.

The effects of CO_2 and pH are better studied on free spawning embryos than encapsulated ones. In the presence of high CO_2 conditions (~1310 µatm), 63-h-old *Mytilus californianus* larvae were found to have a 4% decrease in shell length in comparison to those held at ambient CO_2 levels (~345 µatm) (Kelly et al. 2016). *M. californianus* larvae have consistently been found to have lower shell lengths in the presence of elevated CO_2 and the low aragonite saturation that results from it (Waldbusser et al. 2015). *Argopecten irradians* larvae, however, were found to have

reduced survival but not reduced shell size when exposed to elevated CO_2 during fertilization (White et al. 2014). In studies that have been conducted on encapsulated larvae there have been similar results. In the case of encapsulated *Littorina obtusata* embryos, decreased seawater pH slowed larval development time, decreased spinning and other embryonic movement within the capsule, shortened lateral shell length, and increased spiral shell length (Ellis et al. 2009). However, it is unclear whether such trends are common across a variety of species.

In this study we seek expand upon OA research to address (a) if encapsulated embryos are susceptible to elevated CO_2 and (b) if susceptibility is related to egg mass design. We used a diverse group of egg masses from gastropods collected in or near Friday Harbor Laboratories, WA. By exposing each egg mass to three levels of pCO_2 and measuring the growth of larvae we estimated their response to both present and two possible future marine conditions.

Methods

Study Organisms

We used a diverse array of gastropod encapsulated eggs that included globose, ribbon, string, and bare capsule types (Table 1). Adults and egg masses were collected in and around Friday Harbor Laboratories, WA. Some of these adults were kept in our lab to monitor for egg masses while others were held in other labs as part of separate experiments. Tanks where adults were held were checked daily for new egg masses, and only egg masses < 24 h post-deposition were used in experiments.

Experimental Design

Three pieces were taken from each egg mass so that the same genetic material could be placed in low, medium, and high treatments of CO_2; the exception to this procedure were two trials of *Lacuna sp.*, in which a different egg mass was placed in each treatment due to their small size. For each egg mass the development stage at the start of the experiment was recorded. The egg mass pieces were placed in individual wells of 6-well plates, along with 5 mL of filtered sea water (FSW) bubbled with one of the three levels of CO_2. The three treatments consisted of 600 ppm CO_2 for "low," or current, conditions, 1050 ppm CO_2 for "medium" conditions, and 1500 ppm CO_2 for "high" conditions. Our "low" treatment has a higher pCO_2 than the global average, 400 ppm, because we wished to mimic the conditions that the animals came from on San Juan Island, which has naturally more acidic waters averaging at 650 ppm (Murray et al. 2015).

We bubbled the treatment FSW using three compressed air tanks (Praxair Distribution Inc) containing O_2, N_2, and 1% CO_2/ 99% N_2. Gas from the three tanks was combined in bubbling to mimic natural atmospheric proportions for each treatment, and was fed through tubing into the headspace of glass bottles in preparation for use. Water for each treatment was allowed to equilibrate in the bottles for 48 hours before use. After bubbled FSW was added to the well plates, gases for the three treatments fed through tubes in the bottom of each box to maintain appropriate CO_2 levels. The pH of both a well containing only bubbled water and an adjacent well containing an egg mass sample were tested in each box, in order to determine if pH was remaining consistently as designed across cultures.

FSW was changed once per day for the first 8 days of the experiment, then changed to Mondays, Wednesday, and Fridays due to time constraints. The plates were stacked three to a box in closed 15 x 10 x 10 cm clear 3 x 5 index card boxes. The plates were rotated within their boxes daily to give them equal exposure to different levels in the box, which might have varied slightly in gas conditions or temperatures. All boxes were tied down to plastic louvre, which was submerged with PVC piping and straps to a depth of about 7 cm in flowing seawater to maintain a consistent temperature within boxes. The entire set up was covered in black plastic sheeting to reduce light to the water, and thus to decrease algal growth.

Larvae were checked daily for hatching, and once this occurred their development time to hatching was recorded. They were then photographed in the surface tension of their well using a Nikon Coolpix 4500 camera attached to a dissecting microscope (Olympus SZ60) under full magnification of both camera and microscope (6.3x). The larvae were then collected onto pre-ashed and pre-weighed GFC filters (Glass Microfibre Filter, 696, VWR North American Cat. No. 28333-143) under vacuum and photographed again under minimum magnification. These filters were then washed with reverse osmosis (RO) water with 3.5% ammonium formate in order to replace the FSW with a salt that evaporates. After 3 weeks the filters were washed with RO water to avoid any contamination in the ammonium formate bottle. The filters were then dried in a drying oven for at least 24 hours, weighed, and then ashed at 450°C in a muffle furnace for 6 hours and weighed again.

The responses of larvae to OA were quantified through four measures of growth: organic content, inorganic content, shell length, and hatch time. The organic content of

the hatched larvae was calculated by subtracting the ashed mass of the filter from the dry mass of the filter, and the inorganic content by subtracting the mass of the pre-ashed filter from the ashed mass of the sample and filter. The organic and inorganic masses of each individual larva was calculated by dividing the total values by the number of larvae counted on each filter. The shell length was measured in ImageJ using the most highly magnified of each set of photographs. The hatch time was measured from when masses were started in the treatment until at least 50% of their viable larvae had hatched.

Statistical Analysis

We used a mixed model analysis in SPSS (SPSS, Inc.) with CO_2 treatment as the fixed effect and replicate as the random effect for four analyses: shell length, inorganic mass, organic mass, and hatch time. For shell length we also treated replicate as the subject. For inorganic mass we determined the interaction term between CO_2 concentration and inorganic mass to be insignificant, and thus were able to remove this term and use organic mass as a covariate. In the case of hatch time, we nested replicates within species when analyzing all species as a whole.

Results

Over the course of 8 weeks our conditions maintained three distinct pH levels corresponding to low (current), medium, and high levels of seawater CO_2 (Fig 1). Although the respiring egg mass samples did lower the pH of their own well water, the difference was both consistent and small and thus we maintained the pH conditions that we expected (Fig 2). During the course of this study we also confirmed that pH decreased towards the center of *A. ocelligera* egg masses, also as we expected (Fig 3).

We measured inorganic mass with organic mass as a covariate to account for variation in larval count; in this measurement we expected inorganic mass to decrease with higher CO_2 as calcification became stunted. We found no significant decrease in inorganic mass as CO_2 increased, regardless of egg mass type. We found one significant increase in inorganic mass, in *A. papillosa*, which showed no significant difference between low and medium treatments and then significantly spiked between medium and high treatments (Fig 4).

We then measured both inorganic and organic mass per larva as a separate calculation to account for larval count. We expected inorganic mass to decrease with higher CO_2 as with the previous measurement, while we expected organic mass to remain the same since CO_2 seems to primarily affect calcification. As before we found *A. papillosa* to have the only significant change in inorganic content, increasing between low and high treatments. We found one significant decrease in organic between the low and high CO_2 treatments of *A. ocelligera* (Fig 7, Fig 8).

We expected shell length to decrease consistently with increased CO_2 as calcification became more difficult. We did find this trend to some extent in 6 species/populations of all three egg mass types: one globose, two ribbon, and both strings. In these species/populations we found significant decreases in shell length between low and high CO_2 treatments: -3.85% for *H. japonica*, -11.47% for *H. vesicula* from Argyle Lagoon, -5.28% for *H. vesicula* from Garrison Bay, -6.49% for *A. papillosa*, -1.65% for *Hermissenda crassicornis*, and -1.82% for *D. sandiegensis*. Notably, this decrease in shell length did not correspond to a decrease in inorganic mass for *A. papillosa*, but rather a spike (Fig 5).

We expected an increase in hatching time as larvae were placed under increased CO_2 stress. For the most part we found no significant increase in individual hatching times between treatments, with the exception of one globose and two ribbon species. When all species were taken into account simultaneously, however, there was an increase in overall hatching times with a significant increase between the high treatment in comparison to medium and low (Fig 6).

Discussion

Across all species as a whole we observed only moderate response to elevated CO_2 conditions, indicating that they may be pre-adapted to tolerate OA conditions due to naturally high CO_2 in the egg mass. This pre-adaption may cause species that encapsulate their embryos to have an advantage in early development under future ocean conditions. However, we did observe that certain species across all structure types reacted more strongly to CO_2 changes than others, particularly in terms of shell length. One globose, two ribbon, and both string masses exhibited decline in larval shell length, ranging from -1.65% to -11.47% between low and high CO_2 treatments. This decline supports previous research based on both free-spawning and encapsulated larvae, although it was only evident in 5 of 11 species.

Notably, both string species exhibited a decline in shell length: it is possible that they were both affected because these are the thinnest and least dense structures with the highest surface area of those used in this study. These characteristics may allow CO_s from respiring larvae to escape the mass better than in, for example, a globose mass, and thus these larvae may not have the same natural ability to cope with low pH levels. However, although both string masses were affected, *H. crassicornis* exhibited the least decline of

the 5 species while *A. papillosa* exhibited one of the highest, despite appearing to be the denser of the two. This suggests that although both string masses were affected, they may have differences in structure beyond shape that are important to pH tolerance.

Contrary to previous studies inorganic matter did not exhibit significant decline in any species. This suggests that although shell length declined in many species, it did not correspond to a similar decrease in calcification as we expected from previous studies. In fact, *A. papillosa* exhibited a decrease in shell length but a strong spike in inorganic matter. Shells in these species may therefore be shorter but also wider, suggesting that there was no significant decrease in actual calcification but a morphological response to stress nevertheless. This developmental response was supported by hatch times, which significantly increased with elevated CO_2 concentrations across all species taken as a whole. The increase in hatch time, while relatively small across all species and only significant in a few species taken individually, demonstrates that the stress induced by heightened CO_2 concentrations did make development slower and more difficult, and in the case of *A. papillosa* may have affected shell shape.

As expected most of our species did not exhibit a significant decline in organic matter per larva, with the one exception being *A. ocelligera*. This species showed a significant decline in organic mass between low and high concentrations of CO_2, although no other globose species exhibited significant changes in organic matter between treatments.

For future studies we would like to investigate potential change in shell morphology under OA stress by measuring both length and width of shells under similar conditions to this study. We would also like to compare free swimming larvae of species

with early encapsulation stages to those that free spawn in order to determine whether encapsulation provides a definitive advantage in the context of OA conditions.

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Appendix

Species (sample size)	**Form of Egg Mass**	**Collection Location**	**Mass/ Capsule Type**
Aeolidia papillosa (6)	White string laid in a flat coil, then in a larger spiral. 7 to 10 eggs per capsule.	Eagle Cove	String
Aglaja ocelligera (12)	Bright yellow to pale cream oblong mass (1.5 to 2.5 cm wide) laid in tight coil. 1 to 3 eggs per capsule.	False Bay	Globose
Diaulula sandiegensis (3)	White ribbon (about 0.4 cm wide) laid in 3 to 8 whorls in an oval spiral. 1 to 2 eggs per capsule.	Friday Harbor Labs dock	Ribbon
Doris montereyensis (2)	Cream to yellow ribbon (about 0.5 – 1 cm wide) laid in approximately 3 whorls.	Friday Harbor Labs dock	Ribbon
Fusitriton oregonensis (7)	Bare trapezoidal capsules laid in a continuous whorl. Thousands of egg per capsule.	Friday Harbor Labs touch tank	Bare capsule
Haminoea japonica (7)	Clear mass (about 0.5 – 0.75 cm wide) laid in a dense coil to create an overall parenthesis shape. 1 egg per capsule, eggs large and yellow.	Argyle Lagoon	Globose
Haminoea vesicula (21)	Short yellow ribbon (about 0.5 – 0.75 cm wide) laid in a straight line. 1 egg per capsule.	Garrison Bay, False Bay, Argyle Lagoon	Ribbon
Hermissenda crassicornis (5)	Fine pink string laid in a flat coil, then in a larger spiral. 1 to 4 eggs per capsule.	Friday Harbor Labs dock	String
Lacuna sp. (5)	Cream to yellow ribbon laid in dense donut shape, approximately 0.25 cm in diameter.	Friday Harbor Labs dock	Globose
Melanochlamys diomedea (11)	Clear to white oblong mass (1 – 1.5 cm wide) laid in a tight coil.	Argyle Lagoon, False Bay	Globose

Melibe leonina (7)	Short cream ribbon (about 1.4 cm wide) with attached edge shorter than free, laid in a line in the shape of a fan. 5 or more eggs per capsule.	Picnic Cove	Ribbon
Nucella lamellosa (3)	Bare, pointed oblong capsule (about 0.5 – 0.75 cm long).	False Bay	Bare capsule
Phyllaplysia taylori (4)	Sturdy rectangular ribbon (0.5 – 1 cm wide) laid along eel grass. 1 egg per capsule.	Friday Harbor Labs mesocosm	Ribbon
Triopha catalinae (5)	White or cream ribbon (1.4 cm wide) in loose oval coil, with wavy free edge. 1 to 2 eggs per capsule.	Friday Harbor Labs dock	Ribbon

Table 1. Species used for egg masses and capsules in experiment (Hurst 1966-1967, personal observations).

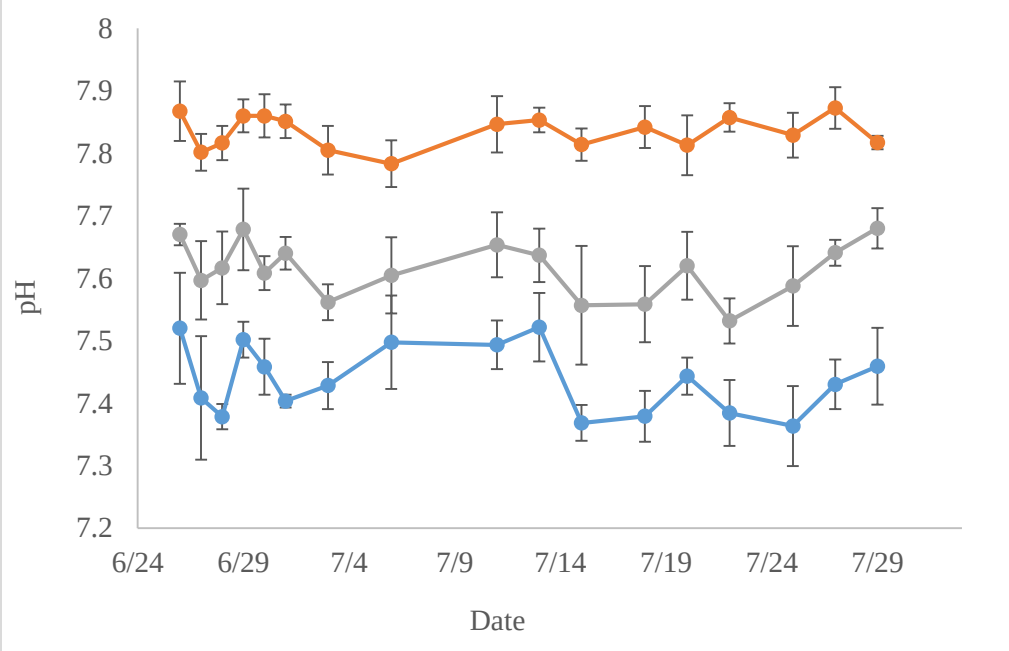


Fig 1. Culture conditions for pH, measured from a well in each box that contained only water, with no egg mass sample.

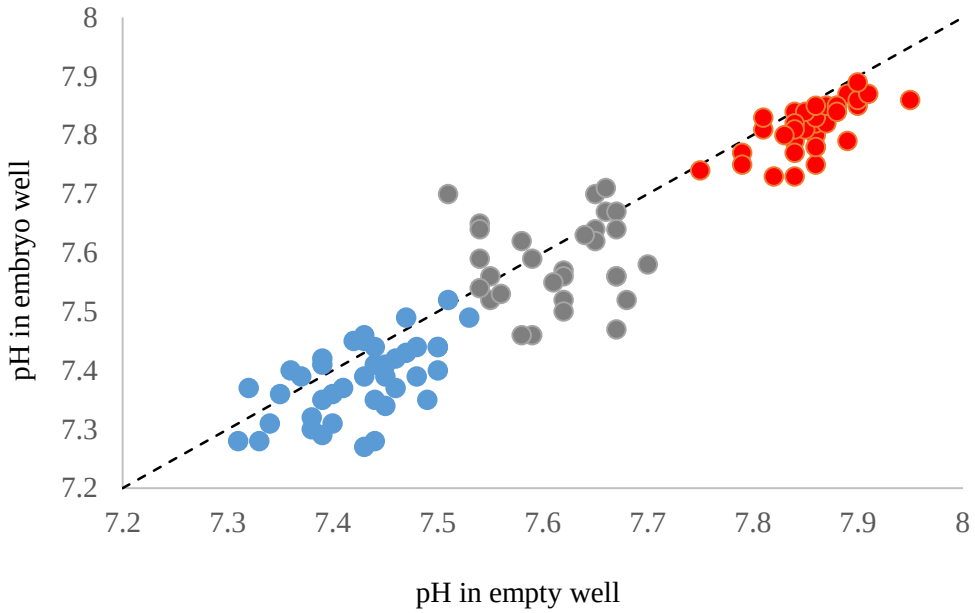


Fig 2. pH conditions in a well containing only water contrasted to conditions in an adjacent well containing an egg mass. This was repeated for each box, and although the egg mass samples caused a slightly lower pH in their water, the culture remained reasonably consistent as a whole.

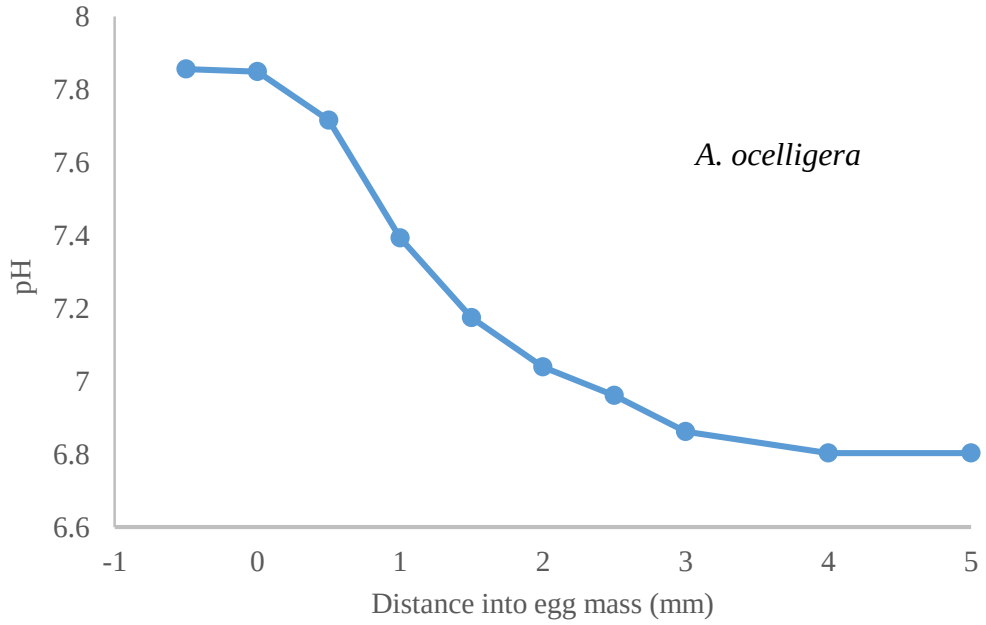
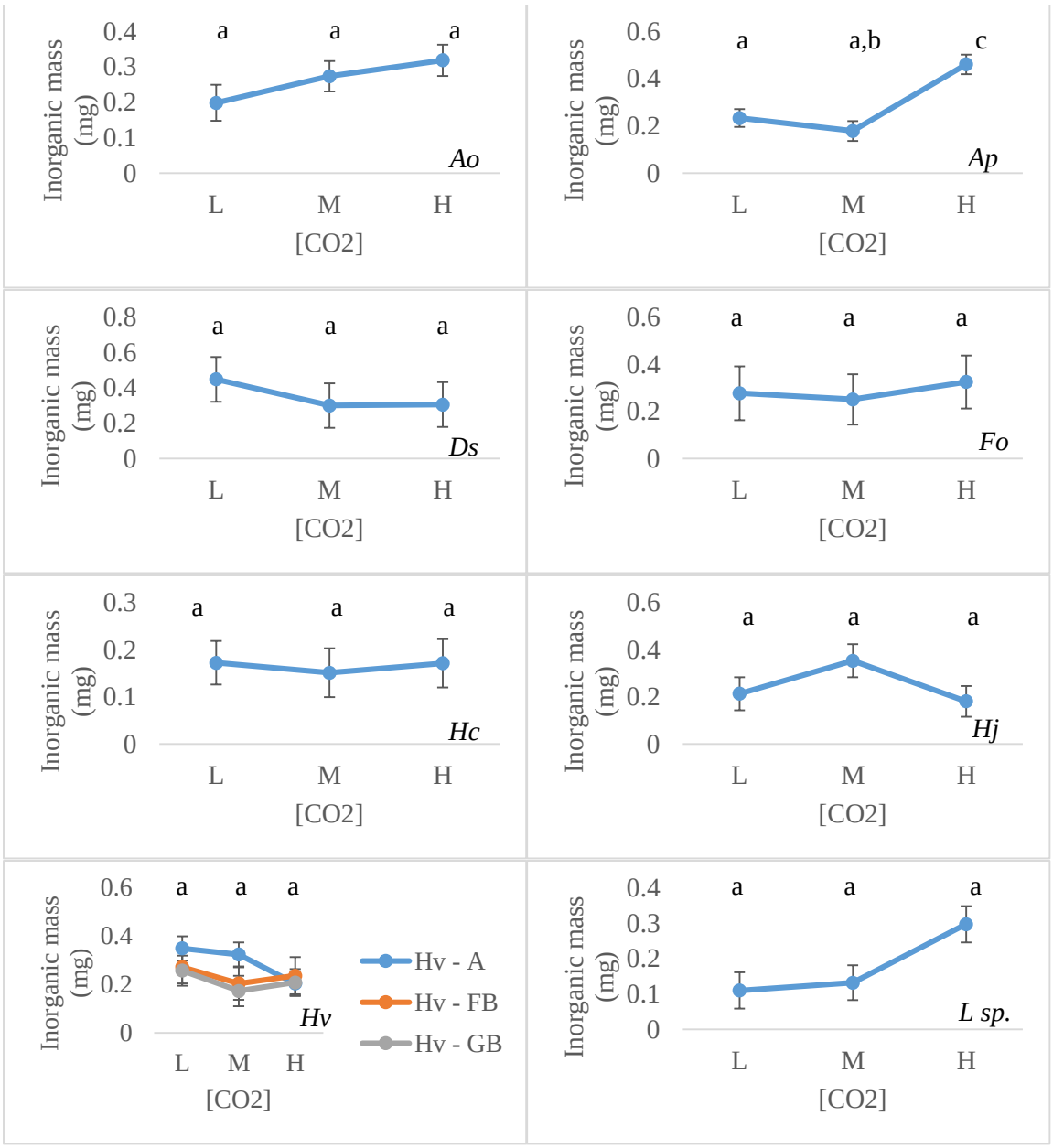


Fig 3. pH decreased towards the center of the egg mass of *A. ocelligera* in comparison with the outer perimeter.


Inorganic mass (mg)
0.4
0.3
0.2
0.1
0
a a a
Ao
L M H
[CO2]
Inorganic mass (mg)
0.6
0.4
0.2
0
a a,b c
Ap
L M H
[CO2]
Inorganic mass (mg)
0.8
0.6
0.4
0.2
0
a a a
Ds
L M H
[CO2]
Inorganic mass (mg)
0.6
0.4
0.2
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a a a
Fo
L M H
[CO2]
Inorganic mass (mg)
0.3
0.2
0.1
0
a a a
Hc
L M H
[CO2]
Inorganic mass (mg)
0.6
0.4
0.2
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Inorganic mass (mg)
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0.4
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a a a
Hv
L M H
[CO2]
Hv - A
Hv - FB
Hv - GB
Inorganic mass (mg)
0.4
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0.2
0.1
0
a a a
L sp.
L M H
[CO2]

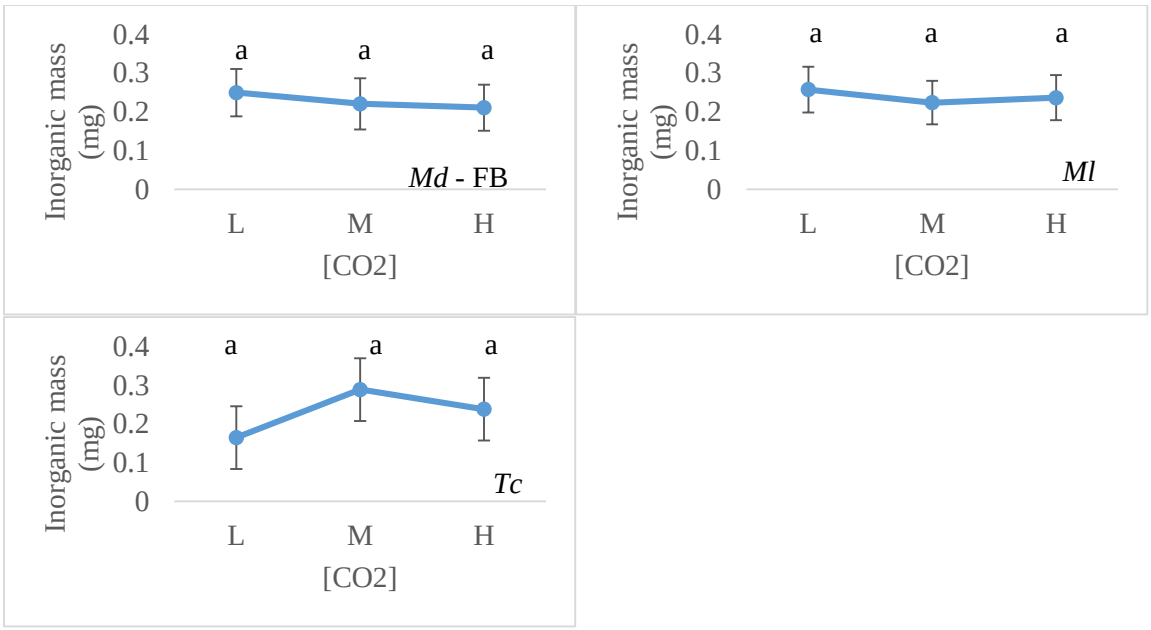
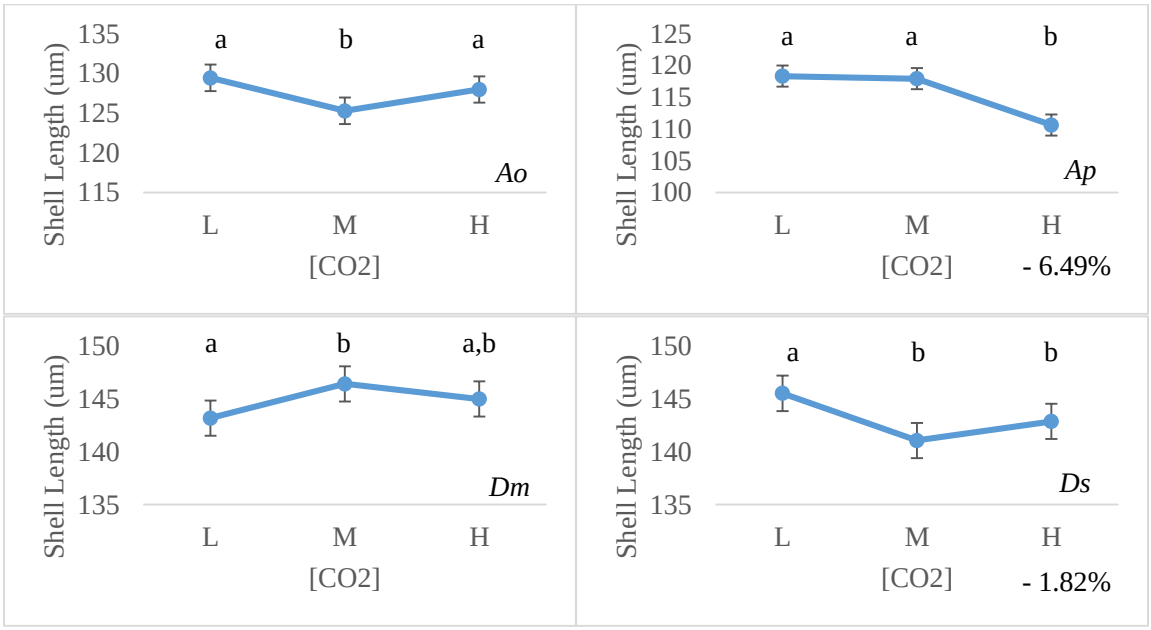


Fig 4. Inorganic mass response to CO_2 change in hatched larvae. Organic mass was used in statistical analysis as a covariate.



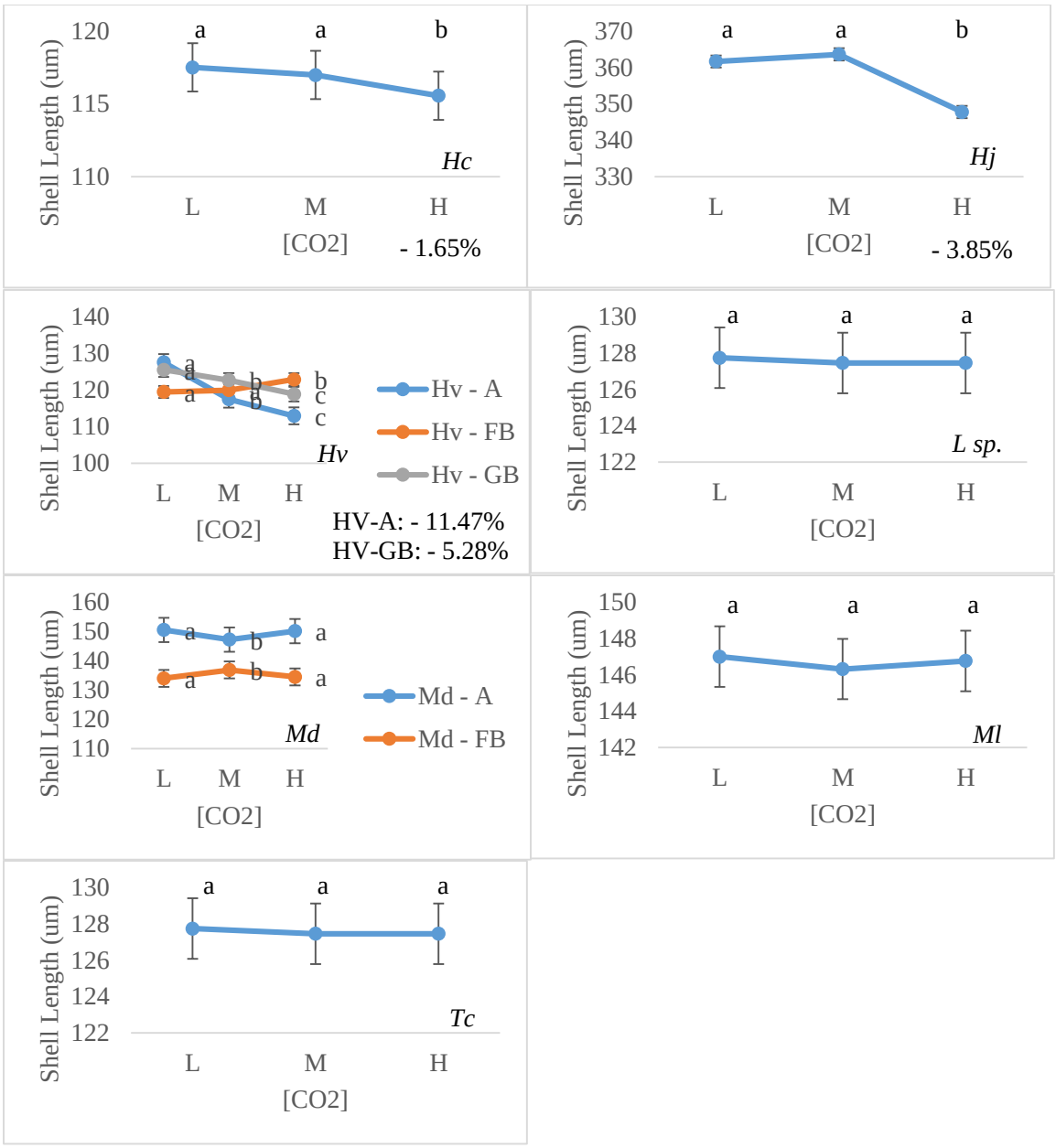
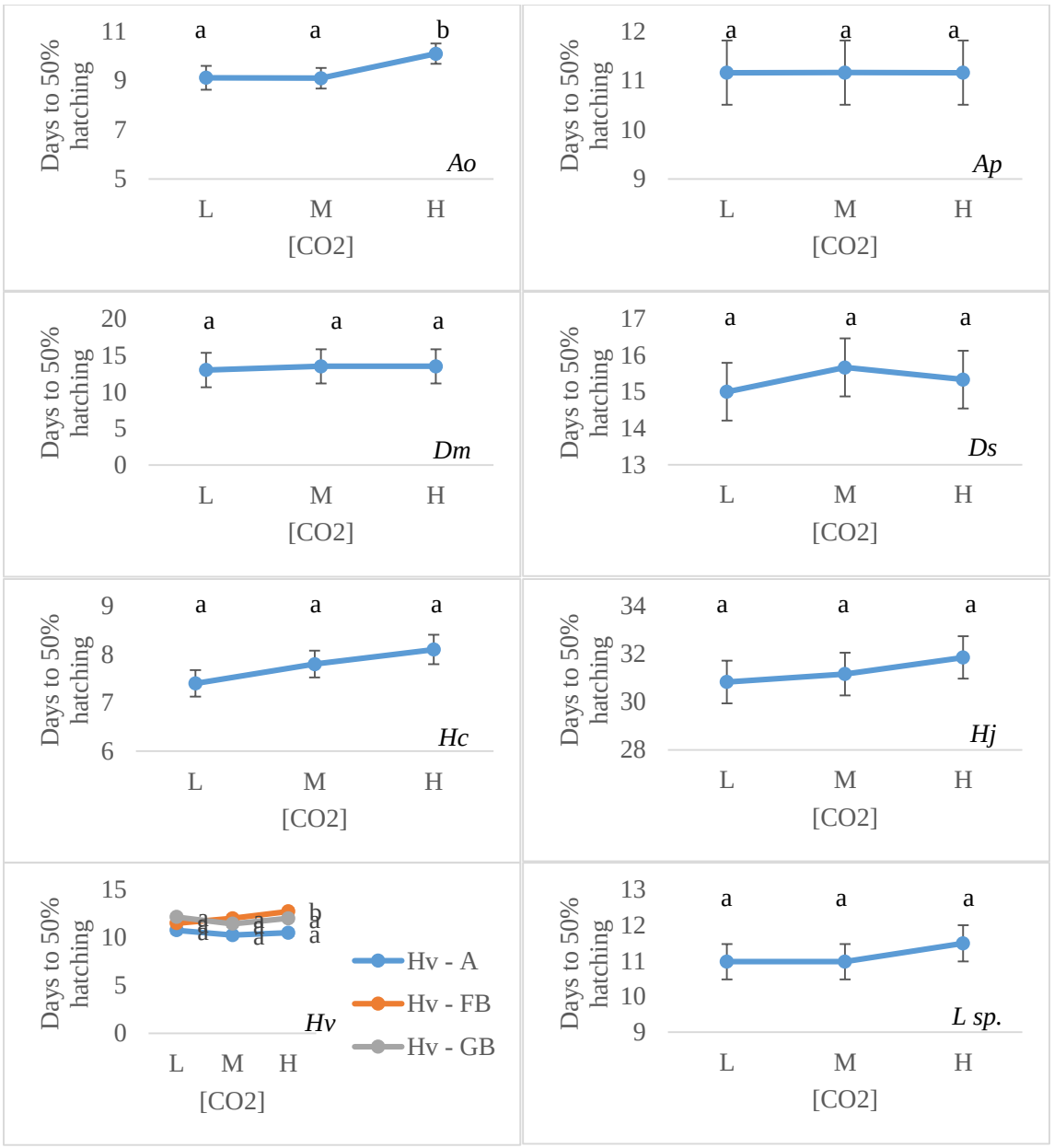


Fig 5. Shell length response to CO_2 change in hatched larvae.


Days to 50% hatching
11
9
7
5
a
a
b
Ao
L
M
H
[CO2]
Days to 50% hatching
12
11
10
9
a
a
a
Ap
L
M
H
[CO2]
Days to 50% hatching
20
15
10
5
0
a
a
a
Dm
L
M
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[CO2]
Days to 50% hatching
17
16
15
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13
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Days to 50% hatching
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Days to 50% hatching
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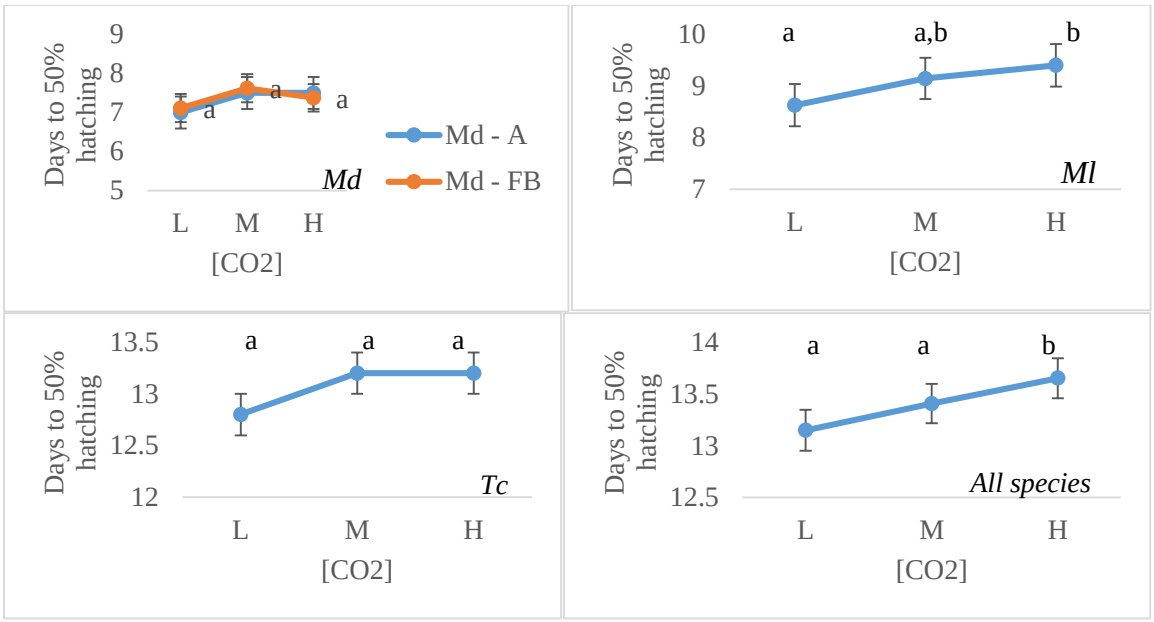
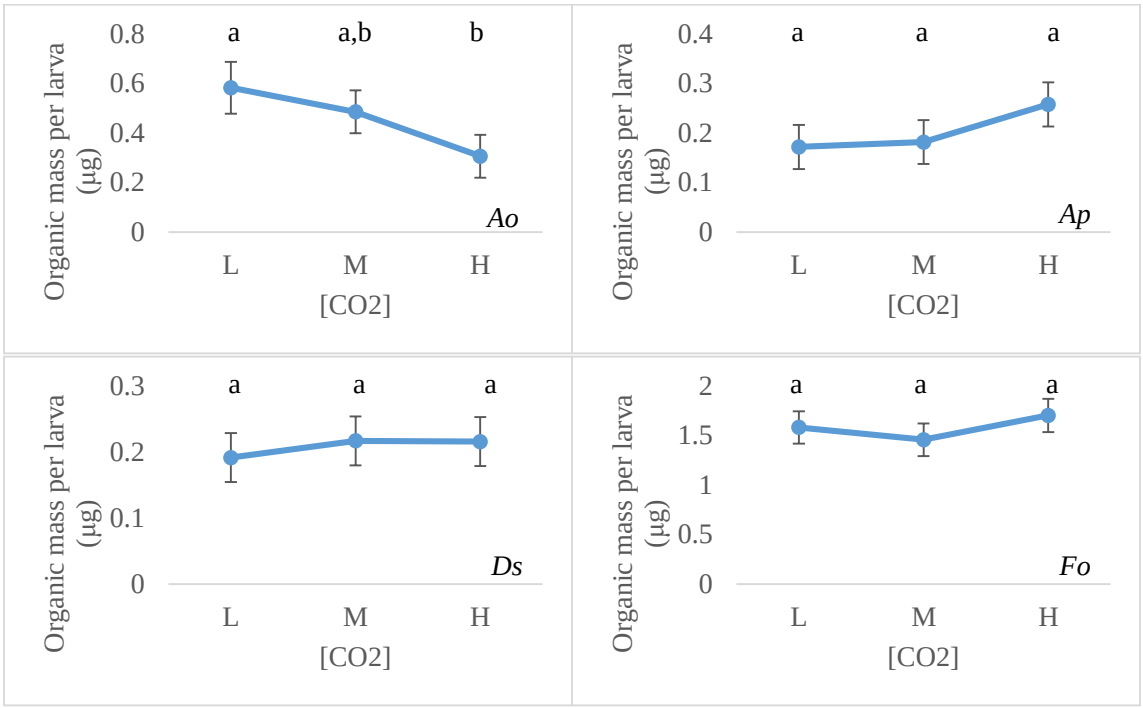


Fig 6. Hatch time response to CO_2 change in larvae.



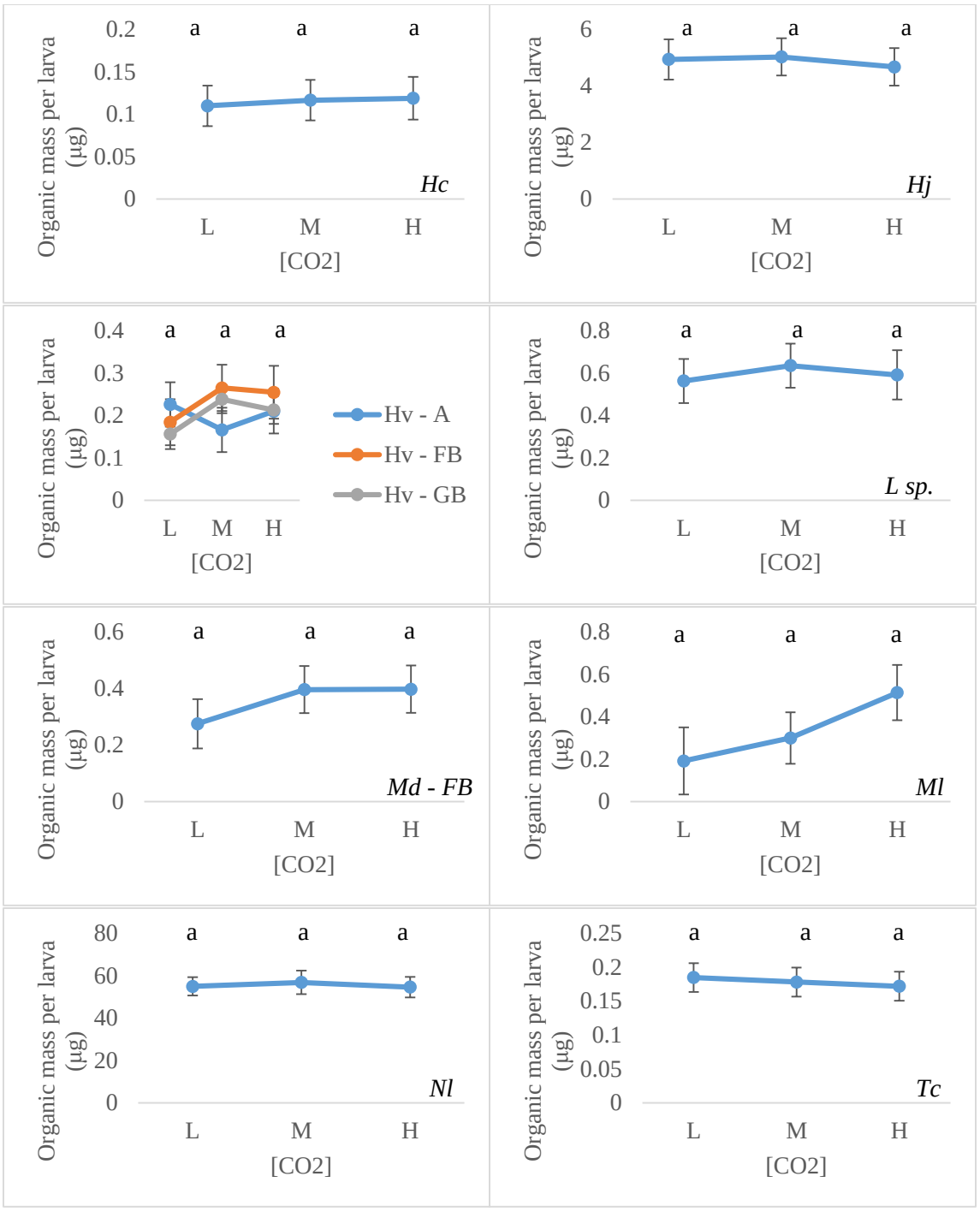
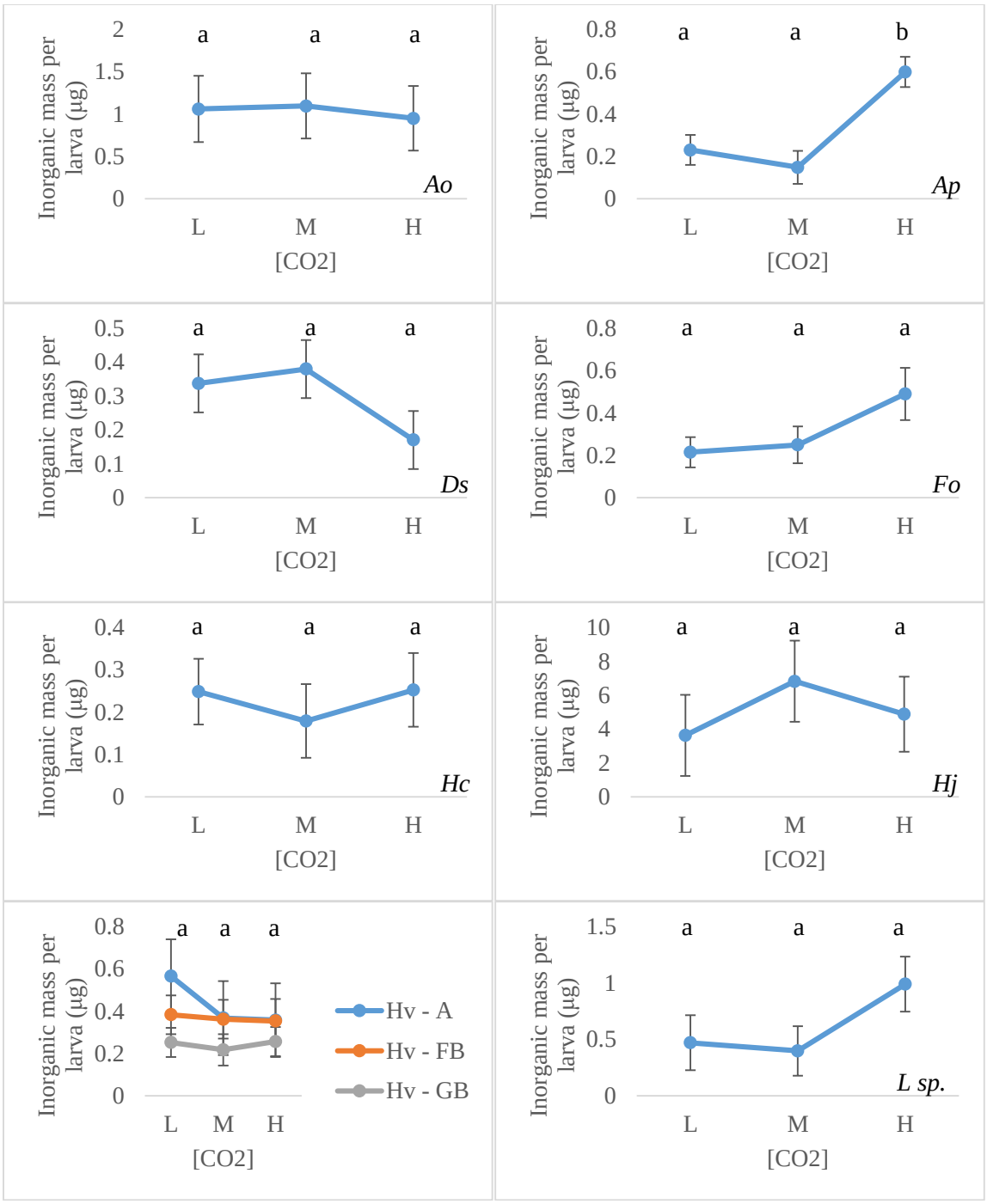


Fig 7. Organic mass response to CO_2 change per hatched larva.


Inorganic mass per larva (µg)
2
1.5
1
0.5
0
a a a
Ao
L M H
[CO2]
Inorganic mass per larva (µg)
0.8
0.6
0.4
0.2
0
a a b
Ap
L M H
[CO2]
Inorganic mass per larva (µg)
0.5
0.4
0.3
0.2
0.1
0
a a a
Ds
L M H
[CO2]
Inorganic mass per larva (µg)
0.8
0.6
0.4
0.2
0
a a a
Fo
L M H
[CO2]
Inorganic mass per larva (µg)
0.4
0.3
0.2
0.1
0
a a a
Hc
L M H
[CO2]
Inorganic mass per larva (µg)
10
8
6
4
2
0
a a a
Hj
L M H
[CO2]
Inorganic mass per larva (µg)
0.8
0.6
0.4
0.2
0
a a a
L M H
[CO2]
Hv - A
Hv - FB
Hv - GB
Inorganic mass per larva (µg)
1.5
1
0.5
0
a a a
L sp.
L M H
[CO2]

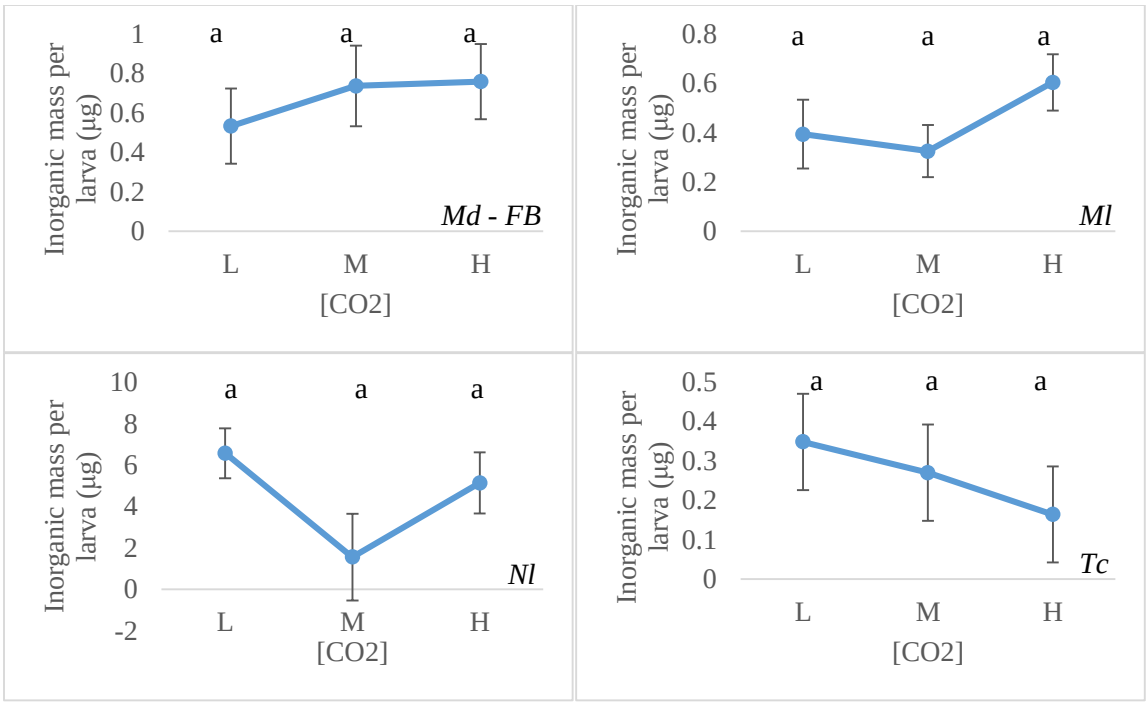


Fig 8. Inorganic mass response to CO_2 change per hatched larva.