Consequences of calcium decline on the embryogenesis and life history of *Daphnia magna*

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**ABSTRACT**

Ambient calcium is declining in thousands of soft-water lake habitats in temperate regions as a consequence of unsustainable forestry practices, decreased atmospheric calcium deposition and acidic deposition. As their exoskeleton is heavily reinforced with calcium, freshwater crustaceans have a high specific calcium requirement relative to other aquatic organisms. *Daphnia*, in particular, is an ideal crustacean for investigating the consequences of calcium decline because it is an abundant and important member of freshwater zooplankton communities. Although it has been established that adult and juvenile *Daphnia* have different tolerances to low ambient calcium as a result of their different life stage-specific calcium requirements, the consequences of declining calcium on embryonic development have never been investigated. Here, we describe the distribution of calcium in embryonic stages of *D. magna* and introduce a novel and easy to use staging scheme. We tested whether calcium can be traced from mothers to their offspring. Finally, we assessed the fitness consequences of maternal provisioning in limiting calcium environments. We found that while embryos require calcium for their development and moulting, they do not equilibrate with environmental calcium levels. Instead, we were able to trace calcium from mothers to their offspring. Furthermore, our data strongly suggest that females are faced with an allocation trade-off between providing calcium to their offspring and using it for growth and moulting. Together, these data provide novel insights into the consequences of calcium decline for freshwater zooplankton.

**KEY WORDS:** Freshwater, Crustacean, Embryonic development, Allocation trade-off, Maternal effects, Acidification, Stress

**INTRODUCTION**

Calcium is an essential mineral required for life and its presence is ubiquitous across the plant and animal kingdoms. Its functions range from structural reinforcement (i.e. fortifying bones, exoskeletons and plant cell walls) to molecular signalling (i.e. coordinating muscular contractions, neuronal cascades and embryonic developmental events) (Webb, 1999; Webb and Miller, 2003). As such, calcium must be exogenously acquired and is generally taken up from the abiotic environment as dissolved calcium, or by ingestion of calcium-containing organisms.

Calcium in Canadian Shield soft-water lakes has been declining in part due to anthropogenic activities that influence calcium cycling (Keller et al., 2001). The parent bedrock of this region (predominantly granite and felsic intrusive rock) is resistant to erosion and is subsequently characterized by shallow, acidic, nutrient-poor soils (Baldwin et al., 2000). This creates an inherent vulnerability of the associated aquatic and terrestrial ecosystems to calcium deficiency (Lawrence and Huntington, 1999; Baldwin et al., 2000). This is further aggravated by processes such as unsustainable forestry practices and acidic deposition, which remove and rapidly liberate calcium stores (respectively) from the ecosystem without sufficient replenishment (Jeziorski et al., 2008b). Hence, once calcium is removed it can take centuries for the calcium concentrations to recover in these environments. In some more extreme cases calcium may be so depleted that the ecosystems may never recover. Ambient calcium concentrations in south-central Canadian soft-water lakes have declined an average of 45% (ranging from 10 to 60%; Jeziorski et al., 2008a) with many of these lakes approaching calcium concentrations as low as 1 mg L−1 (Jeziorski et al., 2008b). This problem is not isolated to one country or ecozone and similar trends have been observed in soft-water lakes in Scandinavia with comparable physical geography (Skjelkvåle et al., 1998; Alstad et al., 1999).

The consequences of calcium decline vary in their severity based on an organism’s specific calcium requirements and their life history strategy. Crustaceans, which undergo cyclic moults that require relatively high amounts of readily available dissolved calcium, are likely to be more dramatically affected by calcium decline than organisms whose calcium requirements are proportionately less. *Daphnia* is a genus of freshwater crustacean that not only undergoes frequent moult cycles which require proportionately high amounts of environmental calcium but also is an integral and abundant component of the freshwater zooplankton community (Jeziorski and Yan, 2006). In fact, *Daphnia* are model organisms for studying gene–environment interactions (Colbourne et al., 2011). As such, they have been used for many decades as biosensors for environmental contaminants (Lampert, 2011). More recently, ecotoxicogenomic approaches have been implemented into these standard practices (Shaw et al., 2008).

*Daphnia* require readily available dissolved calcium to fortify their new carapace post-moult. Tan and Wang (2009a) showed conclusively that the predominant route of calcium acquisition is via active uptake from the environment and that *D. magna* has a limited ability to extend the duration of carapace calcification in response to decreased environmental calcium. Furthermore, there are no known calcium storage mechanisms or organs (Cairns and Yan, 2009). Hence, despite their remarkable capability to persist in changing environments, *Daphnia* appear to have a limited capacity to compensate for low ambient environmental calcium. Because of the important position of *Daphnia* within aquatic food webs (Lampert, 2011), it is critical that we gain a better understanding of how these keystone species physiologically and developmentally cope with this important limitation.

Calcium requirements of crustaceans can vary not only among different species (Rukke, 2002a) but also among different populations (Rukke, 2002b) and life stages within a species (Hessen et al., 2000). The specific calcium requirement of
juveniles is typically higher than that of their adult counterpart (Porcella et al., 1969; Hessen et al., 2000). This means that the calcium required per mass dry weight is higher in juveniles than in adults. This difference is likely related to the different functional requirements of these life stages (i.e. juveniles invest substantial energy into growth and calcification, while adults have the added energy burden of reproduction). Still, the function of calcium in Daphnia embryonic development (Fig. 1), and its temporal and spatial distribution throughout embryogenesis, remains undocumented.

In this study, we set out to investigate the specific calcium requirements of embryos in D. magna Straus 1820 and relate it to the specific calcium requirement of reproductive females. As Daphnia embryos are directly in contact with the external environment while developing in the brood pouch, we hypothesized that Daphnia embryos have the capacity to acquire calcium directly from the environment, similar to what has been shown for female Daphnia (Tan and Wang, 2009a). Alternatively, calcium may be primarily provisioned maternally. In this case, a trade-off may exist between immediate calcium requirements for moulting of the females and provisioning of the offspring, assuming that calcium provisioning during oogenesis is an active process as it is in most other animals (Webb and Miller, 2003). This trade-off would be affected by calcium concentrations in the environment and could result in reduced offspring quality in future generations when calcium concentrations are low. We tested these hypotheses by measuring the consequences of low calcium in the culture medium on embryonic development, reproduction and survival. Note that we developed and employed a novel staging scheme for this study that is suitable for field-based studies and automatic developmental staging using time-lapse recording (Fig. 1). Furthermore, we used radioactively labelled calcium ($^{45}$Ca) to trace calcium from mothers to embryos and tested how reduced calcium affects growth and brood size of Daphnia from one generation to the next.

**RESULTS**

We present results from a series of experiments that were designed to (1) analyse the distribution of calcium in embryonic tissue, (2)
analyse the effects of environmental calcium changes on embryonic development, (3) trace calcium from mothers to their offspring and (4) assess the consequences of reduced calcium in the environment on the development and survival of embryos and mothers, and the reproductive output of mothers.

**Calcium distribution in D. magna embryos**

Our analysis of calcium in embryos confirms the presence of calcium in embryonic tissue. In order to analyse the distribution of calcium in embryonic tissue, embryos were treated with Alizarin Red-S. These embryos consistently showed staining in the thoracic appendages (Fig. 2F,G), ventral cephalic region (Fig. 2G), ventral neuroectoderm (not shown), gut tube (Fig. 2I) and the carapace (Fig. 2F–H). This staining occurred in peripheral tissues surrounding the body (with the exception of the gut tube). While the staining in embryonic stages was relatively diffuse, we consistently found a lack of staining in the inner cell regions (Fig. 2F–J), which seemed to be predominantly undifferentiated (Fig. 2F–J).

**Environmental and maternal calcium effects on embryogenesis and embryo survival**

*Daphnia* are strongly affected by limited calcium in their environment, in development and reproduction. We tested how different environmental calcium levels (high, low and none) affect embryonic development using both manual and automated time-lapse analysis of isolated embryos. We also assessed embryonic survival under these conditions. We found that embryos that were reared in normal calcium environments, and were subsequently isolated from their mothers and transferred to a no calcium COMBO medium, demonstrated normal survival until approximately 48 h of development (Fig. 3A), which corresponds to stage 5 (Fig. 3B). Once the embryos had passed this point, survival in the no calcium treatment dropped off significantly when compared with the high and low calcium control. This suggests that the absence of calcium can result in a significant increase in mortality ($F=13.71$, $P<0.001$, d.f.=8).

Using our embryonic staging scheme we compared the developmental trajectories of embryos that were reared in normal calcium conditions and then transferred to no, low and high calcium conditions (0, 5 and 25 mg l$^{-1}$). We performed this experiment using manual monitoring for the first six stages of embryonic development (Fig. 3B) and automated monitoring for the first four stages of development (Fig. 3C). Note that a time-lapse video of normal development with the automated system can be found in supplementary material Movie 1. We found that after stage 5 the developmental trajectory of embryos in the no calcium treatment started to diverge from that of embryos in low and high calcium, resulting in increased mortality. This trend was significant at stage 6 between the high calcium and no calcium treatments ($F=12.5$, $P<0.01$). Up to stage 4, no difference between the treatments was detected (Fig. 3C). In contrast, embryos produced by mothers in a reduced calcium environment developed significantly faster compared with embryos from mothers that had been exposed to the high calcium concentration during embryo formation (Fig. 3D; $F_{1,28}=231.04$, $P<0.01$).

The multigenerational experiment was designed to specifically test carry-over effects from one generation to the next as a consequence of calcium availability. The experiment was run over two generations and we measured cumulative clutch size (CCS – sum of all clutches produced by an individual *Daphnia* over the experimental period) as well as growth, measured in body size (base of caudal tail to the top of the head above the compound eye) (Fig. 4). Note that in this experiment embryos were grown isolated from their mothers to avoid any transfer of calcium from mothers to offspring (see below for more information), as further described in
the Materials and methods. Using time (weeks) as a covariate, we tested the effects of generation (first or second) and treatment (low and high calcium) on CCS and body size. We found that both generation and treatment had a significant effect on CCS (generation–CCS: $F_{1,486}=4.8$, $P=0.03$; treatment–CCS: $F_{1,486}=15.4$, $P<0.01$; Fig. 4A) and body size (generation–size: $F_{1,486}=15.4$, $P<0.01$; treatment–size: $F_{1,486}=15.6$, $P<0.01$; Fig. 4B). The interaction between generation and treatment was significant for CCS ($F_{1,486}=11.0$, $P<0.01$) but not for body size ($F_{1,486}=0.8$, $P=0.4$). These results are shown in Fig. 4. Under low calcium conditions, *Daphnia* produced more eggs in the first generation than in the second after 6 weeks. This effect was reversed under high calcium conditions. Females developing and growing under low calcium conditions were similar in size; however, under high calcium conditions, females in the second generation were slightly smaller after 6 weeks.

**Tracing calcium from mothers to offspring using $^{45}$Ca**

While *Daphnia* offspring do not equilibrate with environmental calcium over the first 48 h of development, their mothers can provision them with this important mineral during development. Building on previously published data by Tan and Wang (2009a) that provide conclusive evidence for the presence of an active calcium uptake mechanism in *D. magna* juveniles and adults, we exposed isolated embryos to $^{45}$Ca and were unable to find any evidence that $^{45}$Ca accumulates above background in embryos during the first 48 h of development (low 250 kBq $^{45}$Ca exposure: $F_{2,4}=0.05$, $P=0.95$, Fig. 5A; high 1 MBq $^{45}$Ca exposure: $F_{2,4}=0.65$, $P=0.4$).

**Fig. 4. Reduced calcium in the environment results in trans-generational effects for both clutch size and body size.** (A) *Daphnia* produced their largest cumulative clutch size in response to low (5 mg l$^{-1}$) calcium in the environment. Under normal calcium concentrations (high, 25 mg l$^{-1}$), *Daphnia* produced the largest cumulative clutch size in generation 2 (F1). Mothers reared in a low calcium environment initially produced the largest clutches. Their offspring produced smaller clutches but of comparable size to those produced by offspring from mothers reared in a normal calcium environment. (B) After 6 weeks, *Daphnia* reared under normal calcium concentrations were significantly larger than *Daphnia* reared under limiting calcium concentrations. This pattern was seen for both the parents and their offspring; $N=487$.  

![Image](image_url)
calcium from mothers to offspring via an as yet unknown mechanism. We further elucidated the potential consequences of calcium deficiency on life history and found that calcium limitation can adversely affect \textit{Daphnia} embryos indirectly through maternal effects over at least one generation.

**Presence and distribution of calcium in embryos**

In order to determine which embryonic tissues and cells contain calcium, cryosectioned embryos were stained using Alizarin Red-S. Embryos treated with Alizarin Red-S consistently showed staining in: (1) thoracic appendages, (2) ventral cephalic region, (3) ventral neuroectoderm, (4) gut tube and (5) carapace. This staining seemed to be in peripheral tissues of the body (with the exception of the gut tube). Controls did not demonstrate any pigmentation in these regions. Embryos also consistently lacked staining in their inner cell region, which seemed to be predominantly undifferentiated. This suggests that calcium is primarily present in animal pole cells, and less so in the vegetal cells. The presence of calcium in the animal pole has been seen in both zebrafish and \textit{Xenopus} embryogenesis (Webb and Miller, 2003). Pan-embryonic calcium signalling patterns (i.e. waves of signalling over the entire embryo) have been described in many developmental model organisms during the earliest stages of morphogenesis, and calcium signalling is probably a key component of epiboly, involution, convergence and extension events in \textit{D. magna} at this time and throughout development (Webb and Miller, 2003). However, these functional implications are speculative and require further investigation.

**DISCUSSION**

We tested two alternative but not mutually exclusive hypotheses about the source of calcium for \textit{D. magna} embryos. We found that \textit{D. magna} embryos are unable to equilibrate with environmental calcium levels above background during the first 48 h of development but were able to trace radioactively labelled calcium from mothers to offspring via an as yet unknown mechanism. We further elucidated the potential consequences of calcium deficiency on \textit{Daphnia} life history and found that calcium concentrations in the brood pouch, we detected a significant increase in $^{45}\text{Ca}$ in their bodies (Fig. 5B) compared with controls. Embryos from mothers that were exposed to high concentrations of $^{45}\text{Ca}$ showed higher $^{45}\text{Ca}$ content than embryos from mothers that were exposed to low $^{45}\text{Ca}$ (low 250 kBq exposure: $F_{1,4}=13.64$, $P<0.001$, Fig. 5C; high 1 MBq exposure: $F_{1,4}=41.64$, $P<0.001$, Fig. 5C). As we showed that no $^{45}\text{Ca}$ can enter the embryo directly during this stage of development and we know from previously published data that mothers actively take up calcium (Tan and Wang, 2009a), we interpret these findings as maternal provisioning.

**Direct measurements of calcium in embryos and mothers**

The results outlined above were further supported by Flame Atomic Absorption Spectrophotometry (FAAS) measurements of embryos formed in different maternal environments (Fig. 6). While we were unable to demonstrate a statistical difference in calcium content between mothers that were raised in high and low ambient calcium conditions FAAS ($F_{1,4}=2.17$, $P=0.18$; Fig. 6A), we show that mothers that were exposed to high levels of calcium produced embryos with high calcium content and vice versa ($F_{2,4}=15.12$, $P=0.008$; Fig. 6B).

**Influence of environmental calcium on embryogenesis and embryo survival**

The developmental time-lapse experiments conducted for this study provide evidence that embryos formed in different calcium environments can experience temporal changes in developmental schedule (i.e. rate of development). Embryos that came from mothers in low calcium environments experienced an accelerated rate of development through stage 1 and 2 of embryogenesis.
completing each stage approximately 5 h faster than embryos formed in 25 mg l\(^{-1}\) calcium environments. Although this temporal shift occurred, there were no visual, physical abnormalities observed in the developing embryos. Studies in mice have shown that increased levels of calcium can accelerate early blastocyst morphogenesis processes (Stachecki et al., 1994), demonstrating that temporal shifts in development are possible as a consequence of changes in calcium levels. Our data show specifically that decreased environmental calcium concentration might be affecting processes in early development (i.e. pre-organogenesis) and altering overall developmental schedule. Perhaps, embryos of *D. magna* are forced to complete calcium-dependent developmental processes more rapidly because of limited calcium availability. Because a basal amount of calcium is probably required for proper cell adhesion in *Daphnia* embryos, calcium limitation may reduce the amount of free, signalling calcium in the embryos. It would be interesting to investigate the calcium signalling signatures of early embryo morphogenesis via live-fluorescent or aequorin imaging techniques, to determine whether there are changes in embryonic calcium signalling wave frequency as a result of decreased environmental calcium.

The high mortality we observed in the 0 mg l\(^{-1}\) calcium treatment could be related to the onset of direct environmental calcium interaction with newly formed osmoregulatory organs after hatching the vitelline membrane (Charmantier and Charmantier-Daures, 2001). It is likely that the embryonic calcium resources of *D. magna* are exhausted by stage 5, and must be supplemented with environmental calcium. This would account for the dramatic drop in survival that occurs once embryos in the 0 mg l\(^{-1}\) treatment pass stage 5.

Studies in ecotoxicology, including this study, have shown the usefulness of *D. magna* embryos as bio-indicators for water quality and/or endocrine disrupter contamination (Kast-Hutcheson et al., 2001; Wang et al., 2011). If the embryonic membranes of *D. magna* are involved in early embryonic osmoregulation (Charmantier and Charmantier-Daures, 2001), and the transfer of larger minerals such as calcium across these membranes is regulated by complex transport mechanisms as seen in, for example, the chick embryo (Tuan and Ono, 1986), bioassays that employ simple pharmacological exposures may not demonstrate the true effects of the disruptive compounds on embryos if the compounds are unable to cross these barriers. Properly incorporating compounds into the embryos might require acclimating mothers to the compound, and having them transfer it naturally to the embryos as seen with calcium. Alternatively, it might require microinjection of the compound into the embryo to mimic maternal provisioning. In any case, based on the information gathered from this study, embryonic membrane permeability and maternal provisioning pathways should be taken into consideration when designing pharmacological experiments and bioassays aimed at investigating the effects of endocrine disrupters and biological contaminants on embryos of *D. magna*.

We applied a novel and simple staging scheme in our study that (i) allows the post hoc analysis of time-lapse videos as shown in the present study and (2) can be readily used with minimal laboratory equipment and is therefore potentially useful for field experiments. Previously developed staging schemes including a very detailed recently published scheme (Mittmann et al., 2014) have the obvious advantage of providing a finer temporal and spatial analysis of *Daphnia* development but cannot be easily applied outside of the lab as they rely on expensive and specialized equipment. We view our data as confirmation that our staging scheme can provide sufficiently detailed information on development as a consequence of environmental change, while at the same time allowing the processing of large sample sizes with minimal equipment. Still, we provide a detailed comparison with the most recent and detailed staging scheme by Mittmann and colleagues (2014) in order to show that our staging scheme follows the same landmarks as a much more detailed staging scheme.

**Source of calcium required for embryogenesis and maternal calcium provisioning**

Our \(^{45}\text{Ca} \) radiotracer experiments demonstrated that calcium is transferred to the embryo from the mother, and that isolated embryos do not equilibrate with calcium in the environment. These data were largely confirmed by our FAAS measurements of embryos and mothers. The reproductive mechanism that allows mothers to allocate calcium to their offspring is unknown. However, it is plausible that this accumulation of calcium in embryos occurs via the same resting egg calcium reclamation mechanism serendipitously demonstrated in a study by Jezierski et al. (2008a). It would be interesting to pursue this hypothesis in future research by trying to localize and genetically manipulate (i.e. overexpress and knock-down) the genes that encode calcium mobilization and binding proteins. The 1.7-fold difference in calcium content found between embryos formed by mothers that acquired calcium in low and high calcium environments suggests that *D. magna* are capable of regulating the amount of calcium that is allocated to the embryo. Finally, stage 5 of embryogenesis appears to show a particular sensitivity to the lack of calcium in the environment and calcium transport mechanisms in these embryos should be investigated further. For example, future studies could characterize the transport type (active or passive) in hatchlings and this information could provide important insight into the extent to which these stages can compensate for reduced maternal provisioning and/or lack of calcium in the environment.

Understanding mechanisms underlying life history trade-offs is essential for understanding life history evolution (Flatt and Heyland, 2013). Tan and Wang (2009a) conclusively showed that female *D. magna* use active transport to acquire calcium from the environment. Females reared under low calcium conditions had a higher reproductive output in the first generation, indicating that the overall strategy is to sacrifice long-term reproductive success for short-term gain. In a parthenogenic organism such as *Daphnia*, the trade-off between current and future reproduction is particularly interesting as it potentially compromises the success of subsequent maternal clones (i.e. genetically identical organisms). From a genetic perspective, it would seem most advantageous to invest energy in embryogenesis to promote propagation and mass replication of the genome. However, as juveniles are more vulnerable to calcium deficiency than adults (Porcella et al., 1969; Hessen et al., 2000), perhaps this strategy of maternal preservation is optimal at the calcium concentrations explored in this study. It promotes the survival of a reproductive female, rather than embryos and juveniles that may have reduced fitness as a consequence of reduced environmental calcium concentration. Regardless of the proximate mechanisms driving such a strategy, it is not surprising that females can adjust embryonic calcium provisioning given the frequency with which epigenetic modifications and plasticity are observed in *D. magna*. These findings, however, raise the important question of whether, under natural conditions, *Daphnia* can readily adapt to reduced calcium environments.

**Embryonic carryover effects on growth and brood size**

In this study we set out to explore the consequences of calcium decline on *D. magna* when approaching the suggested reproductive
threshold and within an environmentally relevant range for soft-water lakes. Across Canada, calcium content in lakes ranges from 1 to 336 mg l$^{-1}$ and averages 21.8 mg l$^{-1}$ (Jeziorski et al., 2008b). We found that *D. magna* females in higher calcium environments were generally larger than females in the lower calcium treatment. Additionally, the high calcium females had more offspring in the second generation than in the first. Conversely, the low calcium females had more offspring in the first generation than in the second. These findings are particularly interesting in that the primary response of *D. magna* to increased environmental calcium is to increase growth in generation 1, and then increase reproduction in generation 2; the primary response of *D. magna* to decreased calcium is to increase reproduction in generation 1, while body size is maintained over both generations. Future studies should investigate whether these primary and secondary generational responses are triggered by acute environmental calcium changes (plasticity) or whether they represent microevolutionary changes on a population level. A longer generational duration and a wider range of environmental calcium concentrations may be necessary to tease apart the generational consequences of acute and sustained calcium stress on freshwater cladoceran populations.

Future studies may also want to investigate the effects of specimen acclimatization on CCS and body size over multiple generations. A study investigating the low calcium tolerance of two different populations of *D. galeata* (one from a lake of approximately 10 mg l$^{-1}$ Ca$^{2+}$, and one from a lake of approximately 2–3 mg l$^{-1}$ Ca$^{2+}$) found that *Daphnia* acclimatized to higher calcium concentrations had a higher tolerance to calcium stress when compared with populations acclimatized to low calcium environments. Where many protocols require that *D. magna* are kept in media with calcium concentrations above 25 mg l$^{-1}$ (e.g. reconstituted water), the soft-COMBO medium used in our lab results in a final calcium concentration of 8–10 mg l$^{-1}$. However, even calcium concentrations of 10 mg l$^{-1}$ can buffer the effects of low environmental calcium concentration on *Daphnia* (Rukke, 2002b). This could be further examined by running a series of tests monitoring growth and reproduction in *D. magna* strains from different environmental sources, maintained under different culturing regimes.

**MATERIALS AND METHODS**

**Culturing and handling of *Daphnia magna* embryos and adults**

**General culture methods**

A clone of *D. magna* was acquired from the Environmental Science Department at the University of Guelph. The animals were kept at the Hagen Aqualab. They were maintained at 21±2°C, with a photoperiod of 16:8 h (light:dark) under cool full spectrum lighting (940 lm, 15 W). Animals were fed a ration of ~4.7×10$^5$ cells ml$^{-1}$ *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* (total feed concentration=9.375×10$^5$ cells ml$^{-1}$) on Monday, Wednesday and Friday. Animals were kept in soft-COMBO medium (Kilham et al., 1998). *Daphnia* females were kept in 1600 ml volumes of COMBO medium at population densities of 40–45 females/1600 ml culture. Water was replaced three times per week via reverse filtration, and cultures were discarded after a 6 week lifespan or when reproduction had declined. In order to propagate new cultures, 40 juveniles from pre-existing cultures were collected and reared as formerly described.

For calcium-adjusted culture maintenance, COMBO medium preparation was adjusted to achieve final calcium concentrations of 5 mg l$^{-1}$ (low), 10 mg l$^{-1}$ (medium) and 25 mg l$^{-1}$ (high). This was accomplished by adding either more or less CaCl$_2$·2H$_2$O stock medium. It has been demonstrated in previous studies that *D. magna* do not acquire significant calcium from their food (Tan and Wang, 2009b). Therefore, algae culture COMBO medium was not adjusted. Before feeding algae to calcium-adjusted *Daphnia* cultures, algae were condensed via centrifugation and rinsed in calcium-free COMBO medium 3× before being re-suspended in the appropriate calcium-adjusted medium. After rinsing and re-suspension, the algae were fed to calcium-adjusted cultures in the same concentration as for the standard culturing procedure.

**Algae cultures**

Algae cultures of *C. vulgaris* and *P. subcapitata* were grown in heat-sterilized COMBO medium. All culturing flasks were sealed from the environment using Parafilm, and were oxygenated using standard air bubblers in combination with sterilized Nalgene air filters (0.22 µm) and tubing. Ethanol and flame-sterilized pipettes were used for culture sampling and any open culture flask was surrounded with three to four portable Bunsen burners to create an upward air current and prevent culture contamination. Cell counts of the algae cultures were conducted weekly (using a haemocytometer) to monitor culture growth. Cultures were grown to densities of 30×10$^6$ cells ml$^{-1}$ at which point they were stored at 4°C until they were fed to *D. magna* cultures. Algae stored longer than 4 months were discarded.

**Embryo isolation technique**

In order to examine and assess embryos in isolation, we developed a technique to safely remove the embryos from their mother’s brood chamber. Embryo isolation was performed in a shallow Petri dish (~10 ml capacity). Gravid females were immobilized using the negative pressure created by a 0.5 ml plastic disposable transfer pipette (females were held dorsally to minimize or eliminate any physical damage). A second transfer pipette was used to create a gentle stream of water. When the immobilized female contracted her post-abdomen, the abdominal process was lifted and a stream of water was directed into her exposed brood chamber. The water stream was enough to liberate the embryos from the brood chamber. Embryos were then immediately isolated from their mother as her swimming behaviours can cause mechanical damage to the exposed embryos. Females were returned to their cultures.

**Daphnia staging**

For all studies on embryonic development we applied a new staging scheme, developed by our laboratory and currently in preparation for publication (J.-L.G., unpublished). This staging scheme is complementary to existing schemes as it can be employed on live, developing embryos and has distinct advantages over other staging schemes that, although more detailed, require the use of fluorescent markers and higher magnification microscopes (Mittmann et al., 2014). Furthermore, other existing staging schemes do not allow the post hoc processing of time-lapse recordings of development. Our scheme is applicable for automated developmental analysis using an inverted microscope with a motorized stage (described further below), and for manual visual interpretation of real-time developmental progress. We divided embryogenesis into seven visually discernible stages (0–6). All temporal information is with respect to an average rearing temperature of 21°C. Fig. 1 shows high resolution microphotographs of the seven stages with detailed descriptions of the discernible features. Note that we provide here a detailed comparison to the scheme by Mittmann and colleagues (2014), which is one of the most detailed staging schemes in the literature but is not usable for field and time-lapse data, unlike the one used here. In the following description we refer to their scheme as MS.

Stage 0 embryos (~0–15 h; MS stages 1 and 2) possess essentially no visible distinguishing characteristics. Embryos are spherical to ellipsoidal in shape and have large central fat droplets and many surrounding yolk granules.

Stage 1 (15–25 h; MS stages 3–7) is characterized by gastrulation and morphogenesis. The earliest landmarks of this stage are the cephalic invaginations, which appear as small indentations along the sides of the embryo. When these invaginations occur, the embryo seems to organize itself into two regions: the lighter, motile, developing peripheral cells (ventral) and the inner, darker undifferentiated cells (dorsal). The posterior invagination and the lateral carapace ‘buds’ are two other distinguishing features that arise during this stage.

Entering stage 2 the embryo hatches from the chorion (25–35 h; MS stages 7.3/7.4/7.5 and 8). This event is followed by organogenesis. Antennal...
and maxillary appendage rudiments are distinct and forming. Thoracic appendage partitioning is evident. The posterior invagination gives way to a distinct furrow that runs centrally and bilaterally, demarcating the left and right hemispheres sagittally.

During stage 3 (35–40 h; MS stage 9) the shape of the embryo begins to change from the round precursor to something more fusiform, and cephalic structures become more recognizable (i.e. the early compound eye and early neural/brain organization). The darker patch of yolk granules and fat droplets that was once rather uniform and circular follows the changing shape of the embryo with an out-pocketing developing anteriorly in the cephalic region. The early post-abdomen begins to protrude in the posterior region of the embryo. This protrusion is an important defining characteristic of this stage. The carapace becomes visually more robust and prominent. Bifurcation of the secondary antennae becomes clear, and the inner developmental envelope (the vitelline membrane) is still present and visible. Near the end of stage 3, the carapace buds begin a rapid extension posteriorly. The stage ends with the completion of this rapid carapace extension and the rounding of the posterior.

During stage 4 (40–50 h; MS stage 10, ‘hook-shaped abdomen’) the thoracic appendages and the secondary antennae continue to become more sophisticated and clearly demonstrate their segmentation. Setae are now visible on the antennae and appendages. The embryo begins to vigorously move and twitch; however, this movement is restricted to the confines of the vitelline membrane. Organogenesis is well underway, and the cephalic region (rostrum/labrum) becomes much more prominent. The organism appears ovoid, and is now recognizable as the precursor to the familiar juvenile appearance.

Entry into stage 5 (45–55 h) is signified by the hatching of the embryo from the vitelline membrane. The secondary antennae are now extended and motile, as are the thoracic appendages. Neuronal tissue seems to be specializing and there is increased neural tissue investment in the ommatidia of the compound eye. Dorsally, the ostium is maturing and beating. The carapace nearly fully encloses the body of the embryo in the bivalve fashion characteristic of these crustaceans. The caudal spine is still curved and follows the contours of the posterior of the embryo. The gut does not appear to be fully developed, or is veiled by an opaque wall of maternally provisioned yolk granules.

At stage 6 (55+ h) the embryo is considered a juvenile. Completion of the transition into the juvenile stage is marked by several features: little or none of the maternally provisioned yolk remains. The gut is completely formed. The compound eye is a unified collection of ommatidia rather than a partitioned developing structure. The caudal spine has extended and the post-abdomen features a sophisticated, armoured, chitinous post-abdominal claw. The antennal setae are fully extended.

**Analysis of calcium distribution in Daphnia embryos using Alizarin Red-S**

We employed cryosectioning and calcium positive staining in order to determine where calcium localizes throughout *D. magna* embryogenesis. This was done in order to enhance the interpretive power of complementary experiments, and because no record of calcium distribution in embryogenesis, embryos formed by mothers in a common calcium environment were isolated from their mothers (see above) and exposed to 0, 5 or 25 mg l⁻¹ excess calcium. Their progress through embryogenesis in *vitro* was monitored hourly for 72 h using manual monitoring and automated time-lapse analysis. For manual monitoring experiments, embryos were isolated (see above) and rinsed 3× with approximately 15 ml of calcium-free COMBO medium. They were then distributed individually into the wells of 24-well plates. Each well contained 2 ml of COMBO medium (eight wells each of 0, 5 or 25 mg l⁻¹ calcium-adjusted COMBO medium) and *Daphnia* embryos were carefully staged before they were added to the well. Subsequent visual analysis was conducted at 24 h intervals until the embryos had reached stage 6. For the automated time-lapse analysis, five embryos were distributed into separate wells containing either 0, 5 and 25 mg l⁻¹ calcium, totalling 15 individuals. The embryo-containing well plate was then mounted and fixed to the microscope stage and sealed to prevent evaporation during the 72 h experimental duration. The embryo locations were mapped using NIS-Elements Advanced Research Software™. Images were taken every hour for 72 h, or until the embryos had reached stage 5 (at which point the embryos became motile and moved from their mapped position, making it impossible to capture images beyond this time; see also Fig. 1). A time-lapse video of *Daphnia* embryonic development can be seen in supplementary material Movie 1. In the high calcium treatment, embryos achieved >90% survival indicating that the isolation procedure had minimal impact on the survival of embryos in this experiment. Also, we were able to return individuals from these experiments to a larger culture at stage 6 to carry out juvenile and adult lives.

**Maternal exposure experiments**

In order to determine whether embryonic developmental schedule was affected by changes experienced by the mothers as a consequence of different ambient environmental calcium concentrations, embryos were collected from mothers that were kept in either a 5 or 25 mg l⁻¹ calcium environment, and were individually redistributed into a calcium environment that matched the environment in which they were formed. Specifically, eight embryos were distributed into separate wells containing either 0, 5 or 25 mg l⁻¹ calcium, totalling 16 individuals. Embryo developmental trajectories were analysed using the automated time-lapse system described above.

**Multi-generational exposure experiment**

In order to assess the consequences of embryonic exposure to low and high calcium environments for the subsequent generation, embryos were taken from females cultured in 10 mg l⁻¹ calcium COMBO medium and were raised in 24-well plates of either 0 or 25 mg l⁻¹ calcium COMBO medium. Once the embryos had become juveniles they were transferred to a larger pooled culture of the same calcium concentration where they maintained as previously described. Juveniles were collected from the
calcium-adjusted cultures (generation 1) and were transferred to their respective calcium environments. They were maintained as previously described. Every week growth and reproduction (clutch size) were measured. We used CCS (sum of all clutches produced by an individual Daphnia over the experimental period) and body size (base of caudal tail to the top of the head above the compound eye) as fitness-related outputs of the experiment. To measure size, females were removed from culture and placed in a clean shallow Petri dish. Excess water in the dish was removed temporarily to immobilize the female. An image was taken and an on the spot evaluation of brood size and embryo stage was conducted. The process took no more than 60–90 s per female. Once females were measured they were returned to culture. Size was measured using ImageJ analysis software.

**Radiation experiments**

Calcium radiotracer experiments were conducted to explore the potential pathways of acquisition by embryos (i.e. direct exposure/maternal exposure).

**General processing**

Several radiation experiments were conducted to trace calcium from the environment into embryos and mothers as well as from mothers to embryos. All radiation experiments used 10 ml scintillation vials for processing and 45Ca as a calcium isotope. All collected samples were digested and combined with 5 ml of prepared scintillation fluid (0.004% 2,5-diphenyloxazole; 0.0002% 1,4-bis (5-phenyl-2-oxazolyl) benzene; dissolved thoroughly in 2:1 toluene: Triton X-100), prior to rigorous mechanical agitation. Samples were given at least 8 h to fully activate the scintillation fluid before analysis in a Beckman LS6500 scintillation counter. COMBO medium with 45Ca is generally referred to as incubation fluid.

**Timing of maternal exposure to calcium**

Tan and Wang (2009a) conclusively showed that D. magna is actively taking up calcium and these authors established detailed uptake kinetics for the process. We used the basic published parameters of the uptake process to ‘lead’ Daphnia with radioactive calcium for subsequent tracing experiments.

**Embryonic exposure**

Embryos were exposed to 45Ca to determine whether embryos acquire calcium directly from the environment during embryogenesis and whether this process is influenced by environmental calcium concentration. Isolated embryos (stage 0 and 1) from the same cold calcium environment were rinsed 3× (~15 ml volume total) in calcium-free COMBO before being distributed into either a 250 kBq or 1 MBq 45Ca incubation treatment. Embryos were left overnight (16 h) in the calcium treatment. Each replicate of 20 embryos was then rinsed 3× with COMBO (~15 ml total volume) and digested in 0.5 ml of 30% HNO3 at 60°C for 6 h before being processed.

**Test of maternal calcium provisioning using 45Ca tracer**

Pre-gravid females were exposed to 45Ca until they produced a clutch of embryos. Radioactivity was then measured in embryos to determine whether embryos receive calcium from their mothers and whether this transfer of calcium from mother to embryo is influenced by environmental calcium concentration. Freshly moulted females were collected and placed into either a 1 MBq or 1 MBq 45Ca environment. Females were fed algae rations (as per D. magna culture maintenance and algae culture maintenance) for the duration of the experiment. When the females became gravid, their broods were collected (as per embryo isolation), rinsed in COMBO medium thoroughly and digested in 0.5 ml of 30% HNO3 at 60°C for 6 h. Mothers were also rinsed and digested.

**Measurement of calcium in Daphnia using FAAS**

FAAS was performed to determine and confirm the actual calcium concentration in culture medium of embryos and females. Embryos were raised in a 5, 10 or 25 mg l−1 calcium medium. Once they were sexually mature, mothers and their embryos (stage 0–2) were prepared for FAAS analysis. Mothers were processed individually. Embryos were pooled into samples of approximately 50 individuals each. The samples were digested in a small volume of 80% HNO3 (50 µl) for 2–3 h, or until totally dissolved. The samples were diluted to 1% HNO3 (4 ml) and were filtered using sterile syringes and 0.22 µm syringe filters. Approximately 0.04 g of CaCl2 was added to each of the filtered samples (~3.5 ml, 0.5 ml lost in filtration) to reduce the amount of noise that non-calcium divalent cations may cause in the read.

**Statistical analysis**

The statistical program IBM SPSS Statistics 21 and 22, was used to analyse all datasets. ANOVA analyses were used for all treatment group comparisons unless otherwise indicated. Interactions and differences were considered significant when P<0.05. For post hoc comparisons, Bonferroni post hoc analysis was used in SPSS. Errors in graphs are presented as ±1 s.e.m.

**Acknowledgements**

We would like to thank Melania Cirstescu for valuable input into our study.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

A.H. designed and contributed to the conception, interpretation and writing of the study. J.L.G. performed experiments and contributed to the conception, interpretation and writing. N.D.Y. inspired the study, and contributed to the conception, interpretation and revision of the results.

**Funding**

This work was supported by NSERC (Natural Sciences and Engineering Research Council of Canada) Discovery Grant to A.H. (http://www.nserc-crsng.gc.ca/index_eng.asp) and an NSERC CREATE (Collaborative Research and Training Experience) program grant [397997-2011] to J.L.G.

**Supplementary material**

Supplementary material available online at http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.123513/-/DC1

**References**


Movie 1. Time lapse recording of *Daphnia magna* development until hatching (approx. 48 h).