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# Crystalline Calcium Phosphate and Magnetic Mineral Content of *Daphnia* Resting Eggs

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**ABSTRACT**—*Daphnia* is a key crustacean zooplankton of freshwater food chains. One factor that ensures successful propagation is the *Daphnia* resting eggs, which are able to retain structural integrity under extreme conditions. Until recently little was known about the chemical composition, microanatomy, and physical properties of the egg itself. The current study demonstrates that the resting eggs: (1) have shells that are made up of crystalline calcium phosphate and include a honeycombed structure, and (2) contain magnetic material having properties consistent with magnetite. These properties of the resting eggs may ensure *Daphnia* survival in harsh environments.

**Key words:** *Daphnia*, biomineralisation, magnetite, resting egg

## INTRODUCTION

Under normal conditions, *Daphnia* reproduce by parthenogenesis. In unfavourable environments, however, *Daphnia* switch to sexual reproduction and produce robust resting eggs (Kleiven *et al*., 1992; Alekseev and Lampert, 2001). Each resting egg usually encases two resting embryos. When the environment becomes favourable again, the eggs hatch and *Daphnia* reproduce by cyclic parthenogenesis again. Unique adaptive and survival abilities of *Daphnia* resting eggs have been previously reported. For example, resting eggs can remain viable for decades or centuries, and can withstand freezing and drying (Cáceres, 1998). Resting eggs can also survive in the harsh environment of a predator's digestive system (Mellors, 1975), eventually being excreted intact. If the environment condition in which the resting eggs are excreted is favourable, the eggs will hatch, eventually forming a new colony.

It is not clear what confers the resting eggs its resistance to protect the internal embryos. The composition of the resting eggs is reported to be mainly a substance related to chitin (Seidman and Larsen, 1978). Although the resting eggs have been described as "robust", there have been few reports concerning detailed structural, physical and chemical properties of resting eggs. Interestingly, Mellors reported a selective predation of *Daphnia* carrying resting eggs over

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those not carrying resting eggs (Mellors, 1975). It is not clear what attracts predators to maternal *Daphnia.* Although it has been suggested that selective predation of maternal *Daphnia* is due in part to the pigmentation of the eggs (Mellors, 1975), it is possible that non-visual cues may confer increased predatory susceptibility to maternal *Daphnia*.

The current study characterised *Daphnia* resting eggs by using a projection X-ray microscope, analytical X-ray microscope, X-ray diffraction (XRD), and superconducting quantum interference device (SQUID). The projection X-ray microscope is useful, particularly in the field of entomology, for non-destructive visualisation of internal structures (Yoshimura *et al.*, 2000). The analytical X-ray microscope is useful for obtaining fluorescence information related to chemical composition of specimens at ambient conditions via non-destructive means. SQUID is a conventional device that is used to study the magnetic property of materials. Understanding the characteristics of *Daphnia* resting eggs obtained by these methods will give some insight into the ability of *Daphnia* to adapt and propagate in severe environments.

## MATERIALS AND METHODS

### *Daphnia magna*

*Daphnia magna* were raised based on OECD criteria (1998) with *Chlorella vulgaris,* maintained at 20±2°C and exposed to 16-hr period of light followed by an 8-hr period of darkness per day. To obtain resting eggs, 60 three- to four-week-old *Daphnia* were cultured in one liter of medium. When the population density in this medium approached the overcrowding level of 480 per liter, the *Daphnia* began to produce resting eggs, which were collected, rinsed with de-ionised water, and air-dried for analysis. Collection procedures were conducted with non-magnetic instruments.

### Microscopy

A projection X-ray microscope was constructed by modifying a scanning electron microscope (SEM, S-2500CX, Hitachi, Tokyo, Japan), described in detail by Yoshimura *et al.* (2000). This microscope was used to take X-ray micrographs of air-dried *Daphnia* resting eggs. A 5-mm square silicone plate of 0.2-mm thickness with a 1-mm square opening in the centre, on which a SiN membrane (Silson Ltd., Northampton, UK) of 100-nm thickness was pasted, was used as a specimen support plate. A whole air-dried resting egg was placed on this silicon support plate. An aluminium film of 3-μm thickness was used as the target of the electron beam. The film was excited by the focused electron beam of the SEM, with an acceleration voltage of 12 keV. The image was recorded on Kodak 4489 electron microscope film for 120 seconds of exposure time.

To prepare for cross-sections of resting eggs, fixation of the eggs was for 1 hr at room temperature in 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4). The eggs were then rinsed in cacodylate buffer, postfixed 1 hr in 1% $OsO_4$ buffered with 0.1 M cacodylate, dehydrated in a graded ethanol series, and embedded in Epon 812 (TAAB Laboratories Equipment Ltd, Aldermaston, UK). Thin sections of the eggs were cut with a glass knife, mounted onto a glass slide, and coated with carbon. A dissecting scanning electron micrograph of a resting egg cross-section was obtained by an electron probe microanalyser (EPMA, JXA-733, JEOL, Tokyo, Japan), operating at 15 kV and 0.4 nA.

### Fluorescence maps

Fluorescence maps of an air-dried resting egg under ambient conditions were made by an analytical X-ray microscope (XGT-2700, Horiba, Kyoto, Japan), operating at 30 kV and 1.0 mA. The X-ray beam was 100 μm in diameter. Analyses were performed on a 1-mm square area, that covered almost the entire egg. To obtain one map, analyses of 1,000 seconds each were repeated 30 times.

### XRD

XRD patterns of an air-dried resting egg were obtained using a D/max-RAPID diffractometer (Rigaku, Tokyo, Japan). The device included a monochromator and a collimator with a diameter of 100 μm. Cu Kα radiation was used as the X-ray source. Analysis was performed on part of a resting eggshell where its thickness was *ca*. 100 μm. The specimen was irradiated for ten minutes under ϕ and ω oscillation conditions, and diffraction was detected on an imaging plate.

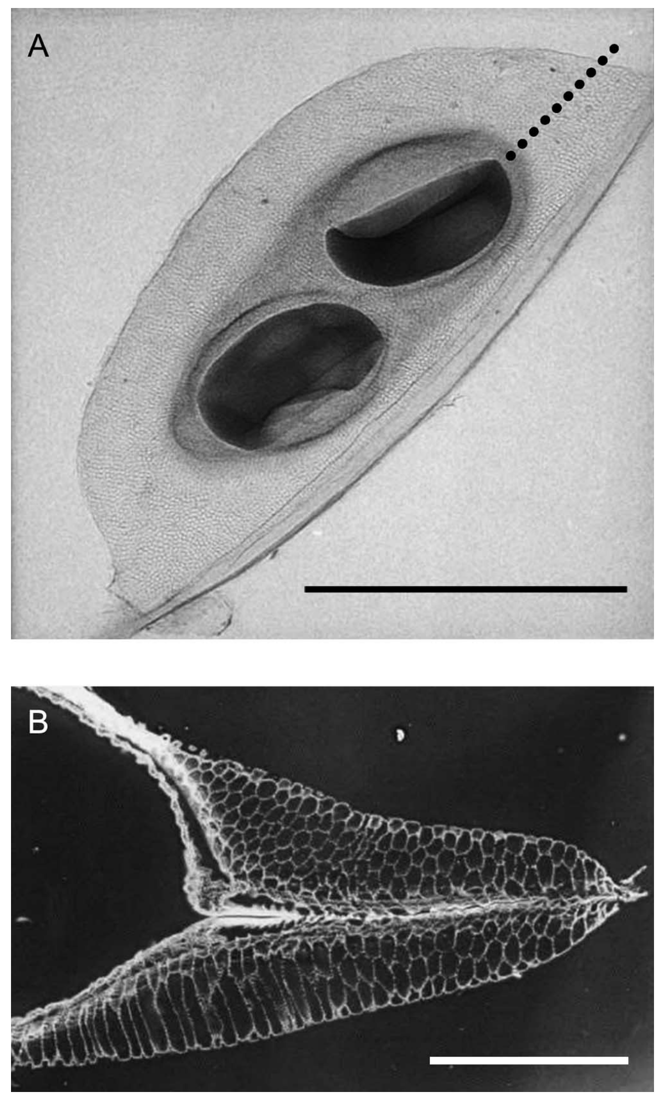


**Fig. 1.** Structure of a *Daphnia* resting egg observed by two different methods. **(A)** X-ray micrograph of an air-dried egg taken by a projection X-ray microscope. Scale Bar, 0.5 mm. **(B)** Dissecting scanning electron micrograph of a resting egg cross-section, cut along the dotted line shown in Fig. 1 A. Scale Bar, 100 μm.

**Magnetisation**

A superconducting quantum interference device (SQUID: MPMS-7, Quantum Design, CA, USA) was used for magnetic measurement of resting eggs with applied fields that ranged from -1 T to 1 T at 300 K. Forty air-dried eggs with a total mass of 1.054 mg were analysed. To minimise noise from containers, a polypropylene sample tube (diameter 4 mm, length 18 cm) was used to hold the specimen. The resting eggs were set on an outer surface of a central position of a sample tube by non-magnetic tape. To minimise noise from the non-magnetic tape, the length of tape used was also 18 cm, to match the length of the sample tube.

## RESULTS

Fig. 1A shows a micrograph of a *Daphnia* resting egg, taken by a projection X-ray microscope. The micrograph shows clear features of the two embryos, which are encased in the resting egg and are located in the central region of the resting egg. The microstructure of the eggshell is also clearly revealed. In addition, the micrograph indicates that the eggshell covers a sizeable portion of the total volume of the resting egg. Fig. 1B shows a scanning electron micrograph of a cross section of the resting egg. As seen in this micrograph, the cross-section of the shell has a honeycomb structure.

To determine chemical composition, fluorescence maps of a 1-mm square area covering most of the egg were created with an analytical X-ray microscope. These identified phosphorus (Fig. 2A) and calcium (Fig. 2B) as well as sulphur and potassium. Furthermore, maps depicted nearly identical distributions of phosphorus and calcium, evidence that the inorganic material in *Daphnia* resting eggshells was likely calcium phosphate rather than calcium carbonate typical of most shells (Silyn-Roberts and Sharp, 1985; Weiner and Addadi, 1997).

An XRD showed a pattern of broad peaks, which indicated a relatively low degree of crystallisation, a small grain size, and the existence of several substances (Fig. 2C). The first major peak occurred in the range of 2θ=10–15 (Peak a). According to the Powder Diffraction Files, Peak a is characteristic of three types of calcium phosphate: calcium

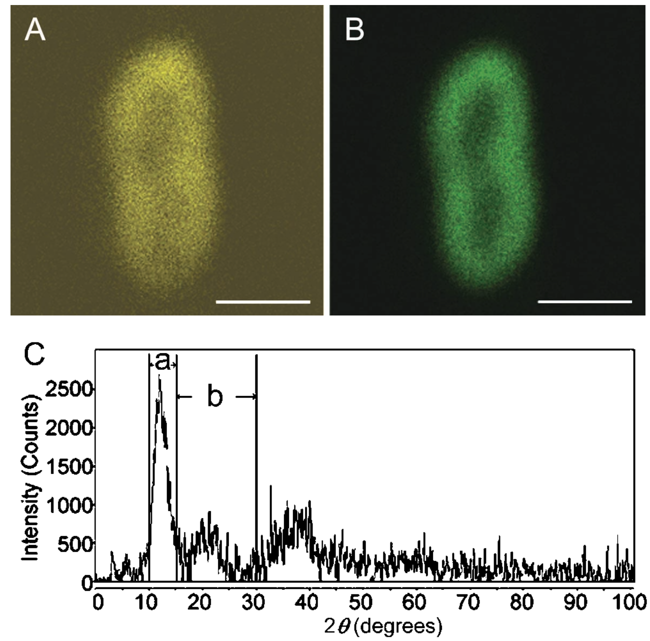


**Fig. 2.** Mineralogical analyses of a resting egg. Fluorescence maps made by an analytical X-ray microscope: **(A)** phosphorous Kα line distribution; **(B)** calcium Kα line distribution. Scale bar, 0.3 mm. **(C)** XRD patterns of a resting eggshell with Cu Kα radiation (λ=1.54050Å).

phosphate ($CaP_4O_{11}$) (Powder Diffraction File: PDF 21–839), calcium phosphate tetrahydrate [$Ca_2(P_4O_{12})\cdot 4H_2O$] (Powder Diffraction File: PDF 41–483), and calcium hydrogen phosphate dihydrate ($CaHPO_4\cdot 2H_2O$: brushite) (Powder Diffraction File: PDF 11–293). Second and third peaks of the calcium phosphates appeared predominantly in the range 2θ=15–30 (Area b). Two polymorphs of calcium carbonate known as calcite and aragonite, common minerals that give exoskeletons and eggshells their strength (Silyn-Roberts and Sharp, 1985; Weiner and Addadi, 1997), are known to have first peaks in the range 2θ=26.2–29.4. Hydroxylapatite, which is a form of calcium phosphate found in vertebrate bones, is known to have a first peak at about 2θ=31.8. None

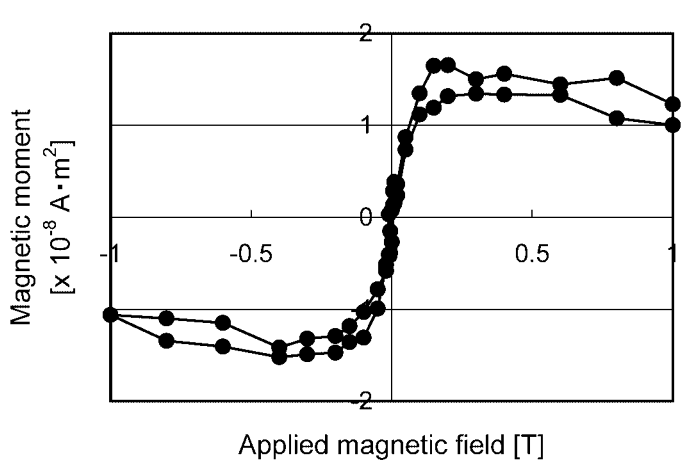


**Fig. 3.** Magnetisation curves at 300 K of resting eggs (n=40, air-dried) with applied magnetic field from –1T to 1 T.

of these were found in the resting eggs, however.

In search of magnetic properties, 40 resting eggs were analysed by a SQUID. A magnetisation curve obtained at 300 K (Fig. 3) revealed saturation of magnetic moment, which indicated the presence of magnetic material. It is reasonable to conclude that magnetite ($Fe_3O_4$) probably accounts for this result, in light of the fact that: (1) the applied magnetic field was *ca*. 0.2 [T] when the magnetic moment was saturated, and (2) magnetic materials existing in nature are mostly iron oxides. The saturation of magnetic moment was $1.5\times10^{-8}$ [A·m$^2$] at 300 K, and the total mass of magnetite in the samples was estimated to be 0.16 μg. Magnetic hysteresis was not apparent, possibly because of the phenomenon of superparamagnetism, resulting from the small particle size of the magnetite. Accordingly, if the size of the magnetite in each resting egg is extremely small, the thermal energy ($k_BT$) may be sufficient to cause random fluctuations in the atomic magnetic moments.

## DISCUSSION

The current results demonstrated that the dormant *Daphnia* embryos are protected by an eggshell made of crystalline calcium phosphate (Figs. 1A and 2), which has a honeycomb structure (Fig. 1B), and that the resting eggs contain magnetic mineral (Fig. 3). These findings may, in part, explain the adaptability of *Daphnia* to various environments.

This is the first study demonstrating that *Daphnia* resting eggshell contain calcium phosphate in crystalline form. Lowenstam *et al.* reported on the phosphatic shell plate of the barnacle *Ibla* (Cirripedia) (Lowenstam and Weiner, 1992). However, reports of crystalline calcium phosphate in invertebrates are rare (Lowenstam and Weiner, 1992; Weiner and Addadi, 1997), and highly unusual for zooplankton (Lowenstam and Weiner, 1989). It is interesting that the genus *Daphnia*, a Cladocerans group zooplankton, produces resting eggs that have an exoskeleton microstructure that resembles vertebrate bone (Bilezikian *et al*., 1996), in that both contain calcium phosphate as an inorganic material and have a honeycomb structure. Such a honeycombed structure may represent an adaptation which resists mechanical forces and acidic conditions of the digestive tracts of predators. Calcium hydrogen phosphate dihydrate (brushite) has been reported to be more stable than hydroxylapatite at pH values lower than 4.0 (Johnsson and Nancollas, 1992). Many fish have two distinct pepsin enzymes that catalyse proteolysis of haemoglobin at a maximal rate in the pH range of 2–4 (Gildberg, 1988). It is possible that these eggs survive in a predator's digestive tract because they contain calcium hydrogen phosphate dihydrate. If so, *Daphnia* resting eggs may offer a new functional perspective of mineralised tissue.

The current data also offer the first evidence that resting eggs contain magnetic mineral (Fig. 3). Similar analyses of bee and butterfly eggs have not shown magnetic saturation (Gould *et al*., 1978; MacFadden and Jones, 1985). Since *Daphnia* have the haemoglobin (Pirow *et al*., 2001) and ferritin gene (GeneBank accession number AJ292556), they are able to internally accumulate iron ions. The role for these ions in *Daphnia* is not clear. Behaviours in some creatures, such as navigation, have been attributed to the ability to detect the Earth's magnetic fields by endogenous microscopic biomagnets, magnetite (Kirschvink, 1997). Magnetotactic bacteria possess linear chains of crystals of magnetite and orient themselves using the Earth's magnetic field (Blackmore, 1975). Honeybees also contain magnetite to detect the Earth's magnetic field, in order to show other bees the direction of food (Gould *et al*., 1978). Spiny lobsters possess magnetic materials (Lohmann, 1984). Although little is known about their detection system, lobsters also undergo an annual mass migration, possibly by utilising the Earth's magnetic fields (Lohmann *et al*., 1995; Boles and Lohmann, 2003). Rainbow trout can detect the Earth's magnetic field with magnetites, which are contained in the olfactory lamellae (Walker *et al*., 1997; Diebel *et al*., 2000), for navigation. Even though it is not clear if magnetite is used by *Daphnia* as a navigational aid, it is possible that *Daphnia* produce magnetite for survival.

Since there is selective predation of *Daphnia* carrying resting eggs over those not carrying resting eggs (Mellors, 1975), one possible function of magnetic material in the eggs is that it reveals the location of resting eggs to potential predators, which leads to their ingestion and distribution to distant environments. As mentioned earlier, rainbow trout, which feed on *Daphnia* (Jahn and Lendman, 1993), recognise and respond to magnetic fields (Walker *et al*., 1997). Paddlefish also feed on *Daphnia* and are able to detect them by using stochastic resonance to amplify weak electrical signals emitted by *Daphnia* (Russell *et al*., 1999). Accordingly, the magnetic material in the resting eggs may act in concert with stochastic resonance (Douglass *et al*., 1993; Wiesenfeld and Moss, 1995) to attract predators. Resting egg production appears to increase predation of maternal *Daphnia*. Nevertheless, the species prevails, since the eggs remain structurally intact until they hatch, after being scattered to more suitable environments. This relationship between *Daphnia* resting eggs and predators resembles that between the seeds of fruits and birds (Moore, 2001). Further studies may attempt to clarify the relation between magnetic material in the resting eggs and predation.

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