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Source: Zoological Science, 21(10) : 1019-1023

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.21.1019>

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Elemental Characterization of *Daphnia* Resting Eggs by X-ray Analytical Microscopy

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ABSTRACT—Resting eggs of *Daphnia*, a key crustacean zooplankton of freshwater food chains, can remain viable for more than a century. These eggs are able to withstand freezing and drying, and can survive the harsh environment of a predator's digestive system. Until recently little was known about the chemical composition, microanatomy, and physical properties of the resting eggs.

The current study utilized a physical technique, the X-ray analytical microscope, to identify and localize component elements of the *Daphnia* resting egg. The analysis demonstrated that phosphorus, sulfur, potassium, and calcium were detected as elemental components of the resting egg, and detection intensities of the four elements differed according to the position of the eggs. Phosphorus and calcium were mostly detected in regions of the eggshell that surrounded the two embryos. In addition, sulfur was distributed throughout the eggshell whereas potassium was localized to the areas that corresponded to where the embryos were encased. Through the use of X-ray analytical microscopy, the current study identifies elemental characteristics in relationship to the structure of the *Daphnia* resting eggs.

Key words: *Daphnia*, resting egg, eggshell, elemental analysis

INTRODUCTION

Daphnia normally reproduce by parthenogenesis. However, in unfavorable environments, robust resting eggs are induced after they switch to sexual reproduction (Kleiven *et al.*, 1992; Alekseev and Lampert, 2001). Previous studies have reported on the kinds of stimuli that induce *Daphnia* to switch from asexual to sexual reproduction (Kleiven *et al.*, 1992; Alekseev and Lampert, 2001). Each resting egg usually encases two resting embryos. When conditions become favorable, the eggs hatch and *Daphnia* begin to reproduce again by cyclic parthenogenesis. Unique adaptive and survival abilities of *Daphnia* resting eggs have been reported. For example, resting eggs can withstand freezing and drying and remain viable for decades or centuries (Cáceres, 1998). Resting eggs are also able to survive in the harsh environment of a predator's digestive system (Mellors, 1975) and are excreted intact and viable to the outside environment.

Insect eggshells, generally described as the cuticle, are complex structures with many functions (Nickles *et al.*,

2002). The cuticle is mainly composed of three parts: a thin and featureless inner vitelline membrane, a honeycomb and trabecular endochorion, and a thick external exochorion. In addition to these structural characteristics, various materials, such as chitin, proteins, and inorganic compounds, are also used to make up the cuticle. Nickles *et al.* (2002) also conducted an elemental analysis of an insect eggshell (blue butterfly). They describe the distribution of the component elements in combination with the eggshell structure and materials. In their study, particle-induced X-ray emission (PIXE) was used to perform elemental analysis. Although PIXE is very sensitive method to analyze elements, it is also a very complex and specialized instrument that uses a microprobe which consists of a Dynamitron particle accelerator and an autoaligned beam line.

Schultz (1977), Seidman and Larsen (1978), and Kawasaki *et al.* (2004) have previously reported on the unique structure of the *Daphnia* resting eggs. *Daphnia* resting eggshells are composed of two pieces of shell, each of which is comprised mostly of a honeycomb structure surrounded by a cuticle layer. A previous study reported on the existence of crystalline calcium phosphate and magnetic minerals in the *Daphnia* resting eggs (Kawasaki *et al.*, 2004). The crystalline calcium phosphate is stable at pH val-

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ues lower than 4.0, similar to that of a predator's digestive tract. However, little is known about the chemical composition of the egg itself.

To further understand the durability of the *Daphnia* resting eggs, the current study characterized the *Daphnia* resting eggs using an X-ray analytical microscope. Like PIXE, the X-ray analytical microscope detects a wide range of elements, from sodium (Na) to uranium (U). Unlike PIXE, however, the microscope uses X-ray intensifier beam exposure, which nondestructively penetrates a sample, and performs fluorescence X-ray analysis at ambient conditions, making sample handling easy. However, the X-ray analytical microscope can not detect light elements such as hydrogen (H), carbon (C), nitrogen (N), and oxygen (O), which are the main component elements of most biological organism.

Our data clearly demonstrates the composition of the component elements and their localization within the *Daphnia* resting eggs.

MATERIALS AND METHODS

Daphnia magna

Daphnia magna were raised based on OECD criteria (1998) with *Chlorella vulgaris*, maintained at $20 \pm 2^\circ\text{C}$ and exposed to 16-hour period of light followed by an 8-hour 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-ionized water, and air-dried for analysis.

X-ray analytical microscopy

Analyses were performed on a 1-mm square area (Fig. 1A) that covered almost the entire egg. Fluorescence X-ray analyses of an air-dried whole resting egg under ambient conditions were carried out using an X-ray analytical microscope (XGT-2700, Horiba, Kyoto), operating at 30 kV and 1.0 mA. Since the X-ray analytical microscope uses X-rays for elemental analysis, there is no need to process samples beforehand. The X-ray beam was 100 μm in diameter.

Three types of analyses (multiple points, line scanning, and maps) were performed on a resting egg. To obtain multiple points analyses, analyses of 100 seconds each on five points on the resting egg were repeated 30 times. Line scanning is useful for investigating and showing the intensity distribution of several elements at a glance along a line traversing the specimen. To perform line scanning, analyses of 1,000 seconds each were repeated 30 times. The position and direction of multiple points analyses and line scanning are marked in Fig. 1A. To obtain 3 dimensional (3-D) distribution images, fluorescence X-ray map scans of 1,000 seconds for each element were performed 30 times in the 1-mm² square outlined in Fig. 1A. Then, each fluorescence X-ray map was analyzed by computer with an image analysis software (Picture Retex Ver1.0R[®]).

In fluorescence X-ray analysis, as well as in other spectroscopic methods related to qualitative analysis, the vertical axis is marked as intensity units. The absolute amount of the elements is not directly implied in the detected fluorescence X-ray spectra.

RESULTS

Fig. 1A shows a light micrograph of an air-dried whole *Daphnia* resting egg taken by a CCD camera attached to the

X-ray analytical microscope at ambient condition. The area of the red square in Fig. 1A is 1 mm². Multiple points analyses (Fig. 1B), line scanning (Fig. 1C), and fluorescence X-ray map scan analyses to obtain 3-D images (Figs. 1D1-4) were performed within this 1-mm² square area. The numbers in Fig. 1A refer to particular points of the eggshell that were subjected to elemental multiple points analysis. Point 1 was situated on the edge of the resting eggshell, Point 2 was sited on the eggshell, Point 3 was sited on the eggshell under which an embryo was encased, Point 4 was sited on the eggshell and Point 5 was sited off of the resting egg and on the sample holder, which served as a control scan.

Fig. 1B shows multiple points analysis performed at the five points marked in Fig. 1A. The horizontal axis is energy (in keV) and the vertical axis is detection intensity in arbitrary units (arb. unit). Fig. 1B shows that four elements were detected as component elements, and the distribution of each element was different at five different points on the resting egg. Phosphorus (P), sulfur (S), potassium (K), and calcium (Ca) were detected at all analyzed points, except at Point 5. The spectra in the higher energy range were omitted from Fig. 1B, because other detected spectra were not different throughout the five points. The main component elements, H, C, N, and O, of biological organisms can not be detected by an X-ray analytical microscope, hence are not shown in the current study. Although the presence of magnetite (Fe₃O₄) in *Daphnia* resting eggs was described in a previous report (Kawasaki *et al.*, 2004), iron (Fe) was not detected in the current study. For Ca, lines at both K α (3.69 keV) and K β (4.01 keV) were detected. The detection intensities of each element were different at each of the five analyzed points. Detection intensity levels of P and Ca were highest at Points 2 and 4 (purple and orange, respectively), followed by Point 3 (light-blue), and Point 1 (yellow-green). S was detected at the highest level at Points 2 and 3, followed by the eggshell at Point 4 and then by the edge of the resting eggshell at Point 1. K was mostly detected at Point 3, followed by Point 2. K was not detected at Points 1 and 4. As expected, there was no detection of any of the four elements at Point 5 (green). It is likely that the two broad peaks between the S and K peaks represents rhodium, since it appears between 2.5 and 3 keV on the energy spectrum and was used as the X-ray source.

Fig. 1C shows the result of line scanning on the resting egg. Line scanning in Fig. 1C was done along the direction of the white arrow that appears in Fig. 1A, initiating on the eggshell edge, passing through the position where an embryo was encased and ending on the opposite eggshell edge. Since P, S, K, and Ca were detected as component elements in a *Daphnia* resting egg as shown in Fig. 1B, these four elements were used as the target elements for line scanning. Intensity distribution of P, S, K, and Ca were yellow, red, blue, and green lines in Fig. 1C, respectively. P and Ca were strongly detected in the eggshell on both sides of the embryo and weakly detected at the center of the embryo. S detection analysis showed a moderate curve

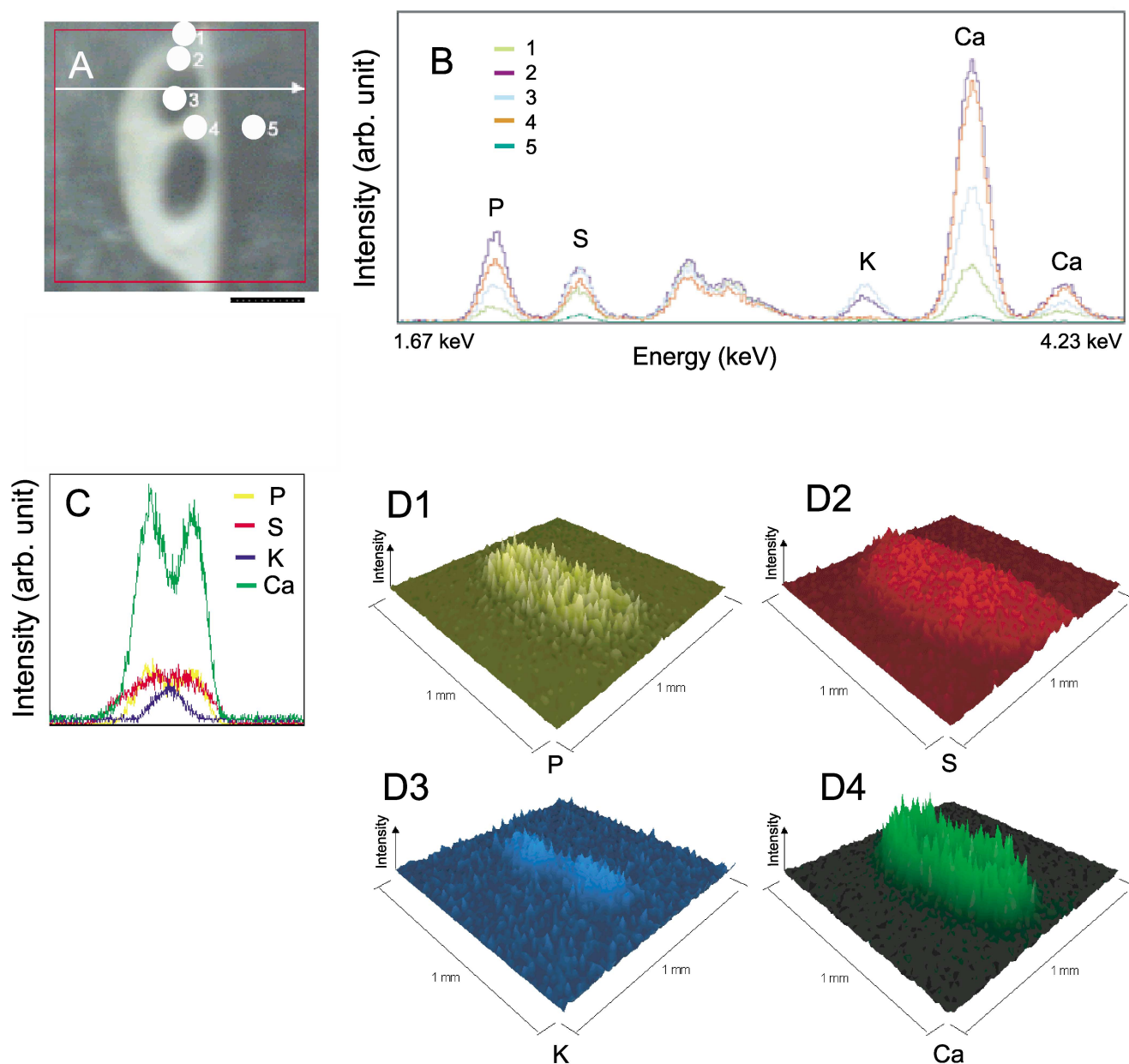


Fig. 1. Results of elemental analyses of a *Daphnia* resting egg with an X-ray analytical microscope. (A) A light micrograph of an air-dried whole *Daphnia* resting egg taken by an X-ray analytical microscope at ambient condition. The area of the red square drawn on the perimeter of Fig. 1A is 1 mm². All analyses (Figs. 1B, C, and D1-4) were performed within this 1-mm² square area. The numbers with white circles in Fig. 1A are multiple analytical points where the X-ray beam of 100 μ m in diameter was exposed. Scale bar is 0.3 mm. (B) Result of elemental multiple-point analysis of the points indicated in Fig. 1A. The results of elemental analysis on 1, 2, 3, 4, and 5 point are yellow-green, purple, light-blue, orange, and green line respectively. Four elements, phosphorous (P), sulfur (S), potassium (K), and calcium (Ca) were detected as components elements. The horizontal axis is energy (in keV) and the vertical axis is detection intensity (arbitrary unit; arb. unit). (C) Elemental results of line scanning. The scan began on the edge of the eggshell and moved to the opposite edge of the eggshell (white arrow in Fig. 1A). The vertical axis is detection intensity (arb. unit). The horizontal axis is corresponded to the position of the white line in Fig. 1A. (D) Results of 3-D distribution images of the four elements, obtained after processing the map scanning in the 1-mm² square area drawn in Fig. 1A. The z-scales are detection intensities. D1, D2, D3, and D4 are P, S, K, and Ca 3-D distribution images, respectively.

intensity from the edge, with the maximum intensity around the center of the embryo. K was weakly detected on the edges of the eggshell and strongly detected at the center.

Figs. 1D1-4 are 3-D distribution images of the four elements. The z-scale is detection intensity. P was detected in the area by which the two embryos were surrounded (Fig.

1D1). S was broadly detected on almost all of the eggshell (Fig. 1D2). In fact, the area of the fluorescence X-ray map of S was greater than the actual area of the eggshell. In general, a fluorescent X-ray map tends to reflect an area larger than the actual subject due to the width of the X-ray beam. In contrast, the distribution of K (Fig. 1D3) was shown as two ovals

at a central position of the eggshell, at the position where the two embryos were encased. Ca was detected in the area by which the two embryos were surrounded (Fig. 1D4).

DISCUSSION

The current study demonstrated that P, S, K, and Ca were detected as component elements of *Daphnia* resting egg using a non destructive X-ray beam. Among these four elements, P and Ca were mostly detected in the regions of the eggshell surrounding the two embryos. According to the 3-D image of S distribution, S was not localized to a particular area of the eggshell. On the other hand, K was clearly localized to the center of the eggshell region, where two embryos were encased. Although a previous study (Kawasaki *et al.*, 2004) suggested the existence of magnetite (Fe_3O_4) in the egg, Fe was not detected in the current study.

The structure of the center of the eggshell region where two embryos are encased is very thin compared to other parts of the eggshell (Fig. 2). Thus it is reasonable to conclude that the detection intensities of P and Ca were weak on the center of the eggshell region. On the other hand, P and Ca, some of which exist as crystalline calcium phosphate as reported in a previous study (Kawasaki *et al.*, 2004), were localized to the area surrounding the two embryos. This area, however, does not have specific structural differences, for example extra eggshell thickness, from the rest of the egg, as far as it can be determined based on previous morphological findings. Further morphological studies combined with elemental analysis are needed to understand why P and Ca were strongly detected. The current study has not confirmed distinct peaks resulting from substances that contain S or K by X-ray diffraction. The likely distribution of S and K is discussed below.

The distribution of S was relatively flat and nonspecific throughout the eggshell, although a slightly strong signal was detected at the center. The 3-D distribution image indicated that S was distributed in a structure with uniform thick-

ness throughout the eggshell. Fig. 2 shows a model structure and cross sections of *Daphnia* resting egg, based on findings of Schultz (1977) and Kawasaki *et al.* (2004). As shown in Fig. 2B and C, structures with a uniform thickness throughout the eggshell are the outer cuticle layer and the inner cuticle layer. Thus, the uniformly spread S observed in Fig. 1D2 is likely to exist mostly in these two cuticle layers, which encases the entire eggshell. The source of the slightly increased intensity around the center may be from proteins that exist in the two embryos. It is possible that there may be S in the honeycomb matrix, but the S distribution around the two embryos should have been more intense on the 3-D image.

Nickles *et al.* (2002) performed elemental analyses of butterfly eggs using PIXE. Consistent with the current findings for the *Daphnia* resting eggs, it was found that S in butterfly eggs was nearly uniform in distribution, giving neither higher spectra nor higher map readings from the ridges than elsewhere throughout the eggshell. Nickles *et al.* (2002) suggested that S is localized to the vitelline membrane. One component common to the cuticle and vitelline membrane is collagen.

The cuticle collagen is composed mainly of glycine-rich repeat residues and characteristically contain three conserved clusters of cysteine residues in the non-Gly rich regions (Page, 2001). The collagens are initially synthesised as pre-procollagen, then extensively cross-linked by reducible disulfide bridges and non-reducible di- and isotriptyrosine cross-links, resulting in the final cuticular matrix. This formation and rearrangement of disulfide bridges is a major feature of collagen assembly at the stages of forming the cuticular matrix. Therefore, the S found in the current study probably originated from cysteine containing cuticle collagen.

In contrast to the other elements, K was specifically localized to the area which corresponded to the position of the two encased embryos (Kawasaki *et al.*, 2004). As mentioned above, the structure of the center of the eggshell region is thin compared to other regions. Therefore the X-ray beam in the current study passed through 1) the outer cuticle, 2) thin honeycomb structure, 3) inner cuticle, 4) embryo membrane, and 5) embryo (See Fig. 2B, C). Levels of K were very low at the edges of the eggshell (Fig. 1B and C), which contains cuticle layers and a thick honeycomb structure. Also, K density is generally higher inside compared to the outside of cells (Sun and Wyman, 1993). Thus, although it is possible that K exists in the center of the eggshell, the K observed in Fig. 1D3 is likely to exist mainly within the embryos. The main purpose of the current study was to perform the elemental qualitative analysis. Further analysis will be needed to identify the chemical substances that include K.

One possible reason why Fe was not detected in the current study is that the amount of Fe is less than the detection threshold of the X-ray analytical microscope. In the future, we hope to establish a more sensitive analytical

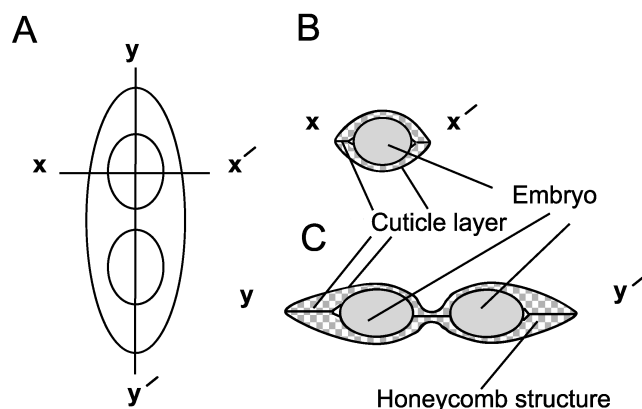


Fig. 2. A diagram of a *Daphnia* resting egg, modified from Schultz (1977) and Kawasaki *et al.* (2004). (A) A whole resting egg. (B) A cross section of a whole resting egg cut along the $x-x'$ axis. (C) A cross section of a whole resting egg cut along the $y-y'$ axis.

method using X-ray to determine the distribution of Fe in *Daphnia* resting eggs and to identify the presence of magnetite. Magnetite may be related to the durability or survival ability of the resting eggs. The thickness of the resting eggshell alone can not fully explain the P and Ca distribution. The structure of the center of the eggshell region, where two embryos are encased, is thin compared to other areas. This non-uniform structure of the *Daphnia* resting eggshell may be related to its durability. We hope to better understand the mechanism of how *Daphnia* resting eggs can adapt and survive in a harsh environment.

ACKNOWLEDGEMENTS

The authors thank K. Yonemochi and S. Tanaka for their technical assistance and discussions, A. Ito for giving us the opportunity to use an X-ray analytical microscope, and also K. Kohn for his helpful discussions, as well as E. Suzuki for his help with the manuscript.

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(Received December 3, 2003 / Accepted August 11, 2004)