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CLADOCERA

Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*

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Abstract *Daphnia* can reproduce through subitaneous and dormant eggs. The production of dormant eggs is induced by stimuli associated with deteriorating growth conditions, and enable *Daphnia* populations to survive temporarily harsh environmental conditions. Dormant eggs are expected to have developed special biochemical adaptations to bridge this long unfavourable period, but little comparative biochemical data are available for dormant and subitaneous eggs. We compared levels of the following molecules between subitaneous and dormant eggs: (a) triglycerides, which are the most abundant energy storage molecules in *Daphnia*, (b) glycerol, a cryoprotectant also involved in energy storage, and (c) the heat shock protein *Hsp60*, a molecular chaperone that may assist in maintaining protein structural integrity and inhibiting cell metabolism during diapause. Unexpectedly, no difference in triglycerides content between egg types was found. As expected, dormant eggs contained more glycerol and relatively more *Hsp60* than subitaneous eggs. The biochemical composition of dormant eggs can therefore be seen as an adaptation to the harsh environmental conditions these eggs encounter.

Keywords Cladocera · Heat shock protein · *Hsp60* · Diapause

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Introduction

Egg quality is a key characteristic with important fitness consequences because it shapes early life-history traits of the offspring (Roff, 1992; Stearns, 1992). In several taxa, like rotifers and cladocerans, different types of eggs are produced: subitaneous eggs and dormant eggs (Gilbert & Williamson, 1983). Egg types differ strongly in the duration of the egg stage, and in environmental conditions eggs and hatchlings encounter. Subitaneous eggs are typically formed during the growing season when conditions are favourable. The resulting offspring hatch within days or weeks, and often encounter similar conditions as when the eggs were produced. Dormant eggs, on the other hand, are frequently formed when growing conditions deteriorate. These eggs pass temporarily unfavourable conditions during dormancy. In cladocerans, the resulting offspring typically hatch into predictably high food level conditions (algal blooms in spring; Caceres, 1998; Hairston et al., 2000).

There is growing evidence that offspring from both egg types differ in physiology, life history and fitness, and these differences have been interpreted as

adaptations of egg types to different growth conditions (cladocerans: Arbaciauskas, 1998; Arbaciauskas & Lampert, 2003; Arbaciauskas, 2004; rotifers: Gilbert, 2004; Gilbert & Schroder, 2004). For example, most hatchlings from dormant eggs of the water flea, *Daphnia magna*, have lower initial mass compared to hatchlings of subitaneous eggs, but have a higher metabolism and growth rate, resulting in a larger size at maturation and an increased energy allocation to reproduction. However, this higher metabolism is traded off against a lower starvation resistance under harsh conditions (Arbaciauskas & Lampert, 2003).

Differences in duration of the egg stage and environmental conditions egg types encounter, together with life history differences between offspring from both egg types, strongly suggest that the two egg types differ in their biochemical composition. One important function of eggs is to store energy. In rotifers, offspring that hatch from dormant eggs have been shown to have more lipid droplets than offspring that hatch from subitaneous eggs (Gilbert, 2004). However, as far as we know, no direct biochemical analysis of storage molecules in eggs has been carried out. In adult *Daphnia*, triglycerides (esters of fatty acids and glycerol) are the key energy storage molecules (Peters, 1987). Half of the accumulated triglycerides between two moults are allocated to their eggs and during development subitaneous embryos consume half of their triglycerides (Tessier et al., 1983).

Furthermore, as dormant eggs have to bridge unfavourable conditions, adaptations are expected which may be absent in subitaneous eggs. Polyhydroxy alcohols, like glycerol and sorbitol, are known to act as cryoprotectants in a wide range of diapausing organisms (Storey, 1997; Denlinger, 2002; Lencioni, 2004). The functions of cryoprotectants are to lower the freezing-temperature, protect macromolecular structures from freezing injuries, stabilize proteins and enzymes against cold denaturation, and limit growth of ice crystals. Glycerol can be formed from glycogen, which is the most abundant carbohydrate in most animals. In insects, high levels of polyols like glycerol and sorbitol are found in diapausing eggs but are absent in nondiapausing eggs (Denlinger, 2002).

Heat shock proteins are another class of molecules that may be important for surviving stressful conditions. These proteins are molecular chaperones that are involved in folding, unfolding, transport and assembly of complex protein units, and prevent proteins from getting into 'inappropriate' aggregations, as well as prevent degradation of misfolded or aggregated proteins (Parsell & Lindquist, 1993; Pockley, 2003). Heat-shock proteins occur under normal cellular conditions, but their expression can increase in response to all kinds of stressors that have the potential to damage cells (Sørensen et al., 2003). In *D. magna*, a common heat-shock protein, *Hsp60*, is constitutively present but also can be induced by predator stress (Pijanowska & Kloc, 2004; Pauwels et al., 2005). There are strong data indicating that heat-shock proteins are an important mechanism to maintain protein structural integrity, and to inhibit DNA synthesis and cell division during diapause in large branchiopods (*Artemia fransicana*; Clegg et al., 1999) and insects (Denlinger, 2002).

In the present study, we compared the biochemical composition of dormant and subitaneous eggs of the cladoceran *D. magna*. More specifically, we focused on triglycerides, glycerol and *Hsp60.* We tested the hypothesis that levels of triglycerides, glycerol and heat shock proteins were higher in dormant eggs than in subitaneous eggs.

Material and methods

Egg collection

Dormant eggs of *D. magna* were collected from a shallow eutrophic pond (OM2 in Heverlee; see Cousyn & De Meester, 1998). Samples of the upper 10 cm of pond sediment were taken in spring 2005 before the hatching peak occurred. Samples were sieved (250 μm) and ephippia with dormant eggs were removed. To prevent hatching, ephippia were kept in the dark at 4°C prior to removal of the dormant eggs out of the ephippia for biochemical analyses. To obtain subitaneous eggs, part of the ephippia were exposed to hatching stimuli (long day photoperiod, high temperature), and hatchlings were kept in culture in the laboratory. This procedure guaranteed that subitaneous and dormant eggs were of genetically similar composition. Hatched animals were cultured under standard conditions (30 individuals per 3 l, 20 ± 2°C; 14 h light/10 h dark photoperiod, and food concentration: *Scenedesmus*

obliquus, 1.5×10^5 cells ml^{-1}or 1.1 g C l^{-1}) for several generations. Subitaneous eggs were removed from freshly killed females, by pushing the upper carapace away from the body and gently flushing the eggs out of the opened brood pouch. Two perpendicular diameters were measured using a calibrated stereoscope on a subsample of 50 eggs of both type. The calculated egg volume was used as an estimate for egg size. All eggs were then transferred to the specific homogenization buffer (see below), homogenized mechanically with a plastic pestle, and stored at −80°C.

Glycerol and triglycerides assay

To quantify glycerol and triglycerides levels, 10 replicates each consisting of 25 subitaneous or 25 dormant eggs (total of 250 eggs per egg type) were analyzed photometrically according to Stibor & Müller Navarra (2000). All absorbance readings of each replicate were done in duplicate and the mean value per replicate was used for further analyses. This assay is based on the phosphorylization and conversion of glycerol to quinoneimine (chromophoric compound). To determine free glycerol content, samples of 25 eggs were homogenized in 25 µl PBS buffer (0.15 M NaCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4 with *o*-phosphoric acid). To ensure that all cell structures were completely degraded, samples were further homogenized ultrasonically at 42 KHz (Branson 2510) five times for 30 seconds. Ten µl of the samples was loaded onto a 96 well plate, and 160 µl Free Glycerol Reagent (F6428, Sigma Aldrich®, St. Louis, USA) was added. This mixture was incubated for 10 minutes at 37°C, and initial absorbance (before adding lipase) was recorded at 540 nm on a microplate reader (Benchmark plus, Biorad®, Hercules, USA). Absorbances were converted to glycerol levels using a calibration curve based on a dilution gradient of a glycerol standard solution, which ran together with the samples.

To quantify triglycerides, a lipoprotein lipase (which degrades triglycerides to glycerol and fatty acids) was added to the samples from the glycerol measurement. We added 40 µl Triglyceride Reagent (Triglyceride reagent T2449, Sigma Aldrich®, St. Louis, USA) to the wells with samples, and after 10 min incubation at 37°C, final absorbance (after adding lipase) was measured at 540 nm. The difference in total glycerol and free glycerol (taking into account the dilution) represents the glycerol that was bound in triglycerides.

Heat-shock protein (Hsp) assay

To quantify *Hsp60* content, 5 replica each consisting of 50 subitaneous or dormant eggs (total of 250 eggs per egg type) were homogenized in 50 µl RIPA buffer (0.15 M NaCl, 1% deoxycholic sodium salt, 1% Triton X-100, 0.1% sodium dodecyl sulphate [SDS], 0.01 M Tris, pH 7.2) to which a general protease inhibitor cocktail was added (P2714, Sigma Aldrich®, Steinheim, Germany). Fifteen µl of each sample was loaded on a SDS polyacrylamide gel (Criterion Precast Gel, 10% Tris–HCl, Biorad®, Hercules, USA) and separated by electrophoresis. Proteins were then transferred from gels to nitro-cellulose membranes by Western Blotting (Lundebye et al., 1995). Membranes were blocked for 1 h in 3% skimmed milk in TBS (25 mM Tris–HCl; 500 mM NaCl). *Hsp60* was detected using a monoclonal antibody (dilution 1:1000, SPA 805, StressGen®, see also Pauwels et al., 2005). Membranes were incubated with the primary antibody for 1.5 h, washed twice in 3% skimmed milk in TBS for 5 min and washed once in TBS with 0.5% Tween 20. Thereafter, membranes were incubated for 1 h with an anti-rabbit, AP-conjugated secondary antibody (polyclonal Goat anti-Rabbit Ig, D0487, DakoCytomation®). Washing was repeated as described above. Colouring of alkaline phosphatase substrate was done by enzymatic reaction using a solution of *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt. Blots were scanned and analyzed with Image Pro plus® v. 5.0. Optical densities were calculated using the density sum (sum of each density measurement in the selected range).

To be able to correct *Hsp* level for the total protein content of each pooled egg samples, we determined protein content of both egg types. For this, we carried out a linear regression analysis of total protein content against egg number. Samples with increasing numbers of subitaneous and dormant eggs (5, 10, 15, 20 and 25; 2 replica of each) were homogenized in 10 µl RIPA buffer. Samples were analyzed photometrically and total protein content was determined using the Bio-Rad protein assay (Bio-Rad®, München, Germany).

Data analysis

Levels of triglycerides, glycerol and *Hsp60* were compared between egg types using *t*-tests. Regression lines of total protein content against number of eggs were used to estimate total protein content of the sample loaded for *Hsp* analysis. We used ANCOVA to test whether regression lines of egg types differed with egg type as an independent variable, total protein content as a dependent variable, and number of eggs as a covariate. Afterwards, we tested for differences in corrected *Hsp60* levels by dividing *Hsp60* levels of each replicate by total protein content, as estimated from the regression lines. To correct for multiple testing, a Bonferroni correction was applied to the four *t*-tests comparing molecule levels between egg types ($\alpha_{adjusted}$ set a 0.05/4 = 0.0125). All analyses were done in STATISTICA 6.0. Assumptions of homogeneity of variance and normality of errors were met in all analysis.

Results

Dormant eggs and subitaneous eggs did not differ in size (mean ± 1 S.E.; dormant eggs: $1.5 \times 10^{-4} \pm 0.3 \times 10^{-4}$ cm^3, $n = 50$ vs. subitaneous eggs: $1.6 \times 10^{-4} \pm 0.7 \times 10^{-4}$ cm^3, $n = 50$, *t*-test: $t_{98} = 1.03$, ns). While considerable concentrations of free glycerol were found in dormant eggs, almost no free glycerol was detected in subitaneous eggs (*t*-test: $t_{18} = -8.62$, $P < 0.01$; Fig. 1a). In contrast, we found no difference between triglycerides levels of the two egg types (*t*-test: $t_{18} = 0.70$, ns; Fig. 1b).

We did not detect a difference in the amount of *Hsp60* per egg between dormant and subitaneous eggs (*t*-test: $t_8 = 1.30$, ns, Fig. 1c). The ANCOVA that tested for the total protein content of both egg types, showed however, a difference in the slopes of the regression lines between egg types for total protein content vs. number of eggs (ANCOVA, Egg number × Egg type: $F_{1,16} = 4.63$, $P < 0.05$). For subitaneous eggs the regression equation was: protein content (µg) = 5.97 + 1.97 × egg number ($F_{1,8} = 25.0$, $P < 0.01$, $R^2 = 0.76$); and for dormant eggs: protein content (µg) = 0.06 + 1.02 × egg number ($F_{1,8} = 25.7$, $P < 0.01$, $R^2 = 0.76$). Based on these regressions, a pooled sample of 50 subitaneous eggs contained a 2.75 fold higher total amount of proteins than a similar sample of dormant eggs. When corrected for total protein content, dormant eggs contained a higher level of *Hsp60* than subitaneous eggs (*t*-test: $t_8 = -4.84$, $P < 0.01$; Fig. 1d), indicating that the relative constitution of *Hsp60* level to the total amount of proteins is higher in dormant eggs than in subitaneous eggs.

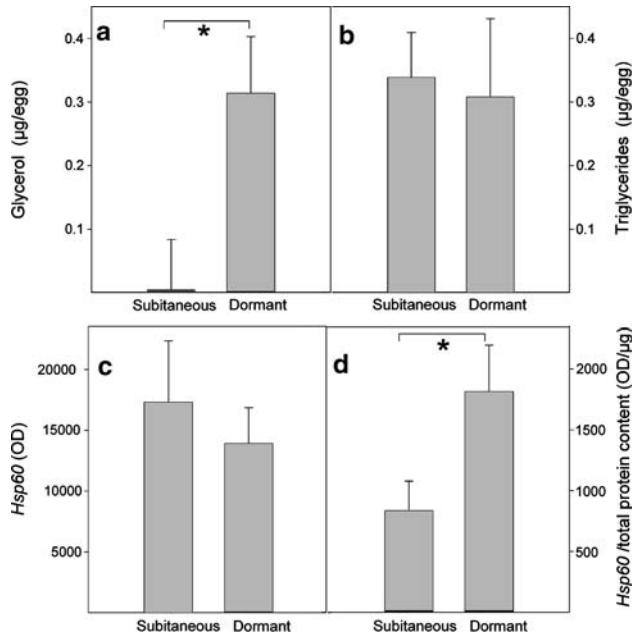

Fig. 1 Levels of glycerol (**a**), triglycerides (**b**) and *Hsp60* [without (**c**) and with (**d**) correction for total protein content] in subitaneous and dormant eggs of *D. magna*. Error bars indicate 1 S.E., asterisks denote significant differences based on a *t*-test ($P < 0.0125$). *Hsp60* is expressed in optical density (OD) units

Discussion

As expected under an adaptive scenario, we found relatively higher *Hsp60* levels and higher glycerol levels in dormant eggs compared to subitaneous eggs. However, similar-sized eggs of both types did not differ in the amount of triglycerides, the most important energy storage molecules in *Daphnia* (Tessier et al., 1983). The higher relative amount of *Hsp60* in dormant eggs is consistent with the fact that heat-shock proteins can play a cryoprotective role during diapause (Denlinger et al., 2001). These proteins also can directly contribute to developmental arrest by helping to shut down the cell cycle (Krebs & Feder, 1997). Our study is the first to report the occurrence of *Hsp60* in diapausing stages. Higher glycerol level in dormant eggs is consistent with the cryoprotectant role this molecule plays in a wide range of diapausing organisms (Storey, 1997; Denlinger, 2002; Lencioni, 2004). Obviously, we cannot exclude the possibility that other protective mechanisms are at work in dormant eggs.

One may argue that the differences in their biochemical content between subitaneous and dormant eggs in our analysis may be due to the fact that they were produced under different conditions. While subitaneous eggs were formed under homogeneous, good conditions in the laboratory, dormant eggs were obtained from an egg bank, and therefore are a mixture of eggs produced in several growing seasons under less favourable and more variable environmental conditions (i.e. food availability, temperature, predator stress etc.). Yet, these differences in conditions reflect the natural situation, and hence, are part of the background selective environment against which differences in egg type composition evolved. Given that only suboptimal conditions trigger the formation of resting eggs (Stross & Hill, 1965), it would be artificial to compare both egg types in females that experienced identical, near optimal conditions. First, the multi-generational mixture of dormant eggs is expected to generate a larger inter-egg variability in biochemical content in dormant eggs. However, this did not result in a higher inter-sample variability in our dataset, as variances were always homogeneous between egg types (see also Fig. 1). Such variability, if present, would only make it more difficult to detect the observed differences in biochemical content between egg types. Second, although dormant eggs were probably made under less ideal conditions (i.e. less resources, lower temperatures, predator stress etc.), they showed higher levels of costly molecules like glycerol and *Hsp*60. Higher abundances of costly molecules, despite less favourable conditions, suggests that the observed differences in biochemical content are not simply a reflection of the inability of females to add enough energy to their eggs, but rather are part of an adaptive response to dormancy.

Predator stress is known to affect *Hsp* levels (Pauwels et al., 2005), yet it is unlikely that predation risk caused the differences in *Hsp* between egg types. First, predation pressure in the studied population has been relatively low during the last 12 years (Cousyn & De Meester, 1998; period after 1998: personal observation). In addition, most dormant eggs are produced in the predation pressure by fish is low. There is also no a priori reason to expect that animals would respond to fish predation by allocating more Hsp in their dormant eggs.

Dormant eggs did not contain more triglycerides than subitaneous eggs. Apparently, triglycerides levels are not responsible for differences in metabolism and starvation resistance of juveniles, after hatching from the two types of eggs (Arbaciauskas & Lampert, 2003). Although triglycerides are the main energy source for development, it may be that other energetic sources such as phospholipids can be used as an energy source during starvation. Further investigations including more detailed analyses of yolk composition and the contribution of phospholipids are needed. However, we did find significantly higher levels of glycerol in dormant compared to subitaneous eggs. In dormant stadia, glycerol also can act as a source of energy reserves (Lencioni, 2004), and it is known that this molecule can easily be used immediately after a period of suppressed metabolism (Kimura et al., 1992). It therefore is possible that glycerol functions both as a cryoprotectant and as an energy source for the egg.

To conclude, the observed biochemical differences between dormant eggs and subitaneous eggs were largely consistent with the different environmental conditions encountered by the egg types. During diapause, dormant eggs suppress their metabolism and need protection against severe stress. In line with this, dormant eggs contained high levels of glycerol and relatively high *Hsp60* levels compared to subitaneous eggs.

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