CRYOPRESERVATION OF *DAPHNIA MAGNA* GENOTYPES

Adinda PUTMAN

Supervisor:
Prof. Dr. Luc De Meester

Co-supervisor:
Dr. Ig. Bart Panis

Members of the Examination Committee:
Prof. Dr. Luc Brendonck
Prof. Dr. Patrick Van Dijck
Prof. Dr. Ellen Decaestecker
Dr. Dominik Martin-Creuzburg
Dr. Joost Vanoverbeke

Dissertation presented in partial fulfillment of the requirements for the degree of Doctor in Science (Biology)

August 2015

Table of contents

Acknowledgements

Het dankwoord is misschien wel het belangrijkste deel van dit boekje. Niet alleen omdat dit het meest gelezen deel van het boekje is, maar ook omdat hierin wordt weergegeven hoeveel steun en hulp een mens nodig geeft om een doctoraat tot een succesvol einde te brengen.

Eerlijkheid gebied mij om te starten met het bedanken van Evelyne en mijn promotoren Luc en Bart. Tijdens mijn masterthesis heeft Evelyne mij aangestoken met de microbe voor wetenschappelijk onderzoek en alhoewel ik vaak heb gevloekt, moet ik haar hiervoor zeer hard bedanken. Zowel Evelyne als Bart waren heel enthousiast over het project om de eitjes van de watervlo te cryopreserveren en alhoewel Luc er eerder sceptisch tegenover stond, mocht ik toch mijn kans wagen en een IWT aanvragen. Luc bedankt om deze kans aan een "fysioloog" te geven en voor de hulp bij de voorbereidingen hiervoor en bij de rest van mijn doctoraat. Ik heb geleerd dat ecologie ook soms leuk kan zijn en dankzij jou kan ik nu het geklieder van mijn dokter lezen. Ook Bart bedankt voor alle ondersteuning! Ik kon altijd bij jou terecht voor cryo-advies (mooi woord vind ik zelf) en ook als ik weer eens in de knoei zat met een berekening.

Ook de rest van mijn juryleden wil ik bedanken voor hun constructieve commentaren op een eerdere versie van dit proefschrift. Luc B bedankt om het voorzitterschap op u te nemen en alles in goede banen te leiden. Ellen bedankt om de moeite nemen om alles te lezen en om het vrouwelijke geslacht en West-Vlaanderen te vertegenwoordigen in mijn jury ;-). Joost bedankt om mij te leren werken in R en om een permanente hulplijn te zijn voor al mijn grote en kleine R problemen. Patrick bedankt dat ik in jouw labo terecht kon voor suikeranalyses en dat ik daar op de steun kon rekenen van Nelson en later Ben. Ook hen moet ik bedanken voor de praktische hulp met de HPLC. Dominik thanks for your hospitality! I really enjoyed my trips to Konstanz! Probably mostly due to the lake, but also because of the good working atmosphere in the lab. I have to thank all people of the institute for that and especially Petra for all the help in the lab and Timo and Nina for fun in the office and to fill my "lonely" evenings. Apart from that, I also have to thank you for all the help on the manuscripts and to show me how ecology can also be fun. I really hope to pass by the lab some time again.

Het grootste deel van de tijd tijdens mijn doctoraat bracht ik door in de kelder van het kolenmuseum. En ik moet zeggen dat dit meestal ne schonen tijd was! Sabine, Ine, Aurora, Evelyne, Mieke, Melissa, Kristien, Jessie en alle andere regelmatige of permanente bezoekers van de daphnialokalen bedankt om het daar zo tof te maken! Sabine ik denk dat ik het meeste uren met jou heb gesleten in het oud daphnialokaal of in de doomkrocht, ne shift meer of minder daar keken wij niet naar é! Als er maar koffie was (bedankt trouwens om mij in te wijden in de geneugeden van de wondere wereld van de koffie!) Ik heb mij kapot gelachen tijdens al die uren en ik hoop echt dat niemand ooit onze conversaties heeft opgenomen! Ine en Sabine bedankt voor het ontbijt als ik op mijn verjaardag moest werken! Sabine, Mieke, jobstudent Ruben en Melissa bedankt voor de hulp bij experimenten waarvan de design weer eens uit de hand was gelopen of op van die dagen waarop al mijn beestjes ineens allemaal baby's maakten! Melissa nen helen dikke merci voor alle hulp bij het potjes kuisen en om mij de teksten van de vlaamse klassiekers bij te brengen! Ook mijn masterstudent Niels verdiend

een vermelding in dit dankwoord. Samen hebben we voor het eerst een *Daphnia* eitje gecryoperserveerd! Eén van de mooiste momenten in mijn doctoraat!

Naast deze Daphnia-mensen zijn er ook nog een aantal andere belangrijke personen die ik niet mag vergeten. Jane (Die ROSSE SISSIE!) je staat al in het dankwoord van mijn masterthesis en ook hier mag je zeker niet ontbreken! Nen hele dikke merci voor alle steun en fun op en naast het labo! Ine, nog eens, ik moet u ook ontzettend hard bedanken om mijn persoonlijke 'google handwriting translate' te zijn!

Ronny en Geert, ook altijd te vinden ergens in de kelder, sorry dat we soms een beetje te luidruchtig waren en bedankt voor alle hulp bij technische problemen. Melissa ook bedankt om ons altijd te voorzien van algen en Eddy bedankt om de flessen voor mijn 'andere' algen te kuisen.

Ook de rest van de LDM-groep en de Brennies moet ik bedanken voor de leuke sfeer. Het was altijd leuk om een bezoekje te brengen aan jullie bureaus of om een bureau met jullie te delen! Koen en Aurora jullie waren mijn laatste bureaugenootjes. Koen bedankt voor alle koekjes! En Aurora merci voor alle zever en om mij van mijn 'dreams and hopes' vanaf te helpen ;-)

Ook Conny en Ria verdienen een woordje van dank. Zonder hen zou het labo niet draaien! Conny ontzettend bedankt voor alle hulp bij alle paperassen en voor de alle andere dingen die je doet om alles vlot te laten verlopen! Ria bedankt voor alle hulp in het labo en om mijn berekeningen na te kijken.

Ook naast het kolenmuseum beleefde ik nog ontzettend veel fun! Soms met dezelfde mensen: Jane, Sabine, Ine en Aurora zonder jullie had ik dit hier nooit afgemaakt en ik ben jullie dus ontzettend dankbaar! En ik hoop echt dat we nog veel fun mogen beleven samen! Maar ook met andere mensen: Holsbeek Ladies ik ben super blij dat ik deel mocht uitmaken van dit fantastische team! Ik heb er enorm van genoten en voetbal was voor mij de ideale manier om mijn frustraties uit te werken! Ik ga jullie heel hard missen en je mag mij zeker een aantal keer verwachten op de matchen volgend seizoen! En ik wil met plezier mee als kookmama op weekend voor in het geval Jazzke weer valt ;-) Bea ook bedankt om mijn zwemmaatje te zijn en Sabine bedankt om mijn loopmaatje te zijn! Het was aangenaam om ook nog eens iets anders te doen dan achter mijn pc zitten in de laatste maanden van mijn doctoraat.

Naast mijn leventje hier in Leuven pendelde ik in het weekend vaak naar vrienden en familie in Waregem of ergens daartussen. Ookal begrepen zij niet altijd helemaal wat er hier allemaal aan de hand was en hoe je zolang iets nuttig kon doen met zo een klein beestje, ook zij hebben ervoor gezorgd dat ik dit tot een goed einde bracht. Mama, papa, oma en opa bedankt voor de steun! Zonder jullie steun was het zelfs niet mogelijk geweest om hieraan te starten, ik ben blij dat ik de kans heb gekregen om te studeren in het "verre" Leuven. Denne en Aline, Féli en Annelies, Jana en Kevin, Danel, Vereecke en Sofie, Hannes en Kathleen, Wouter en Niels, Marlies ontzettend bedankt voor alle steun en vooral voor de nodige ontspanning! Jana en Vereecke bedankt voor de aanmoedigende smsjes in de laatste maanden! Ook alle andere vrienden en familie die ik niet heb opgesomd wil ik bedanken voor de ongetwijfeld zeer leuke momenten die we hebben weleefd!

En dan als laatste moet ik nog de belangrijkste persoon in mijn leven bedanken: Gunther zonder u was dit nooit gelukt! Ik moet u bedanken voor het vele geduld dat je met mij had! Je hebt mij

geholpen met potjes kuisen, je hebt het huishouden overgenomen, je hebt mij ’s nachts vergezeld naar het labo omdat ik niet graag alleen ging in het donker, je hebt mij eten gebracht en opgewacht als ik weer eens in het weekend moest werken en nog zo veel meer. Maar het allerbelangrijkste is misschien wel dat je er voor hebt gezorgd dat ik heb volgehouden, op je eigen manier maar het heeft dan toch gewerkt! We zijn twee rare samen (zoals vele mensen zeggen), waarschijnlijk omdat we alletwee onszelf kunnen zijn bij elkaar! Nen hele dikke merci voor alles! Speciaal voor mij zijn we blijven plakken in Leuven, maar nu beginnen we samen aan een ongetwijfeld spetterend nieuw hoofdstuk in Waregem!

Adinda

Introduction

Cryopreservation or preservation of biological tissue for the long term at ultra-low temperatures is gaining interest for several reasons. The technique is an important tool for the conservation of genetic resources using a minimum of space and maintenance and thereby provides an alternative for live breeding. Cryopreservation has many applications in biotechnology, biomedicine, agriculture, aquaculture and biodiversity conservation. The best-known medical application is probably the use of cryopreserved sperm and egg cells to overcome fertility issues. Another example is the use of cryopreserved bone marrow stem cells or blood cells to cure leukemia and melanomas (Mazur et al., 2008). In addition, the construction of cryobanks may be an important supporting tool to preserve the biodiversity of the planet, as an increased number of species are going extinct or are endangered. According to the red list of the International Union for Conservation of Nature (IUCN) 23.000 of the 76.000 species they have assessed are threatened with extinction, including 26% of all mammals, 13.5% of all birds and up to 40% of all flowering plants (IUCN 2014). The extinction of a species also means a loss of their putative applications in for instance food- or pharmaceutical industry. In order to prevent the loss of his potential, big projects have been developed over the past decades to store the remaining biodiversity. For example, Kew's Millennium Seed Bank stores almost 2 billion species of plant seed, which corresponds to 13% of the world's wild plant species. The Svalbard Global Seed Vault, established in 2008 and located on Spitsbergen, stores every variety of all of earth's 21 major food crops. Additionally, cryobanks would be very useful to maintain the genetic material of the enormous amount of mutant strains of model organisms, such as mice, fruit flies, *Daphnia* and zebrafish that have been created for scientific research. Unfortunately, for many of these animals an optimized protocol is not yet available (Mazur et al., 2008).

A well-established model organism in ecological and evolutionary biological research is the water flea *Daphnia* and especially *D. magna* as it is one of the most widely used model organism in ecotoxicology (Lampert, 2006; 2011; Walker et al., 2012; Walker, 2014). For example, in the US over 20.000 ecotoxicological studies of pesticide-related chemicals have been conducted on *Daphnia*, whereof almost 18.000 used *Daphnia magna* (PAN pesticides database). Publication of the first complete *Daphnia* genome by the 'Daphnia genome consortium' (http://daphnia.cgb.indiana.edu/; Colbourne et al., 2011) led to an even more intensive use of *Daphnia* as an eco-genetical model organism and an official recognition as model species for biomedical research (http://www.nih.gov/science/models; Tautz, 2011; Seda and Petrusek, 2011). *Daphnia* show a wide geographical distribution, they are easy to culture and handle and they have short generation times (Benzie, 2005; Lampert, 2006; Seda and Petrusek, 2011). Additionally, *Daphnia* are a keystone species in aquatic ecosystems due to their central link in the food web, as a grazer of phytoplankton and as preferred prey for macro-invertebrates and fishes (Carpenter et al. 1987; Lampert, 2006; Miner et al., 2012).

Another key asset of *Daphnia* that has been central to the wide use as a model organism is their parthenogenetic reproduction cycle, altering sexual and asexual reproduction (Hebert, 1978; Benzie, 2005; Figure 4). Asexual reproduction gives researchers the opportunity to work with large numbers of genetically identical individuals (De Meester et al., 2004). This allows analysis of the effects of multiple traits on the same set of clones and straightforward analysis of genotype x genotype and genotype x environment interactions and phenotypic plasticity (Tollrian and Leese, 2010; Simon et al., 2011). Dormant eggs form an archive in the sediment were they can stay viable for decades (Hairston et al., 1995; De Meester et al., 2004) and are used in resurrection ecology to reconstruct the evolutionary changes of the past (Kerfoot et al., 1999; Cousyn, 2001; Angeler, 2007; Decaestecker, 2007), while clones are the ideal tool to explore the capacity for evolution in real-time experimental approaches (Van Doorslaer et al., 2009; 2010; Jansen et al., 2015).

For many of the above mentioned applications, using genetically well characterized lines is important. To date, maintenance of the clonal lines can only be achieved through continuous culturing. This is a labor-intensive process and entails the risk of losing important lines because of contamination, disease or accidents. It would be a big step forward if these clonal lineages could be stored safely by cryopreservation, the long-term preservation of living organisms at ultra-low temperatures of liquid nitrogen (-196°C). Also for *Daphnia*, being an aquatic organism, so far there are no successful protocols for cryopreservation, such a protocol would be an enormous help as it would allow increase repeatability in time and space and reliability.

1 Cryopreservation

1.1 Introduction

In cryobiology three categories of cold temperatures are distinguished. Chilling temperatures above 0°C constitute the first category, which are already detrimental for many animals, especially homeotherms, and cold sensitive plants but will be survived by most organisms, depending on the length of exposure. The second category consists of temperatures between 0°C and -40°C. In this range cells may incur freeze damage and only few organisms are able to withstand these temperatures. At temperatures lower than -40°C cryopreservation can occur, because of a drastically decreased metabolic rate at these very low temperatures (Wolfe and Bryant, 2001). For long term storage it is preferable to store biological tissues at temperatures below the glass transition temperature of water (between -120°C and -130°C), since then all metabolic processes are arrested (Hodgson, 1994). If cells can reach these temperatures undamaged, samples can in principal be retained indefinitely as preservation of cells at such temperatures is not harmful per se. The biggest challenge for the cryopreservation of cells is, therefore, spanning the critical cooling process to achieve these temperatures without damage.

1.2 Cryopreservation protocols

Cryopreservation can be performed in different ways. Broadly protocols are divided in two groups: conventional or two-step methods (2.2.1) and vitrification protocols (2.2.2). To get familiar with some common terms used in cryobiology a glossary was added in Box I.

Box I: Definitions related to cryopreservation

Supercooling: a situation in which a liquid is cooled few degrees under its freezing point without a solid phase transition (Wolfe and Bryant, 1999)

Nucleation: the start of ice crystal formation and solid phase transition

Homogeneous nucleation: water molecule(s) as nucleus of the ice crystal

Heterogeneous nucleation: other molecule(s) as nucleus of the ice crystal (Wolfe and Bryant, 1999)

Glass transition: transition to a glass, this is no phase transition. A glass is a metastable amorphous state of a liquid characterized by an extreme high viscosity ($>10^{14}$ Pa.s) (Wolfe and Bryant, 1999).

Vitrification: the process of glass formation, achieved by adequate rapid cooling.

Droplet vitrification: In this technique, the tissue to be preserved is placed in in a drop of vitrification solution on aluminum foil strips prior to plunging in liquid nitrogen. The method is based on advantages high thermal conductivity of aluminum increasing the cooling and thawing rates (Kulus and Zalewska, 2014).

1.2.1 Conventional cryopreservation

Conventional protocols use a step-wise approach to freeze the samples, in which ice crystals are tolerated but strictly controlled via the cooling rate. First, cells are slowly cooled and kept at a constant subzero temperature for a while, allowing for freeze-dehydration to occur. Subsequently samples are rapidly cooled to -196°C by plunging them in liquid nitrogen. During the first step of slow cooling intracellular ice crystal formation is avoided by adequate osmotic dehydration, while in the second step of rapid cooling the damage caused by toxic concentrations of the internal solutes is avoided by minimizing the exposure time (Farrant et al., 1977).

One major factor determining whether cells will survive conventional cryopreservation is the cooling rate (Figure 1). Both too slow and too high cooling rates have adverse effects on the survival of cells,

so an optimal cooling rate has to be found that can be different for every cell type and every organism (Leibo and Mazur, 1971; Mazur, 1984).

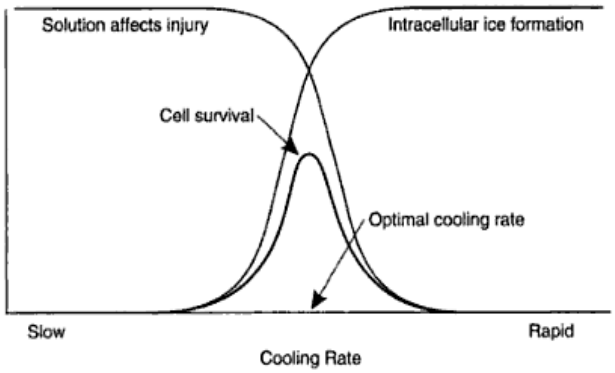

Figure 1: Inverted U-curve of cell survival at different cooling rates. At slow cooling rates cells are mainly affected by high concentrations of intracellular solutes and at rapid cooling rates intracellular ice formation is the most damaging factor (Muldrew et al., 2004).

Slow cooling can be defined as the cooling rate at which cells are able to lose just enough water so that the intracellular solution can stay in osmotic equilibrium with the extracellular medium. As temperatures decreases, ice crystals are initially formed in the extracellular solution which can have several negative effects on the survival of cells. First, extracellular ice crystals can mechanically damage important cell components, like the cell membrane, especially in multicellular tissues (Taylor and Pegg, 1983; Pegg, 1987). Second, due to the osmotic equilibrium across the membrane, cells become progressively dehydrated with an increasing concentration of intracellular solutes as a consequence. A higher viscosity of the cytoplasm, for example due to penetrating cryoprotectants (see Box II), can mitigate the damage caused by slow cooling, as osmotic equilibrium can be achieved while less dehydration of the cell is needed (Muldrew et al., 2004). Not only the toxic concentrations of solutes are a threat, but also the strong decrease in volume caused by the dehydration. This can lead to a phenomenon called ‘expansion-induced lyses’ in which cells can burst upon thawing because they lost a proportion of their plasma membrane via endocytosis during the freezing process (Muldrew et al., 2004). Apart from the above, there are some more problems caused by severe cell dehydration involving the cell membrane. As it is impossible for ice crystals to form in the interlamellar water, due to an intrinsic higher chemical potential compared to the extracellular matrix, this water is attracted to the extracellular ice crystals. However, this would force the membrane to compress and cause it to undergo a ‘gel-liquid crystal’ phase transition (Figure 2A). Implications of this transformation are a reduced membrane permeability and increased risk of leakage upon thawing. Moreover, low hydration forces the membrane components to segregate based on their hydration degree, leading to clearly divided groups of lipids and protein free membrane parts (Figure 2B), which undermines proper membrane functioning (Wolfe and Bryant, 1999; 2001).

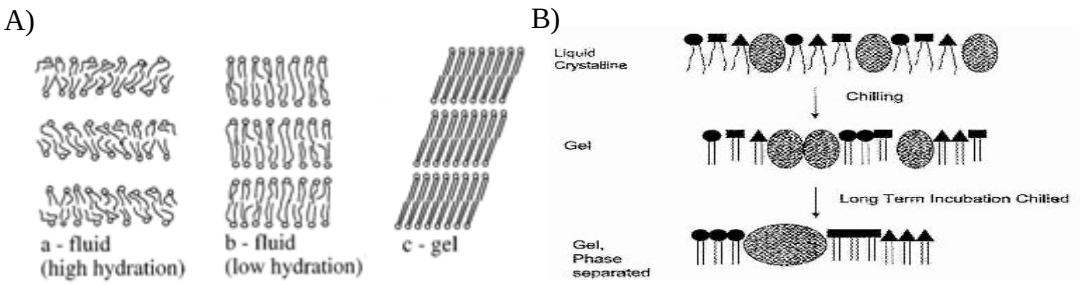

Figure 2: Effects of dehydration on the cell membrane. A) shows a 'gel-liquid crystal' phase transition and B) shows a phase separation. In figure 2B grey circles represent proteins and shapes of the phospholipid head groups depict hydration degree (Wolfe and Bryant, 1999; 2001)

Which of the responses occur and to what extent depends on the lipid composition of the membrane. Cold-acclimated organisms tend to have a higher degree of unsaturation in the fatty acids of their phospholipids to prevent the transition to a gel phase (Farkas et al., 1984; Pruitt, 1990; Schlechtriem et al., 2006).

As is shown in figure 1 also fast cooling can have harmful effects on the survival of cells undergoing cryopreservation. At rapid cooling rates cells are not able to maintain osmotic equilibrium across the membrane because the rate at which ice crystals are formed is higher than the rate at which a cell can lose water. Consequently, more supercooled intracellular water has the opportunity to form damaging intracellular ice crystals (Mazur, 2004; Muldrew et al., 2004)

1.2.2 Vitrification protocols

Vitrification protocols are aiming at ice-free cryopreservation of biological tissues by the formation of a glass. This can be achieved by a fast cooling rate and high viscosity (Fahy et al., 1984). Viscosity increases as cooling progresses, while the vitrification temperature (T_g) raises and the temperature at which homogeneous nucleation (T_h) occurs decreases (Figure 3). At a certain concentration (Cv) the formation of ice crystals in impossible and a glass is formed (Fahy et al., 1984; 2004). Thermodynamically a glass is a liquid, but as molecular motions are inhibited by the high viscosity (> 10^{14} Pa.s), it has properties of a solid without undergoing a phase transitions (MacFarlane, 1987; Wolfe and Bryant, 1999). Both the temperature of vitrification and homogeneous nucleation are dependent on the cooling rate. The graph in figure 3 is made for the minimal cooling rate needed for vitrification (10°C min^{-1}). Cooling rates applied in vitrification protocols are usually much higher, for example a sample that is plunged directly into liquid nitrogen (-196°C) is cooled at 2500°C min^{-1} (Towill and Bonnart, 2003). At these extreme fast cooling rates, vitrification (i.e. the crossing between T_g and T_h), occurs at much lower concentrations of the solutes. This is important because in practice it is very hard to reach high concentrations of intracellular solutes without damaging the cells (See Box

II; Fuller, 2004). These high and potentially toxic concentrations of solutes are the biggest challenge to achieve successful vitrification of cells.

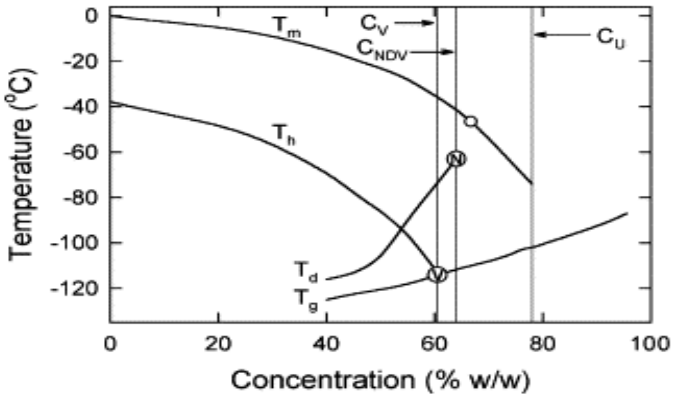

Figure 3: Phase diagram of glycerol in water. T_m is the melting temperature, T_h is the temperature of homogeneous nucleation, T_d is the devitrification temperature and T_g is the vitrification temperature. C_v is the minimum concentration needed for vitrification, C_{NDV} is the concentration where devitrification no longer takes place, C_u is the maximum concentration that can achieved during freezing, N is the point where T_d vanishes and V is the point where vitrification starts (Fahy et al. 2004).

The vitrification approach has many advantages relative to the conventional freezing protocols. First of all, vitrification avoids the damaging effects of intracellular and extracellular ice crystals. Secondly, as cooling happens faster cells are exposed to the high concentrations of solutes for shorter periods of time. Third, there is no need for an optimum cooling and warming temperature in vitrification protocols. In order for vitrification to take place cooling only has to be fast enough to prevent crystallization. In contrast to the controlled freezing of conventional protocols, this can be achieved without specialized freezing equipment, as plunging into liquid nitrogen suffices. For these reason it is believed that vitrification is the most suitable cryopreservation technique for multicellular tissues, like embryos (Rall, 1987; Kulus and Zalewska, 2014).

Generally vitrification protocols consist of multiple consecutive steps divided in two main parts. The first part of the protocol aims to concentrate the cellular solutes and the second part involves the freezing and thawing (Kulus and Zalewska, 2014). In principal there are two ways to increase the cellular viscosity: water can be withdrawn from the cells or additives can be added to the cellular solution. Dehydration can be achieved by air drying or osmotically, via the exposure to a vitrification solution consisting of non-penetrating cryoprotectant substances (See Box II). In addition, cells can be loaded with extra solutes by exposure to penetrating cryoprotectant substances entering the cell trough the cell membrane (See Box II) or by an adaptive metabolism. Changing the culturing conditions, for instance temperature or food, can lead to higher incorporations of certain cryoprotective substance.

1.3 Cryopreservation of aquatic organisms: an additional challenge

Cryopreservation of aquatic species is particularly challenging because of their high water content, high chilling sensitivity (See Box III for *Daphnia*; Zhang, 2004; Zhang and Rawson, 1995) and high sensitivity towards toxic components (Coors and De Meester, 2008; Coors et al., 2009). Although many successful cryopreservation protocols of reproductive tissue of aquatic invertebrates are published (Table 1), only limited numbers of them deal with the preservation of embryos or whole individuals (Gwo, 2000, Zhang, 2004). Although many attempts, also for fish embryos there are no successful cryopreservation protocols yet (Zhang, 2004; Mauger et al., 2006; Tsai et al., 2009; Neves et al., 2014).

Table 1: Overview of successful cryopreservation protocols for reproductive materials of aquatic species

Species	**Reproductive material**	**References**
Fish		Reviewed in:
200 species including:	spermatozoa	Zhang, 2004;
Salmonids		Tiersch and Mazik, 2000;
Cyprenids		Suquet, 2000
Perciformes		Suquet, 2000
Echinoderms		
Sea urchin	spermatozoa	Dunn and Mclachlan, 1973
Sea urchin	embryo	Bellas and Paredes, 2011
San dollar	spermatozoa	Dunn and Mclachlan, 1973
Molluscs		
Pacific oyster	spermatozoa	Adams, 2004
Pacific oyster	embryo	Chao et al., 1997; Paredes et al., 2013
Blue mussel	embryo	Paredes et al., 2013
Greenshell mussel	embryo	Paredes et al., 2012
Crustacea		
White shrimp	spermatozoa	Castelo-Branco et al., 2015
Marine shrimp	spermatozoa	Diwan and Joseph, 2000
Mud crab	spermatozoa	Bhavanishankar and Subramoniam, 1997
Rotifer	embryo	Toledo and Kurokura, 1990

Box II: Cryoprotectants

A cryoprotectant is defined as a chemical substance enhancing the survival of cells to freezing and thawing. They are added to cells before the process of cryopreservation is applied (Fuller, 2004; Muldrew et al., 2004). Cryoprotectants have the capacity to reduce ice crystal formation and ice crystal growth for two reasons. The first reason is that they simply do not fit in the tight structure of an ice crystal and therefor physically disturb ice crystal formation. Secondly, they increase the viscosity of the solution and thereby decrease the mobility of water molecules and slow down the process of ice crystal formation (Wolfe and Bryant, 1999).

Cryoprotectants can be divided in two classes: penetrating and non-penetrating cryoprotectants. Penetrating cryoprotectants are small molecules that are able to penetrate through the cell membrane with low cellular toxicity. Apart from the above mentioned general functions of cryoprotectants, some of these penetrating substances have the ability to interact with and stabilize macromolecules, such as proteins and phospholipids. Additionally, penetrating cryoprotectants can have a mild anti-bacterial effect and they can act as a radical scavenger and secondary solvent for salts, mitigating toxic effects caused by the increasing concentration during cooling (Fuller, 2004; Muldrew et al., 2004). Examples of penetrating cryoprotectants are glycerol, dimethyl sulfoxide, methanol, propylene glycol and ethylene glycol (Denniston et al., 2000). Non-penetrating cryoprotectants are unable to enter the cell through the plasma membrane, accordingly fulfilling their function outside the cell. Because of their high osmotic coefficient they are used to dehydrate cells before freezing, by osmotically withdrawing intracellular water (Fuller, 2004; Muldrew et al., 2004). Sugars, sugar alcohols and polymers belong to the category of non-penetrating cryoprotectants.

Cryoprotectants have to be used carefully, especially when applied at high concentrations. Both non-penetrating and penetrating cryoprotectants can cause lethal cell volume changes due to osmotic dehydration. Penetration of cryoprotectants into the cell is a time consuming process, too slow in order to maintain osmotic equilibrium across the membrane. Consequently, during freezing water is extracted from the cells and during thawing water is penetrating in the cells faster than cryoprotectants leave the cells. This can be eliminated by stepwise administration of the cryoprotectants (Fuller, 2004).

Apart from this osmotic toxicity, depending on its chemical characteristics cryoprotectants can also be chemically toxic depending on the temperature and exposure time. To overcome this problem mixtures are used, combining the beneficial effects of the different cryoprotectants and reducing the negative effects linked to the high concentrations of individual cryoprotectants needed for successful cryopreservation (Fuller, 2004; Muldrew et al., 2004). There are even some synergistic combinations, such as sugars and ethylene glycol. Sugars lower the vitrification temperature of ethylene glycol-based vitrification solutions (Kuleshova et al., 1999). Additionally, sugars, and some other components, have the advantages that they are cellular metabolites naturally synthesized by many organisms when exposed to stress, making them less toxic at high concentrations (Kuleshova et al., 1999; Fuller, 2004).

2 *Daphnia* reproduction cycle: dormant and subitaneous eggs

The life cycle of many Cladocera (Figure 4) is characterized by an alternation between sexual and asexual reproduction, called cyclical parthenogenesis (Bell, 1982; Hebert, 1987).

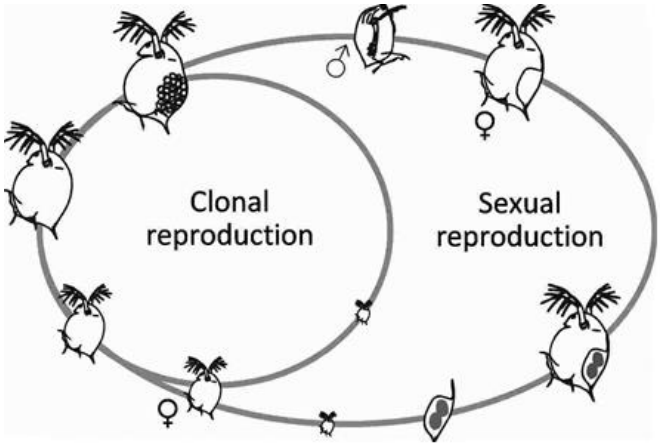

Figure 4: Schematic overview of the cyclic parthenogenetic reproduction of *Daphnia* as an example of a cladoceran life cycle. (Drawing by Kathleen Van der Gucht)

During favorable conditions, water fleas reproduce asexually. Asexual eggs develop immediately within a short period of time (3-4 days for *D. magna* at 20°C), accounting for a fast population growth during the growing season. At the end of the growing season females start to produce males and sexual eggs, although there are some obligate parthenogenetic populations (Hebert and Crease, 1983; Hebert et al., 1988; Colbourne et al., 1997). The switch to sexual reproduction is determined by environmental cues (reviewed in Alekseev and Starobogatov, 1996; Gyllström and Hansson, 2004). Indications of deteriorating conditions triggering sexual reproduction of *D. magna* are a shorter photoperiod, lower temperatures, crowding, decreasing food level and the presence of predation

(Hobaek and Larsson, 1990; Kleiven et al., 1992; Alekseev and Lampert, 2001). Most cladoceran species deposit their sexual eggs in a protective envelope, called ephippium (Figure 5), although there are also some species that shed their eggs freely (Zaffagnini, 1987; Fryer, 1996,). The ephippium contains one or two eggs and is formed by a part of the maternal carapace (chitine) with divergent shapes for different species. Pigmentation with melanin provides a brown color (Zaffagnini, 1987; Fryer, 1996; Hairston and Càceres, 1996).

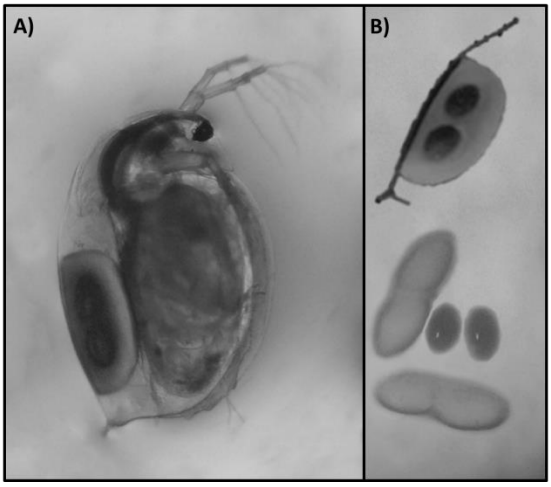

Figure 5: A) Female *Daphnia magna* bearing ephippium and B) Dissected ephippium of *D. magna*: outer protective envelope, inner protective envelope and two dormant eggs.

In contrast to asexual eggs, sexual eggs undergo a period of dormancy before they hatch in spring (Carvalho and Wolf, 1989; Càceres, 1998). Dormancy encompasses a wide spectrum of physiological states characterized by a strong reduction of metabolism (Brendonck and De Meester, 2003). Dormant eggs form a sediment egg bank analogous to plant seed banks (De Stasio, 1989; Hairston and Càceres, 1996). The densities of these egg banks vary between 10^3 and 10^6 eggs m^{-2} (Hairston, 1996; Cousyn and De Meester, 1998; Brendonck and De Meester, 2003) and they can have a major impact on ecological and evolutionary dynamics (Càceres, 1997; Brendonck and de Meester, 2003; Gyllström and Hansson, 2004). Due to their ability to survive harsh environmental conditions and digestion by fish and/or birds dormant eggs are the ideal vector for dispersal in space and time (Proctor, 1964; Mellors, 1975; Hairston et al., 1995; Radzikowski, 2013). In contrast, earlier studies reported no survival of subitaneous eggs digested by fish (Saint-Jean and Pagano, 1995; Bartholmeé et al., 2005). Additionally, results from exploratory experiments performed by myself showed low drought and freeze resistance of subitaneous eggs compared to dormant eggs (See Box III).

Box III: Resistance of dormant and subitaneous *Daphna* eggs to drought and freezing

To our knowledge there are only very limited quantitative data available on the limits of drought and freeze tolerance of dormant and subitaneous eggs of *Daphnia*. Radzikowski (2013) reports that dry dormant eggs are able to withstand -84°C for 3 hours and Doma (1979) reports that dormant eggs can survive 24 hours of air-drying. Therefore, we compared the difference in dehydration and freezing tolerance of sexual and asexual eggs.

Material and methods

EGG COLLECTION

Sexual eggs of *D. magna* were collected from 'Langerode vijver', a pond in Neerijse, Belgium (0°49'42.32"N, 4°38'21.49"O). Individual eggs were placed on a thincert™ (Ref 665 638, Greiner Bio-one, Germany) and fifty replicate eggs were subjected to each of the different treatments (see below).

Asexual eggs were collected from 10 clones that were hatched from the sediment bank of 'Langerode vijver' by exposing the dormant eggs to hatching stimuli (De Meester & Jager, 1993) and subsequently they were cultured under standard laboratory conditions (20°C and 16h light/8h dark cyclus) for several generations. All cultures were fed 150.000 cells ml^{-1} of the green alga *Scenedesmus obliquus*, which corresponds to approximately 2.5 mg C l^{-1}. Fifty replicate individual asexual eggs in the first stage of embryonic development (according to Kast-Hutcheson et al. 2001) were placed on thincerts™ (Ref 665 638, Greiner Bio-one, Germany) and exposed to the experimental conditions (see below). Clones and clutches were randomized over the treatments.

EXPERIMENTAL METHODS

The resistance to air-drying of sexual and asexual eggs was compared by exposing eggs to the air for different periods of time: 0 min (control), 2 min, 5 min, 10 min, 30 min, 12 h, 24 h. The resistance of sexual and asexual eggs towards freezing was tested with and without prior air-drying for 24 h. The asexual eggs because drying resulted in damage and disappearance of the eggs, making it impossible to weigh them after air-drying.

DATA ANALYSIS

The effects of the different treatments on the hatching success of the eggs were tested using generalized linear models (GLM) with a logit-link function and a binomial distribution, followed by sequential Bonferroni-correction (Holm 1979) to correct for multiple testing. To test whether differences between clones have an effect on the survival of the eggs when they were exposed to air-

drying, we used a generalized linear mixed model. All analyses were performed using the packages car, lme4 and multcomp in the statistical software R (version 3.0.2).

Results

There is a significant difference between the desiccation resistance of sexual and asexual eggs (Table 1). Sexual eggs can withstand air-drying for 24 hours without significant loss of survival, while for asexual eggs survival at all drying times from 5 minutes onward is significantly lower than in the control conditions (Figure 1). Asexual eggs were derived from 10 different clones, but this diverse genetic background does not affect survival of the eggs during the experiment ($p = 0.3577$).

Table 1: Results of GLM comparing the survival of sexual and asexual eggs of *Daphnia magna* (egg type) exposed to air-drying for different periods of time (drying time).

Variables	Df	Chi^2	P-value
Egg type	1	433.83	**$<2.2\ E^{-16}$**
Drying time	7	66.71	**$6.8\ E^{-12}$**
Egg type : drying time	7	43.15	**$3.121\ E^{-07}$**

There were no asexual eggs surviving one of the freezing treatments, while sexual eggs were capable of surviving -20°C without significant loss of survival and almost 20% even survived -196°C. During the process of air-drying the eggs lose water. Twenty sexual eggs weigh at the start of the experiment on average 0.32 mg and lose 0.20 mg during 24 hours of air-drying, corresponding to 61.75% of the egg weight that evaporates. This loss of water has a beneficial effect on the survival of sexual eggs exposed to severe freezing conditions, such as -80°C and -196°C (Figure 2, Table 2).

Table 2: Results of GLM of the effects of freezing conditions (freezing temperature), a priori drying (pre drying) and their interaction on the hatching success of sexual eggs of *Daphnia magna.*

Variables	Df	Chi^2	P-value
Freezing temperature	3	46.607	**$4.123\ E^{-10}$**
Pre drying	1	21.889	**$2.889\ E^{-06}$**
Freezing t°: pre drying	3	31.666	**$6.154\ E^{-07}$**

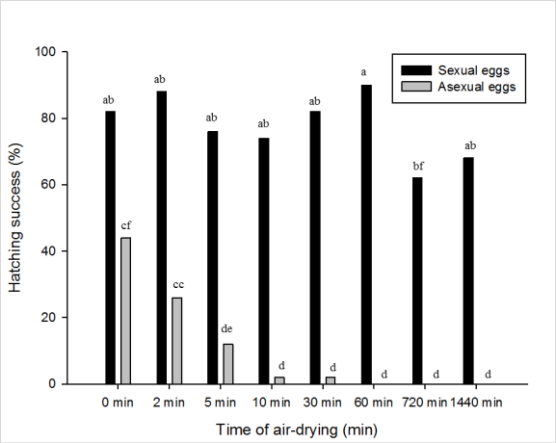

Figure 1: Effect of time of exposure to air-drying on the survival of sexual (black bars) and asexual eggs (grey bars) of *Daphnia magna.* Distinct letters indicate significant differences (n = 121, $p < 0.05$, generalized linear model, followed by sequential Bonferroni-correction).

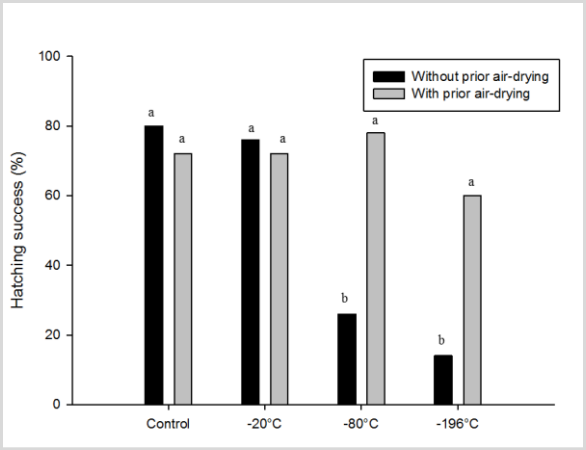

Figure 2: Effect of exposure to freezing temperatures with (grey bars) or without (black bars) prior 24 h air-drying on the survival of sexual eggs of *Daphnia magna.* Distinct letters indicate significant differences (n = 28, $p < 0.05$, generalized linear model, followed by sequential Bonferroni-correction).

Discussion

Our results confirm the expected differences between sexual and asexual eggs of *Daphnia magna* regarding the adaptive resistance towards drought and freezing required during diapause. Sexual eggs are used as a means of dispersal in time and space, so they often encounter stressful environments and must be able to survive for long periods of time (Hairston et al., 1995; Brendonck & De Meester, 2003). Our results showed a high resistance of sexual eggs, they can withstand dehydration via air-drying for 24h and severe freezing temperatures as low as -196°C. Asexual eggs, which are typically produced during favorable conditions, only survive air-drying for 2 minutes and do not survive any freezing conditions.

Survival of the sexual eggs under freezing conditions that *Daphnia* encounter in their natural habitat (until -20°C) is not affected by the water content of the eggs, but survival at lower temperatures (-80°C and -196°C) rises substantially as water content decreases. A lower amount of water and a higher viscosity reduce the formation of damaging intracellular ice crystals (Bryant and Wolfe, 1999; Mazur, 2004;).

2.1 Adaptation to dormancy

The long term survival of dormant stages due to a high level of stress resistance requires some adaptations. Diapause is characterized by a low metabolic rate (Hahn and Denlinger, 2007; Storey and Storey, 2007) and energy used for cellular metabolism in active states can now be redirected to other processes, such as the production of stress protectant cellular metabolites (Denlinger, 2002; Lopes et al., 2004; Tunnacliffe and Wise, 2007; Tunnacliffe et al., 2010) and the production of morphological protective structures (Liu et al., 2009). We identified adaptation at the level of protective envelopes, yolk structure and biochemical composition.

2.1.1 The epphippial case and protective membrane structures

For many cladocerans, the most obvious difference between subitaneous and dormant eggs is the fact that dormant eggs are deposited in a protective envelope, the ephippium, while subitaneous eggs are shed freely or in the brood pouch of the female (Zaffagnini, 1987; Fryer, 1996). Additional to the ephippium (Figure 5), dormant eggs are additionally individually covered by an outer envelope (Figure 6). Subitaneous eggs are surrounded by an outer wall of only 0.35 µm thick and a plasma membrane, whereas dormant eggs have an outer wall of 2.2 µm thick, comprising of 2 layers, and a plasma membrane (Seidman and Larsen, 1979; Zaffagnini, 1987). Both the ephippium and the outer envelope protect dormant eggs against mechanical damage and abiotic factors like drying, freezing, UV radiation or chemicals (Seidman and Larsen, 1979; Kawasaki et al., 2004).

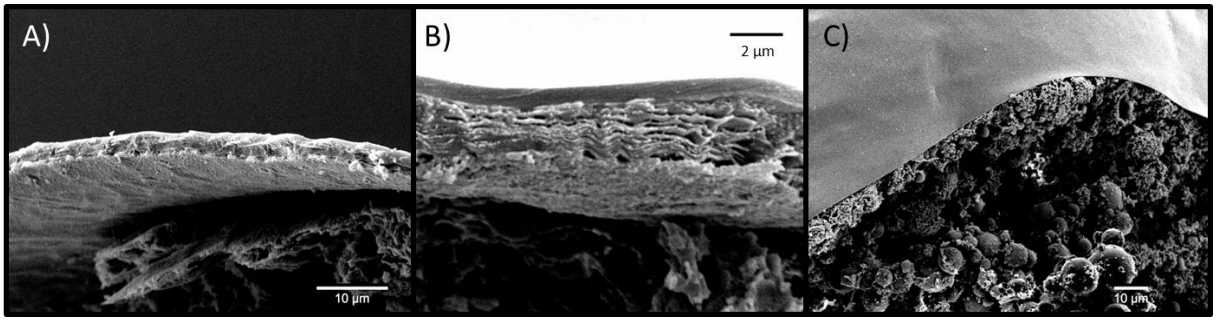

Figure 6: Scanning electron microscopy of dormant egg envelope (A and B) and subitaneous egg envelope (C).

2.1.2 Yolk structure

These envelopes slow down the process of desiccation, but cannot prevent it. Consequently, dormant eggs themselves need additional mechanism to cope with drought and freezing. A first mechanism to survive these conditions is probably the structure of the egg yolk. Resistant eggs tend to have a more homogeneous finely grained yolk structure without vacuoles, while non-resistant eggs have vacuoles

and the structure of the yolk is irregular with more pronounced granulation (Makrushin, 1978). Zaffagnini (1987) observed similar properties in the eggs of *Daphnia*. The cytoplasm of asexual eggs contains yolk globules, while the cytoplasm of sexual eggs is characterized by pronounced granulation.

2.1.3 Biochemical composition

Dormant stages accumulate stress protectant substance, of which part of the current knowledge is summarized in Table 2.

Table 2: Cryoprotective components found in dormant stages of several invertebrates

Biochemical component	**Species**	**References**
Lipids		
PUFA	*Daphnia pulicaria*	Abrusan et al., 2007
Sugars and sugar alcohols		
Trehalose	*Anguina tritici*	Womersley and Smith, 1981
	Artemia franciscana	Clegg, 1965
	Bombyx mori	Ikeda et al., 1993
	Cristatella mucedo	Hengherr and Schill, 2011
	Ditylenchus dipsaci	Womersley and Smith, 1981
	Daphnia magna	Hengherr et al., 2011
	Daphnia pulex	Hengherr et al., 2011
	Polypedilum vanderplanki	Kikawada et al., 2005; Watanabe et al., 2002
	Triops longicaudatus	Hengherr et al., 2011
	Triops cancriformis	Hengherr et al., 2011
	Triops australiensis	Hengherr et al., 2011
	Steinermena feltiae	Solomon et al., 2000
Glycerol	*Artemia franciscana*	Clegg et al., 1997
	Bombyx mori	Denlinger, 2002
	Daphnia magna	Pauwels et al. 2007b
	Pangrellus redivivus	Womersley and Smith, 1981
	Turbatrix aceti	Womersley and Smith, 1981
Proteins		
Hsp 60	*Daphnia magna*	Pauwels et al. 2007b
Hsp 26, 70, Artemin	*Artemia franciscana*	Clegg and Campagna, 2006
	Paratemia	Clegg and Campagna, 2006
LEA proteins	Review	Tunnacliffe et al., 2010
	Adineta ricciae	Pouchnkina-Stantcheva et al., 2007
	Artemia franciscana	Watts et al., 1994
	Aphelenchus avenae	Browne et al., 2000

3 Objectives and outline thesis

There are many applications using specific clonal lines of *Daphnia*, so it would be good if these clonal lines can be safely stored and repeatedly used with minimal effort in terms of maintenance. Therefor the main goal of this thesis was to develop a cryopreservation protocol for asexual eggs of *Daphnia magna*, the most widely used *Daphnia* species. Aquatic organisms tend to be very sensitive to cold temperatures due their high water content. The advantage of working with *Daphnia* for this research is that they can alter between sexual and asexual reproduction, meaning that they besides the production of sensitive asexual eggs are also able to produce drought and freezing resistant sexual eggs. Their resistance to extreme freezing temperatures up to -196°C (See Box III) makes them suitable for techniques like cryopreservation, but as dormant eggs are already able to stay viable for decades it is not necessary to develop special protocols to preserve them. However, the features used by dormant eggs to cope with drought and low temperatures can provide valuable information for the development of a cryopreservation protocol for the asexual eggs.

To achieve this we first wanted to increase our knowledge on the physiology of sexual eggs compared to asexual eggs. Second, we tried to establish a suitable protocol for cryopreservation and third we combine both and test the capacity of manipulated asexual eggs to survive cryopreservation (Figure 7).

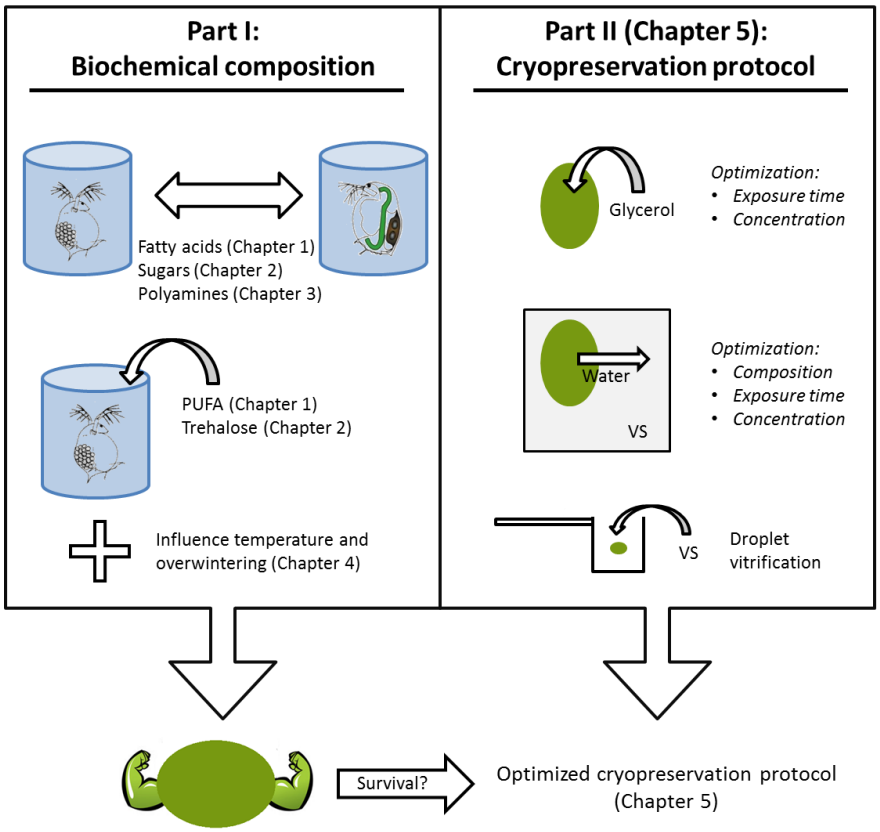

Figure 7: Overview of the experiments conducted in this study distributed over two parts and five chapters.

In the first part of this thesis we study the biochemical composition of asexual eggs and decapsulated (no ephippium, see Figure 5) sexual eggs of *D. magna*. We compare the composition of fatty acids, sugars and polyamines between sexual and asexual eggs (Chapter 1-3). Additionally, we look at the plasticity of these traits under different food conditions in an attempt to alter the biochemical composition of asexual eggs towards a higher content of putative cryoprotectant substances and consequently a higher stress resistance (Chapter 1-3). Apart from the differences between the two types of eggs, *Daphnia* populations also show a big genetic variation resulting in variable egg characteristics (De Meester et al., 2004; Pauwels et al., 2007). In chapter 4 we study the naturally occurring differences in biochemical composition of parthenogenetic eggs and life history characteristics of their hatchlings between genetic lines of different seasons (winter vs. summer conditions) and their responses to temperature changes.

The second part of the thesis deals with the development of the cryopreservation protocol (Chapter 5). Because of the multicellular nature of asexual eggs, we have chosen to use a vitrification protocol as it is currently considered the most suitable method for freezing of embryos (Kulus and Zalewska, 2014; Fahy and Wowk, 2015). More specifically we will use droplet-vitrification, in this method the eggs are placed in a drop of vitrification solution on an aluminum "spoon" to be plunged in liquid nitrogen (Kulus and Zalewska, 2014). Prior to the freezing step, the viscosity in the eggs had to be maximized. We tried to attain this via loading with a penetrating cryoprotectant, glycerol, and osmotic dehydration in a vitrifying solution. Both steps needed optimization in terms of exposure time and concentration in order to avoid toxic effects and mortality before freezing occurs. Finally, the 'enhanced' more stress resistant asexual eggs, in terms of fatty acid and sugar composition, derived from the experiments in part one are subjected to the vitrification protocol.

References

Abrusan, G., Fink, P. and Lampert, W. (2007) Biochemical limitation of resting egg production in *Daphnia*. *Limnology and oceanography,* **52,** 1724-1728.

Adams, S. L., Smith, J. F., Roberts, R. D., Janke, A. R., Kaspar, H. F., Robin Tervit, H., Anne Pugh, P., Webb, S. C. and King, N. G. (2004) Cryopreservation of sperm of the Pacific oyster (Crassostrea gigas): development of a practical method for commercial spat production. *Aquaculture,* **242,** 271-282.

Alekseev, V. and Lampert, W. (2001) Maternal control of resting-egg production in *Daphnia*. *Nature,* **414,** 899-901.

Alekseev, V. and Starobogatov, Y. (1996) Types of diapause in Crustacea: definitions, distribution, evolution. *Hydrobiologia,* **320,** 15-26.

Angeler, D. G. (2007) Resurrection ecology and global climate change research in freshwater ecosystems. *Journal of the North American Benthological Society,* **26,** 12-22.

Bartholmeé, S., Samchyshyna, L., Santer, B. and Lampert, W. (2005) Subitaneous eggs of freshwater copepods pass through fish guts: Survival, hatchability, and potential ecological implications. *Limnology and oceanography,* **50,** 923-929.

Bell, G. (1982) *The masterpiece of nature: the evolution and genetics of sexuality.*, CUP Archive.

Bellas, J. and Paredes, E. (2011) Advances in the cryopreservation of sea-urchin embryos: Potential application in marine water quality assessment. *Cryobiology,* **62,** 174-180.

Benzie, J. A. (2005) The genus Daphnia (including Daphniopsis)(Anomopoda: Daphniidae). In: H. J. F. Dumont (ed) *Guide to the identification of the microinvertebrates of the continental waters of the world*. Ghent: Kenobi productions and Leiden: Backhuys, pp 376

Bhavanishankar, S. and Subramoniam, T. (1997) Cryopreservation of spermatozoa of the edible mud crab Scylla serrata (Forskal). *Journal of Experimental Zoology,* **277,** 326-336.

Brendonck, L. and De Meester, L. (2003) Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia,* **491,** 65-84.

Browne, J., Tunnacliffe, A. and Burnell, A. (2002) Anhydrobiosis: Plant desiccation gene found in a nematode. *Nature,* **416,** 38-38.

Cáceres, C. E. (1997) Temporal variation, dormancy, and coexistence: A field test of the storage effect. *Proceedings of the National Academy of Sciences,* **94,** 9171-9175.

Cáceres, C. E. (1998) Interspecific Variation in the Abundance, Production, and Emergence of *Daphnia* Diapausing Eggs. *Ecology,* **79,** 1699-1710.

Carpenter, S. R., Kitchell, J. F., Hodgson , J. R., Cochran, P. A., Elser, J. J., Elser, M. M., Lodge, D. M., Kretchmer, D. and He, X. (1987) Regulation of lake primary production by food web structure. *Ecology,* **68,** 1863-1876.

Carvalho, G. R. and Wolf, H. G. (1989) Resting eggs of lake- Daphnia I. Distribution, abundance and hatching of eggs collected from various depths in lake sediments. *Freshwater biology,* **22,** 459-470.

Castelo-Branco, T., Batista, A. M., Guerra, M. M. P., Soares, R. and Peixoto, S. (2015) Sperm vitrification in the white shrimp Litopenaeus vannamei. *Aquaculture,* **436,** 110-113.

Chao, N. H., Lin B, T. T., Chen A, Y. J., Hsu A, H. W. and Liao, I. C. (1997) Cryopreservation of late embryos and early larvae in the oyster and hard clam. *Auquculture,* **155,** 31-44.

Clegg, J. S. (1965) The origin of threhalose and its significance during the formation of encysted dormant embryos of Artmia salina. *Comparative Biochemistry and Physiology,* **14,** 135-143.

Clegg, J. S. (1997) Embryos of *Artemia fansciscana* survive four years of continous anoxia: the case for complete metabolic rate depression. *The Journal of Experimental Biology,* **200,** 467-475.

Clegg, J. S. and Campagna, V. (2006) Comparisons of stress proteins and soluble carbohydrate in encysted embryos of Artemia franciscana and two species of Parartemia. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **145,** 119-125.

Colbourne, J., Pfender, M., Gilbert, D., Thomas, W., Tucker, A., Oakley, T., Tokishita, S., Aerts, A., Arnold, G., Basu, M., Bauer, D., Caceres, C., Carmel, L., Casola, C., Choi, J., Detter, J., Dong, Q., Dusheyko, S., Eads, B., Frohlich, T., Geiler-Samerotte, K., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E., Kultz, D., Laforsch, C., Lindquist, E. and Lopez, J. (2011) The ecoresponsive genome of Daphnia pulex. *Science,* **331,** 555 - 561.

Colbourne, J. K., Hebert, P. D. and Taylor, D. J. (1997) Evolutionary origins of phenotypic diversity in Daphnia. In: T. J. Givnish and K. J. Sytsma (eds) *Molecular evolution and adaptive radiation.* Cambridge university press, pp. 163-188.

Coors, A. and De Meester, L. (2008) Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in Daphnia magna. *Journal of Applied Ecology,* **45,** 1820-1828.

Coors, A., Vanoverbeke, J., De Bie, T. and De Meester, L. (2009) Land use, genetic diversity and toxicant tolerance in natural populations of Daphnia magna. *Aquatic Toxicology,* **95,** 71-79.

Cousyn, C. and De Meester, L. (1998) The vertical profile of resting egg banks in natural populations of the pond-dwelling cladoceran Daphnia magna Straus. *Archiv für Hydrobiologie,* **52,** 127-139.

Cousyn, C., De Meester, L., Colbourne, J. K., Brendonck, L., Verschuren, D. and Volckaert, F. (2001) Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. *Proceedings of the National Academy of Sciences,* **98,** 6256-6260.

De Meester, L., Gomez, A. and Simon, J. (2004) Evolutionary and ecological genetics of cyclical parthenogens. In: A. Moya and E. Font (eds) *Evolution from molecules to ecosystems.* Oxford university press, pp. 122-134.

De Meester, L. and Jager, H. (1993) Hatching of *Daphnia* sexual eggs. I. Intraspecific differences in the hatching responses of D. magna eggs. *Freshwater biology,* **30,** 219-226.

De Stasio, B. T. (1989) The seed bank of a freshwater crustacean: copepodology for the plant ecologist. *Ecology,* **70,** 1377-1389.

Decaestecker, E., Gaba, S., Raeymaekers, J. a. M., Stoks, R., Van Kerckhoven, L., Ebert, D. and De Meester, L. (2007) Host-parasite `Red Queen' dynamics archived in pond sediment. *Nature,* **450,** 870-873.

Denlinger, D. L. (2002) Regulation of diapause. *Annual Review of Entomology,* **47,** 93-122.

Denniston, R. S., Michelet, S., Bondioli, K. R. and Godke, R. A. (2000) Principles of embryo cryopreservation. In: T. R. Tiersch and C. C. Green (eds) *Cryopreservation in aquatic species.* Vol. 7. World aquaculture society, pp. 274-290.

Diwan, A. and Joseph, S. (1998) Cryopreservation of spermatophores of the marine shrimp Penaeus indicus H. Milne Edwards. *Indian Journal of Fisheries,* **46,** 159-166.

Doma, S. (1979) Ephippia of *Daphnia magna* Straus: A technique for their mass production and quick rivival. *Hydrobiologia,* **67,** 183-188.

Dunn, R. S. and Mclachlan, J. (1973) Cryopreservation of echinoderm sperm. *Canadian journal of zoology,* **51,** 666-669.

Fahy, G. and Wowk, B. (2015) Principles of Cryopreservation by Vitrification. In: W. F. Wolkers and H. Oldenhof (eds) *Cryopreservation and Freeze-Drying Protocols.* Vol. 1257. Springer New York, pp. 21-82.

Fahy, G. M., Macfarlane, D. R., Angeli, C. A. and Meryman, H. T. (1984) Vitrification as an Approach to Cryopreservation'. *Cryobiology,* **21,** 407-426.

Fahy, G. M., Wowk, B., Wu, J. and Paynter, S. (2004) Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology,* **48,** 22-35.

Farrant, J., Walter, C. A., Lee, H. and Mcgann, L. E. (1977) Use of two-step cooling procedures to examine factors influencing cell survival following freezing and thawing. *Cryobiology,* **14,** 273-286.

Fryer, G. (1996) Diapause, a potent force in the evolution of freshwater crustaceans. *Hydrobiologia,* **320,** 1-14.

Fuller, B. J. (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryoletters,* **25,** 375-388.

Gwo, J. C. (2000) Cryopreservation of aquatic invertebrate semen: a review. *Aquaculture Research,* **31,** 259-271.

Gyllström, M. and Hansson, L.-A. (2004) Dormancy in freshwater zooplankton: Induction, termination and the importance of benthic-pelagic coupling. *Aquatic Sciences,* **66,** 274-295.

Hahn, D. A. and Denlinger, D. L. (2007) Meeting the energetic demands of insect diapause: Nutrient storage and utilization. *Journal of Insect Physiology,* **53,** 760-773.

Hairston, N., Jr. and Cáceres, C. (1996) Distribution of crustacean diapause: micro- and macroevolutionary pattern and process. *Hydrobiologia,* **320,** 27-44.

Hairston, N. G. (1996) Zooplankton egg banks as biotic reservoirs in changing environments. *Limnology and oceanography,* **41,** 1087-1092.

Hairston, N. G., Van Brunt, R. A., Kearns, C. M. and Engstrom, D. R. (1995) Age and Survivorship of Diapausing Eggs in a Sediment Egg Bank. *Ecology,* **76,** 1706-1711.

Hebert, P. D., Ward, R. D. and Weider, L. J. (1988) Clonal-diversity patterns and breeding-system variation in Daphnia pulex, asexual-sexual complex. *Evolution,* **42,** 147-159.

Hebert, P. D. N. (1978) The population biology of *Daphnia* (Crustacea, Daphnidae). *Biological Reviews,* **53,** 387-426.

Hebert, P. D. N. (1987) Genotypic characteristics of cyclic parthenogens and their obligately asexual derivatives. In: S. Stearns (ed) *The Evolution of Sex and its Consequences.* Vol. 55. Birkhäuser Basel, pp. 175-195.

Hebert, P. D. N. and Crease, T. (1983) Clonal diversity in populations of Daphnia pulex reproducing by obligate parthenogenesis. *Heredity,* **51,** 353-369.

Hengherr, S., Heyer, A. G., Brümmer, F. and Schill, R. O. (2011) Trehalose and Vitreous States: Desiccation Tolerance of Dormant Stages of the Crustaceans Triops and Daphnia. *Physiological and Biochemical Zoology,* **84,** 147-153.

Hengherr, S. and Schill, R. O. (2011) Dormant stages in freshwater bryozoans—An adaptation to transcend environmental constraints. *Journal of Insect Physiology,* **57,** 595-601.

Hobaek, A. and Larsson, P. (1990) Sex determination in *Daphnia magna*. *Ecology,* **71,** 2255-2268.

Hodgson, J. (1994) The Culture of Cryopreservation. *Nat Biotech,* **12,** 82-82.

Ikeda, M., Su, Z.-H., Saito, H., Imai, K., Sato, Y., Isobe, M. and Yamashita, O. (1993) Induction of embryonic diapause and stimulation of ovary trehalase activity in the silkworm, Bombyx mori, by synthetic diapause hormone. *Journal of Insect Physiology,* **39,** 889-895.

Jansen, M., Coors, A., Vanoverbeke, J., Schepens, M., De Voogt, P., De Schamphelaere, K. a. C. and De Meester, L. (2015) Experimental evolution reveals high insecticide tolerance in Daphnia inhabiting farmland ponds. *Evolutionary Applications,* **8,** 442-453.

Jaromir, S. and Petrusek, A. (2011) Daphnia as a model organism in limnology and aquatic biology: introductory remarks. *Journal of limnology,* **70,** 337-344.

Kast-Hutcheson, K., Rider, C. V. and Leblanc, G. A. (2001) The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environmental Toxicology and Chemistry,* **20,** 502-509.

Kawasaki, T., Yoshimura, H., Shibue, T., Ikeuchi, Y., Sakata, M., Igarashi, K., Takada, H., Hoshino, K., Kohn, K. and Namiki, H. (2004) Crystalline Calcium Phosphate and Magnetic Mineral Content of Daphnia Resting Eggs. *Zoological Science,* **21,** 63-67.

Kerfoot, W. C., Robbins, J. A. and Weider, L. J. (1999) A new approach to historical reconstruction: Combining descriptive and experimental paleolimnology. *Limnology and oceanography,* **44,** 1232-1247.

Kikawada, T., Minakawa, N., Watanabe, M. and Okuda, T. (2005) Factors Inducing Successful Anhydrobiosis in the African Chironomid Polypedilum vanderplanki: Significance of the Larval Tubular Nest. *Integrative and Comparative Biology,* **45,** 710-714.

Kleiven, O. T., Larsson, P. and Hobæk, A. (1992) Sexual Reproduction in Daphnia magna Requires Three Stimuli. *Oikos,* **65,** 197-206.

Kuleshova, L. L., Macfarlane, D. R., Trounson, A. O. and Shaw, J. M. (1999) Sugars Exert a Major Influence on the Vitrification Properties of Ethylene Glycol-Based Solutions and Have Low Toxicity to Embryos and Oocytes. *Cryobiology,* **38,** 119-130.

Kulus, D. and Zalewska, M. (2014) Cryopreservation as a tool used in long-term storage of ornamental species – A review. *Scientia Horticulturae,* **168,** 88-107.

Lampert, W. (2006a) Daphnia: model herbivore, predator and prey. *Polish J Ecol,* **54,** 607 - 620.

Lampert, W. (2006b) Daphnia: Model herbivore, predator and prey. *Polish journal of ecology,* **54,** 607-620.

Lampert, W. (2011) Daphnia: development of a model organism in ecology and evolution. International Ecology Institute Oldendorf/Luhe.

Leibo, S. P. and Mazur, P. (1971) The role of cooling rates in low-temperature preservation. *Cryobiology,* **8,** 447-452.

Liu, Y.-L., Zhao, Y., Dai, Z.-M., Chen, H.-M. and Yang, W.-J. (2009) Formation of Diapause Cyst Shell in Brine Shrimp, *Artemia parthenogenetica*, and Its Resistance Role in Environmental Stresses. *Journal of Biological Chemistry,* **284,** 16931-16938.

Lopes, F. L., Desmarais, J. A. and Murphy, B. D. (2004) Embryonic diapause and its regulation. *Reproduction,* **128,** 669-678.

Macfarlane, D. R. (1987) Physical aspects of vitrification in aqueous solutions. *Cryobiology,* **24,** 181-195.

Makrushin, A. V. (1978) Anhydrobiosis and yolk structure of eggs in cladocera. *Zoologichesky zhurnal,* **57,** 364-374.

Mauger, P. E., Le Bail, P. Y. and Labbé, C. (2006) Cryobanking of fish somatic cells: Optimizations of fin explant culture and fin cell cryopreservation. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **144,** 29-37.

Mazur, P. (1984) *Freezing of living cells: mechanisms and implications.* Vol. 247.

Mazur, P., Leibo, S. P. and Seidel, G. E. (2008) Cryopreservation of the Germplasm of Animals Used in Biological and Medical Research: Importance, Impact, Status, and Future Directions. *Biology of Reproduction,* **78,** 2-12.

Mellors, W. K. (1975) Selective Predation of Ephippal Daphnia and the Resistance of Ephippal Eggs to Digestion. *Ecology,* **56,** 974-980.

Miner, B. E., De Meester, L., Pfrender, M. E., Lampert, W. and Hairston, N. G. (2012) Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society of London Biological sciences,* **279,** 1873-1882.

Muldrew, K., Acker, J. P., Elliott, J. and Mcgann, L. E. (2004) The water to ice transition: implications for living cells. In: B. J. Fuller, N. Lane and E. E. Benson (eds) *Life in the frozen state.* CRC Press LLC, pp. 67-108.

Neves, P. R., Ribeiro, R. P., Streit, D. P., Jr., Natali, M. R. M., Fornari, D. C., Santos, A. I. and Godoy, L. C. (2014) Injuries in pacu embryos (Piaractus mesopotamicus) after freezing and thawing. *Zygote,* **22,** 25-31.

Paredes, E., Adams, S. L., Tervit, H. R., Smith, J. F., Mcgowan, L. T., Gale, S. L., Morrish, J. R. and Watts, E. (2012) Cryopreservation of Greenshell™ mussel (Perna canaliculus) trochophore larvae. *Cryobiology,* **65,** 256-262.

Paredes, E., Bellas, J. and Adams, S. L. (2013) Comparative cryopreservation study of trochophore larvae from two species of bivalves: Pacific oyster (Crassostrea gigas) and Blue mussel (Mytilus galloprovincialis). *Cryobiology,* **67,** 274-279.

Pauwels, K., Stoks, R., Decaestecker, E. and De Meester, L. (2007a) Evolution of Heat Shock Protein Expression in a Natural Population of Daphnia magna. *The American Naturalist,* **170,** 800-805.

Pauwels, K., Stoks, R., Verbiest, A. and De Meester, L. (2007b) Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*. *Hydrobiologia,* **594,** 91-96.

Pegg, D. E. (1987) Ice Crystals in Tissues and Organs. In: D. E. Pegg and A. M. Karow, Jr. (eds) *The Biophysics of Organ Cryopreservation.* Vol. 147. Springer US, pp. 117-140.

Pouchkina-Stantcheva, N. N., Mcgee, B. M., Boschetti, C., Tolleter, D., Chakrabortee, S., Popova, A. V., Meersman, F., Macherel, D., Hincha, D. K. and Tunnacliffe, A. (2007) Functional Divergence of Former Alleles in an Ancient Asexual Invertebrate. *Science,* **318,** 268-271.

Proctor, V. W. (1964) Viability of Crustacean Eggs Recovered From Ducks. *Ecology,* **45,** 656-658.

Radzikowski, J. (2013) Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *Journal of Plankton Research,* **4,** 707-723.

Rall, W. F. (1987) Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology,* **24,** 387-402.

Saint-Jean, L. and Pagano, M. (1995) Egg mortality through predation in egg-carrying zooplankters. Studies on Heterobranchus longifilis larvae fed on copepods, cladocerans and rotifers. *Journal of Plankton Research,* **17,** 1501-1512.

Seidman, L. A. and Larsen Jr, J. H. (1979) Ultrastructure of the envelopes of resistant and nonresistant Daphnia eggs. *Canadian journal of zoology,* **57,** 1773-1777.

Simon, J.-C., Pfrender, M. E., Tollrian, R., Tagu, D. and Colbourne, J. K. (2011) Genomics of Environmentally Induced Phenotypes in 2 Extremely Plastic Arthropods. *Journal of Heredity,* **102,** 512-525.

Solomon, A., Salomon, R., Paperna, I. and Glazer, I. (2000) Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. *Parasitology,* **121,** 409-416.

Storey, K. B. and Storey, J. M. (2007) Tribute to P. L. Lutz: putting life on `pause' – molecular regulation of hypometabolism. *Journal of Experimental Biology,* **210,** 1700-1714.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J. and Billard, R. (2000) Cryopreservation of sperm in marine fish. *Aquaculture Research,* **31,** 231-243.

Tautz, D. (2011) Not just another genome. *BMC Biology,* **9,** 8.

Taylor, M. J. and Pegg, D. E. (1983) The effect of ice formation on the function of smooth muscle tissue stored at −21 or −60 °C. *Cryobiology,* **20,** 36-40.

Tiersch, T. R. and Mazik, P. M. (2000) *Cryopreservation in aquatic species.*, World Aquaculture Society Baton Rouge, LA.

Toledo, J. D. and Kurokura, H. (1990) Cryopreservation of the euryhaline rotifer Brachionus plicatilis embryos. *Aquaculture,* **91,** 385-394.

Tollrian, R. and Leese, F. (2010) Ecological genomics: steps towards unraveling the genetic basis of inducible defenses in Daphnia. *BMC Biol,* **8,** 51.

Towill, L. E. and Bonnart, R. (2003) Cracking in a Vitrification Solution During Cooling or Warming Does Not Affect Growth of Cryopreserved Mint Shoot Tips. *Cryoletters,* **24,** 341-346.

Tsai, S., Rawson, D. M. and Zhang, T. (2009) Development of cryopreservation protocols for early stage zebrafish (Danio rerio) ovarian follicles using controlled slow cooling. *Theriogenology,* **71,** 1226-1233.

Tunnacliffe, A., Hincha, D., Leprince, O. and Macherel, D. (2010) LEA Proteins: Versatility of Form and Function. In: E. Lubzens, J. Cerda and M. Clark (eds) *Dormancy and Resistance in Harsh Environments.* Vol. 21. Springer Berlin Heidelberg, pp. 91-108.

Tunnacliffe, A. and Wise, M. (2007) The continuing conundrum of the LEA proteins. *Naturwissenschaften,* **94,** 791-812.

Van Doorslaer, W., Stoks, R., Swillen, I., Feuchtmayr, H., Atkinson, D., Moss, B. and De Meester, L. (2010) Experimental thermal microevolution in community-embedded Daphnia populations. *Climate research (Open Access for articles 4 years old and older),* **43,** 81.

Van Doorslaer, W., Vanoverbeke, J., Duvivier, C., Rousseaux, S., Jansen, M., Jansen, B., Feuchtmayr, H., Atkinson, D., Moss, B., Stoks, R. and De Meester, L. (2009) Local adaptation to higher temperatures reduces immigration success of genotypes from a warmer region in the water flea Daphnia. *Global Change Biology,* **15,** 3046-3055.

Walker, C. (2014) *Ecotoxicology: Effects of Pollutants on the Natural Environment.*, CRC Press.

Walker, C. H., Sibly, R., Hopkin, S. and Peakall, D. B. (2012) *Principles of ecotoxicology.* CRC Press.

Watanabe, M., Kikawada, T., Minagawa, N., Yukuhiro, F. and Okuda, T. (2002) Mechanism allowing an insect to survive complete dehydration and extreme temperatures. *Journal of Experimental Biology,* **205,** 2799-2802.

Watts, S. A., Lee, K. J. and Cline, G. B. (1994) Elevated ornithine decarboxylase activity and polyamine levels during early development in the brine shrimp Artemia franciscana. *Journal of Experimental Zoology,* **270,** 426-431.

Wolfe, J. and Bryant, G. (1999) Freezing, drying and/or vitrification of membrane-solute-water systems. *Cryobiology,* **39,** 103-129.

Wolfe, J. and Bryant, G. (2001) Cellular cryobiology: thermodynamic and mechanical effects. *International journal of refrigeration,* **24,** 438-450.

Womersley, C. and Smith, L. (1981) Anhydrobiosis in nematodes—I. The role of glycerol myo-inositol and trehalose during desiccation. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* **70,** 579-586.

Zaffagnini, F. (1987) Reproduction in *Daphnia*. In: R. H. Peters and R. De Bernardi (eds) *Daphnia.* Vol. 45. Memorie dell'Instituto Italiano di Idrobiologia, pp. 245-284.

Zhang, T. (2004) Cryopreservation of gametes and embryos of aquatic species. In: B. J. Fuller, N. Lane and E. E. Benson (eds) *Life in the frozen state.* CRC Press LLC, pp. 415-435.

Zhang, T. and Rawson, D. M. (1995) Studies on Chilling Sensitivity of Zebrafish (Brachydanio rerio) Embryos. *Cryobiology*, **32,** 239-246.

Part I

Chapter 1

A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality

ADINDA PUTMAN, DOMINIK MARTIN-CREUZBURG, BART PANIS AND LUC DE MEESTER

Modified from Journal of plankton research 2015

Abstract

In the cyclical parthenogenetic *Daphnia* asexual eggs develop immediately and enable fast population growth, while sexual eggs are dormant and can survive harmful conditions. We studied whether this different function is reflected in different fatty acid profiles and explored the capacity of *D. magna* to adjust fatty acid provisioning of its eggs depending on food resources. We quantified neutral- and phospholipid content of sexual and asexual eggs produced under different food conditions and compared these too eggs collected from a natural pond. In eggs obtained under different laboratory food regimes, total concentration of neutral fatty acids per unit biomass was not affected by food source or egg type. Both egg types contained lower amounts of fatty acids in the neutral fraction when produced in nature than under laboratory conditions. Fatty acid concentration in the phospholipid fraction was lower in sexual than asexual eggs. Fatty acid composition of eggs largely reflected that of the food of the mothers, albeit with small modifications. Sexual eggs produced on *S. obliquus* diet (no C20-PUFA), contained higher concentrations of EPA and ARA in both fractions than asexual eggs.

1 Introduction

Like many aquatic organisms inhabiting inland waters, the cladoceran *Daphnia* produces eggs that develop immediately and eggs that first go through a dormant stage, the latter as a strategy to cope with temporarily harsh periods (Brendonck and De Meester, 2003). Most *Daphnia* species are cyclical parthenogens, with the dormant eggs being produced sexually while the subitaneous eggs are produced parthenogenetically. Asexual eggs are typically produced during favourable conditions and allow for rapid population growth. By the end of the growing season, when the animals are exposed to deteriorating environmental conditions, *Daphnia* start producing sexual eggs. Sexual eggs are encased in a protective envelope, the ephippium, and are deposited in the sediment where they form a dormant egg bank. These eggs can stay viable for decades and are resistant to desiccation, extreme temperatures and digestion by animals (Decaestecker et al., 2009; Frisch et al., 2014).

Sexual and asexual eggs have very different functions during the life cycle (Brendonck and De Meester, 2003). Dormant eggs are used to disperse in space and time, consequently the hatchlings from sexual eggs experience different growth conditions compared to hatchlings from asexual eggs, which are more likely to encounter similar conditions as the parental generation. The resulting offspring differ in life history traits (Cáceres, 1998; Arbaciauskas and Lampert, 2003) and it is conceivable that this is reflected in differences in the biochemistry of the eggs.

Dormant eggs require special adaptations to survive drought and freezing during diapause, and these adaptations may include different resource allocation strategies. In agreement with the requirements for dormancy, Pauwels et al. (2007) reported that sexual eggs of *Daphnia magna* contain more glycerol and heat shock proteins than asexual eggs. Both glycerol and heat shock proteins play a key role in the protection of cell metabolism during stress conditions and have also been found in anhydrobiotic cysts of other diapausing organisms, such as *Artemia fransicana* (Clegg et al., 1997) and certain insects (Denlinger, 2002). Early studies have reported morphological differences between parthenogenetic and dormant eggs. For instance, oocytes of sexual eggs do not contain free lipid droplets and are enclosed in three membranes, while the oocytes of asexual eggs do contain lipid bodies and only have two membranes (Zaffagnini, 1987). Based on these histological observations, sexual oocytes are expected to contain higher amounts of triglycerides, the main energy storage molecules in *Daphnia* (Peters, 1987). However, Pauwels et al. (Pauwels et al., 2007) did not find significant differences in triglyceride concentrations between sexual and asexual eggs of *Daphnia* magna. In another study, dormant eggs of *Daphnia pulicaria* contained much more fatty acids, especially polyunsaturated fatty acids (PUFA), than parthenogenetic eggs (Abrusan et al., 2007).

The biochemical composition of parthenogenetic eggs of *Daphnia* has been shown to be highly plastic. Predation pressure as well as both food quantity and quality in the maternal generation have a strong influence on the biochemical composition of the eggs (Gliwicz and Guisande, 1992; Stibor, 2002; Wacker and Martin-Creuzburg, 2007; Schlotz et al., 2013). When exposed to low food quantity, *Daphnia* females have been reported to produce smaller clutches with larger eggs and a higher content of protein, lipid and carbon (Gliwicz and Guisande, 1992). In contrast, Tessier et al. (Tessier et al.,1983) observed that females reproducing under low food conditions allocate less maternal lipids to each egg than females of the same genotype (i.e. clone) under high food conditions, and that neonates hatching from eggs with a higher triglyceride content survive longer under starvation. Besides food quantity, resource allocation into the eggs can be affected by food quality. Sterols (Von Elert et al., 2003; Martin-Creuzburg et al., 2005) and polyunsaturated fatty acids (Brett, 1993; Müller-Navarra et al., 2000; Von Elert, 2002; Persson and Vrede, 2006) are both important determinants of biochemical food quality for *Daphnia.* When exposed to poor food quality conditions, total fatty acid concentrations are reduced in both somatic tissue and parthenogenetic eggs, while the concentration of cholesterol is constant in eggs but lowered in somatic tissue (Wacker and Martin-Creuzburg, 2007). Not only the concentration but also the fatty acid composition of the eggs is strongly determined by the maternal dietary supply (Schlotz et al., 2013). It is generally accepted that arthropods are unable or at least have limited capabilities to synthesize PUFA de novo from low molecular weight precursors (Leonard et al., 2004). Five PUFA have been frequently discussed as being essential, of which three are n-3 fatty acids (i.e. α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)) and two are n-6 fatty acids (i.e. linoleic (LIN, 18:2n-6) and arachidonic acid (ARA, 20:4n-6)) (Von Elert, 2002; Kainz et al., 2004; Martin-Creuzburg et al., 2010). PUFA play important roles in cell growth and proliferation and are precursors for other important molecules. For instance, EPA and ARA are precursors for prostaglandins and other eicosanoids, which are important mediators in reproduction, the immune system and ion transport physiology (Stanley, 2006; Heckmann et al., 2008). Along with sterols and proteins, PUFA-containing phospholipids define the physical characteristics of cell membranes (Stillwell and Wassall, 2003; Valentine and Valentine, 2004). Both the saturation degree and the length of the fatty acid chains within phospholipids influence membrane flexibility and permeability (Pruitt, 1990).

In this study, we explored whether the cladoceran *D. magna* is capable of adjusting fatty acid allocation towards both asexual and sexual eggs as a function of the fatty acid profile of the food of the mother. Previous studies on the allocation of fatty acids to eggs in *Daphnia* as a function of food quality focused on parthenogenetic eggs only, while the one study that compared fatty acid content of sexual and asexual eggs in *Daphnia* focused on one diet only (Abrusan et al., 2007). Here we compared the plasticity of fatty acid concentration and composition in asexual and sexual eggs of *D.*

magna, whose mothers were reared on two algae differing strongly in their fatty acid profile and in asexual and sexual eggs produced by mothers in a natural pond. Because of the distinct functions between lipid classes, we separated the total lipid fraction into neutral lipids and phospholipids prior to fatty acid analyses, to distinguish between fatty acids that are primarily used as energy resources and fatty acids that are primarily used as cell membrane components, respectively. We expected that sexual eggs contain more neutral lipids than asexual eggs, as they are able to survive for decades. Regarding phospholipids, we expected to find a higher plasticity in fatty acid profiles in asexual eggs than in sexual eggs as the latter may require a certain fatty acid composition to be able to withstand harsh environmental conditions.

2 Material and methods

2.1 Cultivation and preparation of the food

In our laboratory experiments, we used the green alga *Scenedesmus obliquus* (SAG 276-3a) and the eustigmatophyte *Nannochloropsis limnetica* (SAG 18.99) to rear *Daphnia magna.* These two algae are characterised by highly distinct fatty acid profiles, i.e. *S. obliquus* lacks PUFA with more than 18 carbon atoms, while *N. limnetica* contains high concentrations of C20-PUFA, especially eicosapentaenoic acid (Martin-Creuzburg et al., 2009; Von Elert, 2002).

Algae were grown in batch cultures at 18°C in aerated 10 liter vessels with illumination at 170 µmol quanta m^{-2} s^{-1} and harvested in the late-exponential growth phase. *S. obliquus* was grown in a medium consisting of 10 mL L^{-1} of enriched seawater (ES) nutrients (Provasoli, 1968), 5 mL L^{-1} of Walne nutrients (Walne, 1965) and the vitamins B1, B12 and H dissolved in dechlorinated tap water. *N. limnetica* was grown in modified Woods Hole (WC) medium with the vitamins B1, B12 and H (Guillard, 1975). Food suspensions were prepared by concentrating the cells via centrifugation (2500g, 5 min) followed by resuspension in tap water. Cell densities of the food suspensions were counted with an Attune® acoustic focusing cytometer (Life technologies, Carlsbad, CA, USA).

2.2 Egg collection

To compare the biochemical composition of sexual and asexual eggs of *D. magna* produced under different conditions, we collected sexual and asexual eggs produced by animals in a natural pond and sexual and asexual eggs derived from females fed either *S. obliquus* or *N. limnetica* in the laboratory. The laboratory animals were themselves derived from the same pond as from which the other eggs were collected.

2.2.1 Eggs collected from a natural population

Dormant eggs of *D. magna* were collected from 'Langerode vijver', a pond in Neerijse, Belgium (0°49'42.32"N, 4°38'21.49"E). The upper 5-10 cm of the sediment, corresponding to the active egg bank (Càceres, 1998), was taken during the winter of 2011-2012, when the eggs are in diapause and before the spring hatching peak occurred. Ephippia were collected by sieving the sediment over first 1 mm and then 250 µm mesh-sized sieves. To prevent hatching of the eggs during this process, eggs were kept in the dark and placed on ice. When the sieving was finished, all collected ephippia were stored in the dark at 4°C, awaiting further analyses. Ephippia were manually isolated from the sediment fraction and decapsulated to collect four replicate samples consisting of 100 sexual eggs for fatty acid analysis. These manipulations were performed in a room with only red light (700nm), to prevent unwanted light exposure of the dormant eggs. Samples were stored at -80°C to stop all biochemical processes in the cells.

Asexual eggs from the pond 'Langerode vijver' were collected directly from the brood pouch of animals sampled during the growing season following our winter sampling for dormant eggs. Four samples consisting of 100 parthenogenetic eggs in the first stage of daphnid embryonic development (according to Kast-Hutcheson et al. 2001) were collected by dissection of the females brood chamber and stored at -80°C before freeze drying.

2.2.2 Laboratory cultured eggs

We conducted an experiment in the laboratory to generate sexual and asexual eggs from females cultured under different food conditions. Therefore, from the sediment bank of 'Langerode vijver', 10 clones were hatched by exposing the dormant eggs to hatching stimuli, i.e. a relatively high temperature (20°C), a long-day photoperiod (16L:8D) and fresh medium (tap water dechlorinated and aged for 24h) (De Meester and Jager, 1993). Hatched animals were reared in 0.5 L jars (density: 20 individuals per liter) filled with aged tap water (aerated for 24h prior to use) under standard conditions (20 ± 2 °C and a photoperiod of 16L:8D) for several generations. All cultures were fed 150.000 cells mL^{-1} of *S. obliquus*, which corresponds to approximately 2.5 mg C L^{-1}. After this preconditioning phase, the second clutch of a new generation was subjected to the different experimental conditions.

To obtain asexual eggs, per treatment four replicate one liter jars per clone (10 clones), with 15 individuals in each jar, were cultured under standard conditions (20 ± 2 °C and a photoperiod of 16L:8D). In the first treatment all cultures were fed with *S. obliquus*, whereas in the second treatment all cultures were fed with *N. limnetica* (both at an algal cell density of 150.000 cells ml^{-1}). In both treatments, jars were cleaned every two days and food was renewed daily to keep algal concentration above the incipient limiting level. Females bearing their third clutch were dissected to collect asexual

eggs in the first stage of daphnid embryonic development (according to Kast-Hutcheson et al., 2001). From each replicate, one sample of 100 eggs (10 eggs per clone) was collected and stored at -80°C. In total, eight samples (four replicates per treatment) were put in storage.

Lastly, we induced sexual reproduction using the same clones and under the same food conditions as described above, namely the algae *S. obliquus* and *N. limnetica*. To obtain starting material we reared 20 individuals of the same 10 clones as used for the production of the asexual eggs under standard conditions, i.e. 20 ± 2 °C, a photoperiod of 16L:8D and an algal cell density of 150.000 cells mL^{-1} . After they had released their second clutch, four animals of every clone were mixed in a one liter jar (replicated 4 times) and feeding level was raised to 250.000 cells mL^{-1}. The photoperiod was switched from long-day (16L:8D) during five days to short-day (8L:16D) photoperiod during two days, as this stimulates sexual reproduction in *Daphnia* (De Meester and Jager, 1993). Once a week half the medium was renewed and all dormant eggs were collected. Dormant eggs were stored in Eppendorf tubes in the dark at 4°C for at least one month. After storage, these laboratory-derived ephippia were decapsulated and four replicate samples consisting of 100 sexual eggs each were collected for each treatment. The eight samples were stored at -80°C.

In total, 24 samples (2 types of eggs: sexual vs. asexual eggs, 3 treatments: eggs collected from a natural population and *S. obliquus*- and *N. limnetica*-fed laboratory animals; four replicates per treatment) were freeze dried, weighed (dry mass) and transferred to Eppendorf tubes until further analysis. To reduce lipid peroxidation, egg samples were overlaid with gaseous nitrogen.

2.3 Chemical analysis

2.3.1 Analysis of food quality

To analyse the fatty acid composition of the laboratory-reared algal food source, three replicate samples of 9.5 mg carbon (10mL) for *S. obliquus* and 2.9 mg carbon (15mL) for *N. limnetica* were filtered on precombusted (5h, 550°C) GF/F filters (Whatman, 25mm). For determination of the food composition of the animals in the pond, three random seston samples of 200mL, corresponding to 0.66 mg carbon, were taken from 'Langerode vijver' and filtered on precombusted GF/F filters.

The fatty acid composition of the food sources was analysed as described in Martin-Creuzburg et al. (2010). Briefly, loaded filters were deposited in 7 mL of a mixture of dichloromethane and methanol (2:1) and stored at -20°C. Total lipids were extracted three times (sonication for 30min) with dichloromethane:methanol (2:1). Pooled cell-free extracts were evaporated to dryness using nitrogen. The lipid extracts were transesterified with 4 mL of 3 N methanolic HCl (60°C, 15 min) and 100 µL of internal standard (20 µg mL^{-1} 17:0 ME and 25 µg mL^{-1} 23:0 ME) was added. After cooling down to

room temperature, the fatty acid methyl esters (FAME) were further extracted and analysed as described for the egg samples. The absolute amount of each FAME was normalized to the carbon content of the food suspensions.

As expected, the fatty acid profiles of the food sources differed considerably (Figure 1, Table 1). *N. limnetica* was rich in C20:5n-3 (42.7%) and C20:4n-6 (4.4%). In contrast, no PUFA with more than 18 carbon atoms were detected in *S. obliquus*. The major fatty acid identified in *S. obliquus* was 18:3n-3 (59.5%). The lake seston contained significantly smaller quantities of all fatty acids, but low concentrations of LIN, ALA, ARA and EPA were detected. Docosahexaenoic acid was not present in any of the samples.

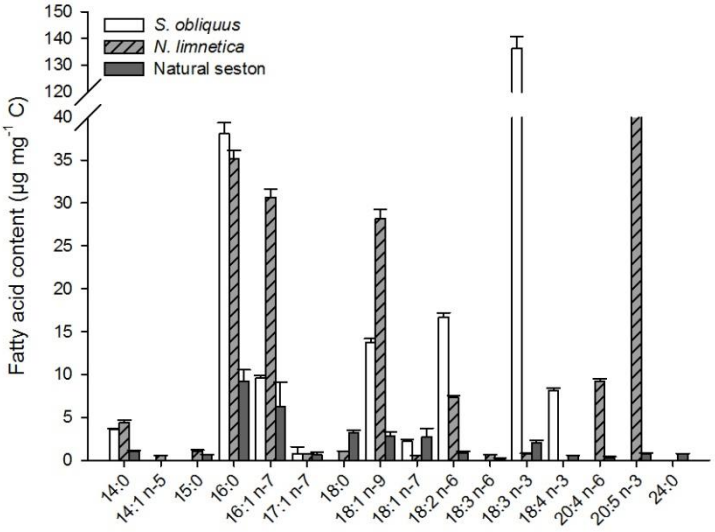

Figure 1: Fatty acid composition of *Scenedesmus obliquus* (white bars), *Nannochloropsis limnetica* (hatched bars) and seston of lake 'Langerode vijver' (dark grey bars), expressed in µg mg^{-1} carbon. Given are means of three replicates; error bars indicate one standard error.

Table 1: Abundances of key fatty acids in *Scenedesmus obliquus*, *Nannochloropsis limnetica* and seston of lake 'Langerode vijver'. The total concentration (µg mg^{-1} carbon) and the percentages of saturated fatty acids (SFA), mono unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and essential fatty acids are listed. Data are means of three replicates.

Fatty acid	*N. limnetica*	*S. obliquus*	Lake seston
∑ FA	209.97	229.13	31.8
SFA	19.82%	18.19%	46.65%
MUFA	28.67%	11.48%	38.84%
(n-3) PUFA	43.09%	63.06%	10.48%
(n-6) PUFA	8.19%	7.27%	4.03%
C18:2 n-6	3.48%	7.27%	2.76%
C18:3 n-3	0.38%	59.51%	6.27%
C20:4 n-6	4.40%	nd	0.09%
C20:5 n-3	42.71%	nd	2.45%
C22:6 n-3	nd	nd	nd

2.4 Analysis of egg samples

Egg samples were analysed using a combination of the methods described in Zhu et al. (2006) and Martin-Creuzburg et al. (2010). In a first step, lipid classes were separated. Total lipids were divided into several classes based on the charge of the head group. Sterols and glycerides have a less polar head group, while glycolipids, phospholipids and sphingolipids contain a more polar head group. In a second step, the composition of the liberated fatty acid chains was analysed.

Freeze-dried egg samples were homogenized in 1 mL of isopropanol. The mixture was sonicated for 30 min at 4°C. After centrifugation at 10.000 g for 10 min, the supernatant was collected in a glass tube and the residue was extracted again with 1 mL of $CHCl_3$:MeOH (2:1) during sonification for another 30 min at 4°C. After centrifugation at 10.000 g for 10 min, both supernatants were combined, and 2 mL of $CHCl_3$ and 1 mL of a 0.88% KCl solution were added. The mixture was thoroughly shaken and centrifuged at 4000 g for 15 min. The upper aqueous layer was aspired and 1 mL of MeOH:0.88% KCl (1:1) was added. After vortexing and centrifugation at 4000 g for 15 min, the upper phase was aspired together with the interphase. The lower phase was evaporated at 40°C under a stream of N_2. The residue (lipid extracts) was dissolved in 2 mL of $CHCl_3$:acetic acid (100:1) and applied to a silica gel column, wetted with $CHCl_3$:acetic acid (100:1), to separate the different lipid classes. First, 5.53 mL of $CHCl_3$ was applied to the column for eluting neutral lipids, such as sterols and glycerides (fraction 1). Secondly, 2.67 mL of acetone, followed by 2.67 mL of

acetone:MeOH:acetic acid (100:5:1) were applied to the column for eluting glycolipids and sphingolipids (fraction 2). Then 4 mL of a MeOH:$CHCl_3$:H_2O mixture (100:50:40) was applied to the column for eluting the phospholipids. To this fraction, 2.25 mL of $CHCl_3$ and 3 mL of H_2O were added. The mixture was vortexed and centrifuged at 4000 g for 2 min. The upper water phase was removed and the lower $CHCl_3$ phase containing the phospholipids was used for further analysis (fraction 3). All fractions were evaporated under a N_2 stream at 40°C (Zhu et al., 2006).

For fatty acid analysis, the dried samples were resuspended in 4 mL of 3 N methanolic HCl (Sigma-Aldrich) together with 100 µL of internal standard (20 µg mL^{-1} 17:0 ME + 25 µg mL^{-1} 23:0 ME) and subsequently incubated for 20 min at 60°C in a sealed vial to transesterify fatty acids into methyl esters. After cooling, FAME were extracted three times with 1.5-2 mL isohexane. The fraction of isohexane was evaporated to dryness under a N_2 stream and the extraction procedure was repeated again with 100 µL isohexane. Afterwards, the isohexane was evaporated under a N_2 stream at 45°C and the FAME were resuspended in a volume of 10 µL isohexane. FAME were analysed by gas chromatography (GC; Hewlett-Packard 6890™) equipped with a flame ionization detector (FID) and a DB-225 (J&W Scientific, 30 m × 0.25 mm inner diameter × 0.25 µm film) capillary column for FAME analysis. Details of GC configurations are given elsewhere (Martin-Creuzburg et al., 2010). FAME were identified by comparison of retention times with those of reference compounds (Sigma-Aldrich). Fatty acids were quantified by comparison with internal standards and by using multipoint standard calibration curves determined for each FAME from mixtures of known composition (Sigma-Aldrich). The absolute amount of each FAME was normalized to the egg dry mass and the egg number (Martin-Creuzburg et al., 2010).

2.5 Data analysis

Data were analysed using the statistical software CANOCO for windows for PCA and the packages car and phia in R (version 3.0.2) for two-way ANOVA, MANOVA and contrast analyses. To visualize the fatty acid distribution, ordination diagrams of principal component analysis (PCA) were made with the interaction factor between food source and egg type plotted as supplementary variable. Effects of the food sources (*S. obliquus*, *N. limnetica*, lake seston) and egg types (sexual and asexual eggs) on the concentrations of the individual fatty acids of the different lipid classes were tested using a multivariate analysis of variance (MANOVA). To test for the effect of egg type within the different food sources and for differences among the laboratory treatments and between these treatments and the natural conditions, contrast analyses were performed. Total amounts of fatty acids were compared between food sources and egg types using two-way analyses of variance (ANOVA); lipid classes were analysed separately. The statistical analyses were performed on both data sets, i.e. the dry mass related data set and the per egg related data set.

3 Results

Overall, the fatty acid profile of both sexual and asexual eggs reflected the fatty acid profile of the food sources encountered by the mothers (Figure 2, see addendum). For example, C18:3n-3 (ALA) and C20:5n-3 (EPA), the principal PUFA in *S. obliquus* and *N. limnetica*, respectively (Table 1), were the principal PUFA in the eggs of mothers reared on these algae (Table 2).

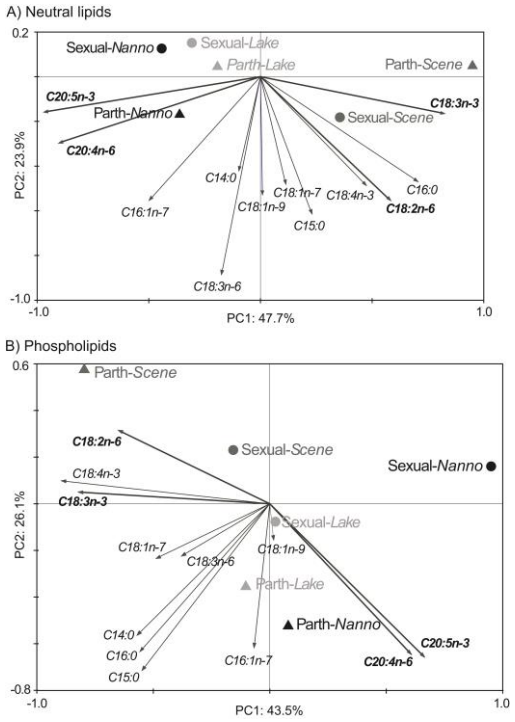

Figure 2: Ordination diagrams of the Principal component analyses (PCA) of the fatty acid composition (upper panel: fraction of neutral lipids; lower panel: fraction of phospholipids) of sexual and parthenogenetic (Parth) eggs of *Daphnia magna* fed *Nannochloropsis limnetica* (Nanno) or *Scenedesmus obliquus* (Scene), and of sexual and parthenogenetic eggs directly isolated from a natural pond (Lake). Concentrations of the different fatty acids (ng mg^{-1} dry weight) were the dependent variables, while type of food (*Nannochloropsis limnetica*, *Scenedesmus obliquus*, lake seston) and egg type (resting versus parthenogenetic) were the independent variable.

Table 2: Fatty acid composition of sexual and asexual eggs of *Daphnia magna* produced on *Nannochloropsis limnetica* or *Scenedesmus obliquus*, and of sexual and asexual eggs directly isolated from a natural lake. The total concentration (µg mg^{-1} dry mass) and the percentages of saturated fatty acids (SFA), mono unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and essential fatty acids of neutral lipid and phospholipid fractions are given. Data are means of four replicates.

Neutral lipid fraction

	Asexual eggs			Sexual eggs		
	N. limnetica	*S. obliquus*	Lake seston	*N. limnetica*	*S. obliquus*	Lake seston
∑ FA	51.07 +/- 11.65	51.7 +/- 6.19	18.03 +/- 2.99	37.61 +/- 17.7	43.67 +/- 12.3	24.97 +/- 3.41
SFA	4.18%	29.10%	13.38%	2.29%	2.29%	5.26%
MUFA	35.32%	14.56%	29.01%	30.69%	30.69%	23.74%
(n-3) PUFA	50.94%	47.15%	43.88%	52.52%	52.52%	55.61%
(n-6) PUFA	9.56%	9.19%	13.73%	14.50%	14.50%	15.40%
C18:2 n-6	4.34%	8.79%	7.27%	6.40%	11.62%	5.39%
C18:3 n-3	0.95%	63.40%	17.99%	0.94%	52.22%	13.24%
C20:4 n-6	4.63%	0.17%	5.44%	6.97%	0.69%	8.62%
C20:5 n-3	48.98%	nd	22.32%	50.76%	1.45%	38.43%
C22:6 n-3	nd	nd	nd	nd	nd	nd

Phospholipid fraction

	Asexual eggs			Sexual eggs		
	N. limnetica	*S. obliquus*	Lake seston	*N. limnetica*	*S. obliquus*	Lake seston
∑ FA	29.43 +/- 3.60	30.02 +/- 7.63	20.64 +/- 1.57	14.46 +/- 2.56	15.37 +/- 5.98	15.33 +/- 6.65
SFA	21.05%	18.78%	23.60%	16.29%	22.26%	22.68%
MUFA	30.62%	21.22%	29.49%	41.81%	29.87%	41.90%
(n-3) PUFA	38.22%	46.82%	34.16%	30.68%	33.47%	26.24%
(n-6) PUFA	10.11%	13.18%	12.75%	11.22%	14.39%	9.17%
C18:2 n-6	3.66%	12.56%	5.22%	5.76%	12.10%	3.88%
C18:3 n-3	6.12%	44.14%	8.72%	nd	27.91%	6.29%
C20:4 n-6	5.06%	0.21%	7.22%	5.45%	2.30%	5.30%
C20:5 n-3	36.49%	0.21%	23.51%	30.68%	3.48%	18.01%
C22:6 n-3	nd	nd	nd	nd	nd	nd

In none of the egg samples fatty acids were observed in fraction 2, which is supposed to contain the fatty acids derived from glycolipids and sphingolipids. For fatty acid concentrations in the other lipid classes distinct patterns were found (Figure 3). There is a significant interaction between food sources and egg type on fatty acid concentration for fatty acids derived from the neutral lipid fraction (fraction 1) and from the phospholipid fraction (fraction 3) (Table 3; the MANOVA on the amount of fatty acids per egg gave similar results, see Table 1, Appendix 1). The main effect of food type is also highly significant for both the neutral lipid and the phospholipid fraction. Egg type is highly

significant for phospholipids but marginally non-significant for neutral lipids (Table 3). Given the significant interaction effects, we also explored the impact of egg type for each food class separately through independent contrasts. Contrast analysis revealed a significant effect of egg type for each food condition (Table 4). Likewise, the fatty acid composition of the neutral and phospholipid fraction differed significantly among the eggs from the *Nannochloropsis* and *Scenedesmus* fed laboratory cultures and among the eggs obtained in the laboratory cultures and from the natural lake (Table 4).

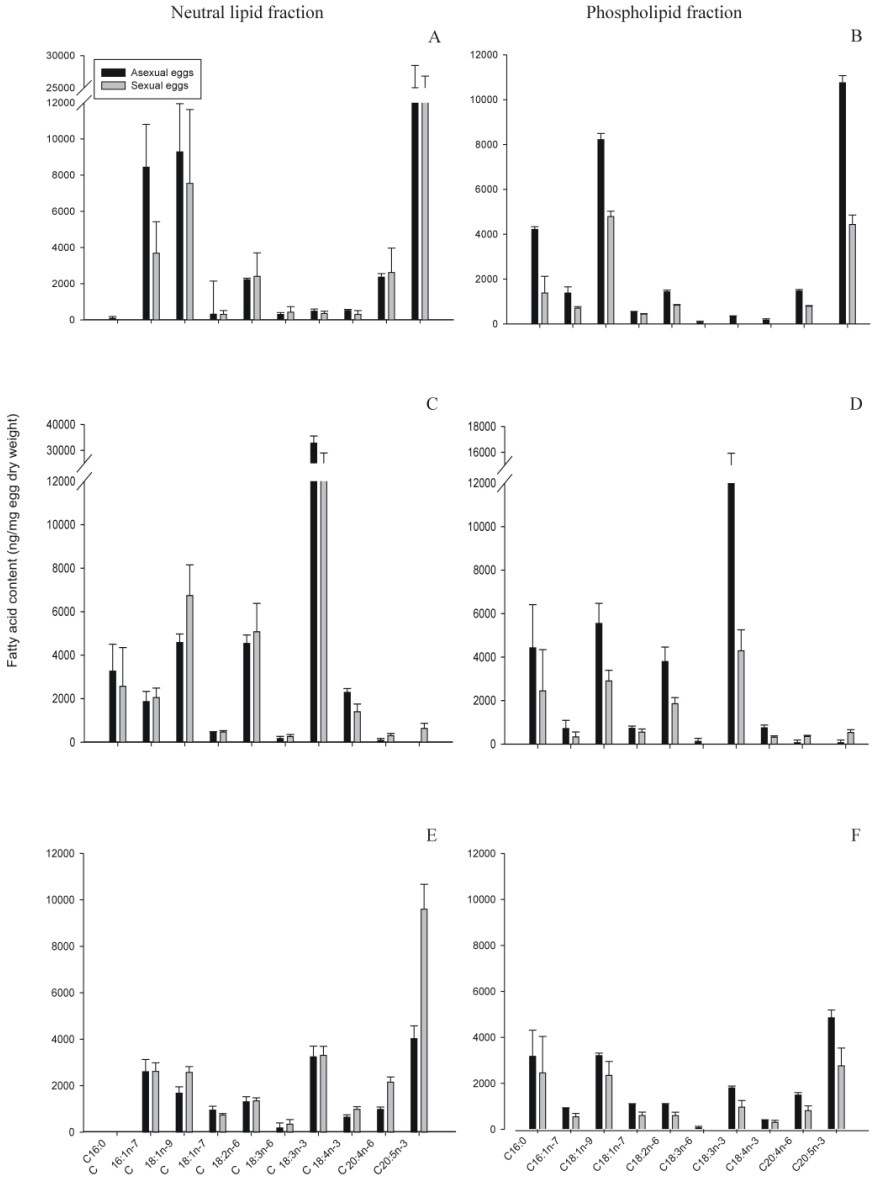

Figure 3: Fatty acid composition of sexual (grey bars) and asexual eggs (black bars) of *Daphnia magna* (ng mg^{-1} dry mass) produced on *Nannochloropsis limnetica* (A and B) and *Scenedesmus obliquus* (c and d), and of sexual and asexual eggs directly isolated from a natural pond (LRV) (E and F). The lipids were separated into a neutral lipid fraction (A, C, and E) and a phospholipid fraction (B, D, and F). Data are means of four replicates; error bars indicate one standard error.

Table 3: Results of MANOVA comparing the fatty acid composition of sexual and asexual *D. magna* eggs produced on *Nannochloropsis limnetica* and *Scenedesmus obliquus*, and of sexual and asexual eggs directly isolated from a natural lake. Concentrations of the different fatty acids (ng mg^{-1} dry weight) were the dependent variables and type of food (*Nannochloropsis limnetica*, *Scenedesmus obliquus*, lake seston) and type of egg (sexual vs. asexual eggs) were the independent variables. Data sets for neutral lipids and phospholipids were analysed separately.

Neutral lipid fraction				**Phospholipid fraction**			
Variables	Df	F value	P value	Variables	Df	F value	P value
Food	2	37.259	**5.80E-10**	Food	2	165.503	**4.88E-15**
Egg type	1	3.142	0.069	Egg type	1	18.864	**0.0004**
Food x egg type	2	6.177	**0.0002**	Food x egg type	2	16.271	**2.95E-07**

Table 4: Results of contrast analyses following the MANOVA (Table 3) comparing the fatty acid composition of sexual and asexual *D. magna* eggs produced on *Nannochloropsis limnetica* and *Scenedesmus obliquus*, and of sexual and asexual eggs directly isolated from a natural lake. Upper part of the table contrasts both egg types for every food source; the lower part of the table contrasts the laboratory treatments with each other and both together against eggs isolated from the natural lake. Data on neutral lipid and phospholipid fractions were analysed separately.

Neutral lipid fraction				**Phospholipid fraction**			
Variables	Df	F value	P value	Variables	Df	F value	P value
Egg type under Scenedesmus	1	11.82	**0.003**	Egg type under Scenedesmus	1	11.816	**0.003**
Egg type under Nannochloropsis	1	3.855	**0.041**	Egg type under Nannochloropsis	1	32.961	**0.0004**
Egg type when isolated from lake	1	13.113	**0.003**	Egg type when isolated from lake	1	7.284	**0.007**
Nanno vs Scene	1	15.495	**0.002**	Nanno vs Scene	1	12.226	**0.002**
Lake vs Lab	1	10.151	**0.003**	Lake vs Lab	1	20.971	**0.0005**

The total concentration of lipids in the neutral fraction expressed per mg dry weight of the eggs is only influenced by food type, not by egg type (Table 5). Posthoc tests (Tukey's HSD) revealed no significant difference between the two laboratory treatments ($p = 0.1$), but the eggs from the laboratory cultures had significantly higher concentrations of fatty acids of neutral lipids than eggs isolated from nature ($p < p = 0.009$). When the data are expressed as concentrations of fatty acids per egg, we also observed a significant difference in the concentration of fatty acids extracted from the neutral fraction (Table 3, Appendix 1). The concentration of fatty acids derived from phospholipids in the eggs was

not affected by the food conditions the mothers were exposed to, but did differ between the two types of egg both for the amount of per unit biomass (Table 5) and per egg (Table 3, Appendix 1). There was also a significant food x egg type interaction for the total amount of phospholipids per egg (Table 3, Appendix 1). In the laboratory treatments, where high concentrations of dietary fatty acids were provided (Table 1), the total concentration of phospholipid-derived fatty acids was twice as high in the asexual eggs compared to the dormant eggs. The asexual eggs sampled from the lake contained 1.35 times more phospholipid-derived fatty acids than the dormant eggs sampled from the sediment (Table 2). Sexual and asexual eggs did not only differ in their total concentration of phospholipids; we also observed higher concentrations of almost every fatty acid we measured in the asexual compared to the sexual eggs. There are only two exceptions, namely C20:4n-6 and C20:5n-3, which were detected in higher concentrations in the sexual eggs of mothers reared on a *S. obliquus* diet (Figure 3, Table 2).

Table 5: Results of ANOVA comparing the total fatty acid concentrations of sexual and asexual *D. magna* eggs produced on *Nannochloropsis limnetica* and *Scenedesmus obliquus*, and of sexual and asexual eggs directly isolated from a natural lake. Concentrations of the total fatty acid contents (ng mg^{-1}) were the dependent variables and type of food (*Nannochloropsis limnetica*, *Scenedesmus obliquus*, lake seston) and type of egg (sexual vs. asexual eggs) were the independent variables. Data sets for neutral lipids and phospholipids were analysed separately.

Neutral lipid fraction				**Phospholipid fraction**			
Variables	Df	F value	P value	Variables	Df	F value	P value
Food	2	8.395	**0.003**	Food	2	2.156	0.145
Egg type	1	0.73	0.404	Egg type	1	33.792	**1.66E-05**
Food x egg type	2	1.154	0.338	Food x egg type	2	2.529	0.108

4 Discussion

D. magna females take up considerable amounts of fatty acids from their food and accumulate them as triacylglycerols or phospholipids (Goulden and Place, 1990; Becker and Boersma, 2005). Less than 2% of the accumulated fatty acids in daphnids have been reported to be synthesized de novo (Goulden and Place, 1990). Consequently, the fatty acid composition of somatic tissue (Von Elert, 2002; Brett et al., 2006; Martin-Creuzburg et al., 2010) and eggs (Abrusan et al., 2007; Schlotz et al., 2013) tend to strongly reflect that of their diet. This is confirmed by our data showing that in the laboratory treatments, in which the animals were cultured under standardized conditions and provided with high food concentrations, both kind of eggs contained fatty acids in relative abundances reflecting those of

the maternal food. This shows that food quality has important consequences for both energy storage (neutral lipids) and membrane composition (with phospholipids as their main component) in both sexual and asexual eggs.

In addition, we found that the concentrations and composition of fatty acids in both neutral lipids and phospholipids differed between the two egg types. As the main energy storage molecule in *Daphnia,* triglycerides (representing neutral lipids) are vital for proper development and for survival under starvation (Tessier et al., 1983; Peters, 1987). Although we observed slight differences in the composition of the fatty acids of these neutral lipids per mg dry weight between asexual and sexual eggs, we did not find differences in the concentration of the total amount of neutral lipids between egg types. On a per egg basis, the total amount of neutral lipids was found to be lower in sexual than in asexual eggs, reflecting that sexual eggs (7.7 µg) were lighter than asexual eggs (9.4 µg). Pauwels et al. (2007) did not find differences in the amount of triglycerides per egg between sexual eggs collected from the sediment and asexual eggs of mothers reared on *S. obliquus* in the laboratory, but their result might have been influenced by the fact that the two egg types had a different history. Asexual eggs always contained more phospholipid-bound fatty acids than sexual eggs when they were produced under the same conditions. The higher concentrations of fatty acids (neutral and phospholipid fraction) we observed in asexual compared to sexual eggs is inconsistent with the results of Abrusan et al. (2007), who reported that the total fatty acid content in sexual eggs of *D. pulicaria* is much higher than in asexual eggs when produced on a *S. obliquus* diet.

Asexual eggs facilitate fast population growth and are produced under favourable conditions, while sexual eggs are the vector for dispersal in time and space. As a result, in contrast to asexual eggs, sexual eggs often encounter stressful environments and must be able to survive for long periods (Hairston et al., 1995; De Meester et al., 2004). This is expected to be associated with a higher concentration of energy storage molecules and with a specific fatty acid composition of the membranes. The results of Pauwels et al. (2007) and Abrusan et al. (2007) reporting differences in biochemical composition between dormant and parthenogenetic eggs are consistent with these expectations, as are the results of Arbaciauskas and Lampert (2003), who showed that the offspring from dormant and parthenogenetic eggs differ in life history traits. In accordance with Abrusan et al. (2007), we found that *D. magna* maintain a certain concentration of long-chain PUFA in their sexual eggs even when they are not provided by the food. In both neutral lipids and phospholipids, we found ARA (C20:4 n-6) and EPA (C20:5 n-3) in higher concentrations in sexual eggs than in asexual eggs when they were produced on a *S. obliquus* diet, which lacks both ARA and EPA. It is already known that ARA and EPA, presumably in their capacity to serve as eicosanoid precursors (Stanley, 2006), are important for reproduction in *Daphnia* (Becker and Boersma, 2005; Wacker and Martin-Creuzburg, 2007; Martin-Creuzburg et al., 2010). The higher allocation of ARA and EPA together with the

finding that EPA supplementation increases resting egg production (Abrusan et al., 2007), suggests that C20 PUFA are very important to produce viable resting eggs. It is well-established that these long-chain PUFA are important in maintaining the integrity of cell membranes. Crustacea are capable of adapting the fluidity of their membranes by changing the proportions of saturated and unsaturated fatty acids (Priutt, 1990). In addition, PUFA are crucial for acclimatization to cold temperatures (Hazel and Williams, 1990; Masclaux et al., 2009) and supplementation of a C20-PUFA deficient diet (*S. obliquus*) with ARA or EPA has been shown to increase population growth rates, in particular at colder temperatures, suggesting that PUFA requirements of *Daphnia magna* increase with decreasing temperature (Martin-Creuzburg et al., 2012). The above functions might play a crucial role in the survival of sexual eggs during harmful conditions and as asexual eggs do not need to cope with these stressors this might also explain the higher allocation of EPA and ARA towards sexual eggs.

The amount and composition of fatty acids in the field differed considerably from that of laboratory grown algae, which contained high levels of key fatty acids, and these differences are reflected in the fatty acids retrieved from the eggs. For fatty acids derived from phospholipids, there were no significant differences between the concentrations in eggs collected from the pond and those produced in the laboratory. Total fatty acid concentration of the neutral fraction was lower in eggs collected from the pond than in eggs produced in the laboratory. In accordance with Tessier et al. (Tessier et al., 1983), these results suggest that the allocation of neutral lipids, i.e. energy reserves, into the eggs increases with the dietary lipid availability. The differences may, however, in part also be due to other conditions that differed in the field compared to the laboratory. For example, temperature encountered by the mothers may have an influence on the fatty acid composition of somatic tissues (Sperfeld and Wacker, 2011; Martin-Creuzburg et al., 2012) and, as a consequence, potentially also on the allocation of fatty acids into the eggs. In addition, food conditions in the field may have differed during periods of dormant egg production and the active growth period. In a lake there are typically two peaks of dormant egg production during conditions of low food quantity (clear water phases) following a population peak, while asexual eggs may be produced during more favourable conditions (Sommer et al., 1986; Alekseev and Lampert, 2001). During these clear water phases the lake seston is dominated by PUFA-rich algae, mostly diatoms and cryptophytes (Alghren et al., 1992; Müller-Navarra et al., 2004; Hartwich et al., 2012), and consequently there might be a higher dietary PUFA availability during the production of sexual eggs than during asexual reproduction.

We conclude that both food quality and distinct allocation strategies influence the fatty acid composition of asexual and sexual eggs of *Daphnia*, with asexual eggs in general having higher concentrations of fatty acids than sexual eggs. The fatty acid composition of both asexual and sexual eggs largely reflected the fatty acid profile of the maternal food, but with an enrichment of specific long-chain PUFA, especially ARA and EPA, in the sexual eggs when the mothers were fed a diet

lacking long-chain PUFA. We propose that these PUFA, presumably together with other factors, such as heat shock proteins and glycerol (Pauwels et al., 2007), are involved in mediating the striking resistance of *Daphnia* dormant eggs to harsh environmental conditions, including exposure to cold temperatures.

Acknowledgments

AP enjoys a PhD fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen), and the work was financially supported by KU Leuven Research Fund project PF/2010/007. We thank P. Merkel for technical assistance and M. Schepens and S. Navis for help during the experiments.

References

Abrusan, G., Fink, P. and Lampert, W. (2007) Biochemical limitation of resting egg production in *Daphnia*. *Limnology and oceanography,* **52**, 1724-1728.

Ahlgren, G., Gustafsson, I.-B. and Boberg, M. (1992) Fatty acid content and chemical composition of freshwater microalgae. *Journal of Phycology,* **28**, 37-50.

Alekseev, V. and Lampert, W. (2001) Maternal control of resting-egg production in *Daphnia*. *Nature,* **414**, 899-901.

Arbaciauskas, K. and Lampert, W. (2003) Seasonal adaptation of ex-ephippio and parthenogenetic offspring of *Daphnia magna*: differences in life history and physiology. *Functional Ecology,* **17**, 431-437.

Becker, C. and Boersma, M. (2005) Differential effects of phosphorus and fatty acids on *Daphnia* growth and reproduction. *Limnology and oceanography,* **50**, 388-397.

Brendonck, L. and De Meester, L. (2003) Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia,* **491**, 65-84.

Brett, M. T. (1993) Resource quality effects on Daphnia longispina offspring fitness. *Journal of Plankton Research,* **15**, 403-412.

Brett, M. T., Müller-Navarra, D. C., Ballantyne, A. P., Ravet, J. L. and Goldman, C. R. (2006) Daphnia fatty acid composition reflects that of their diet. *Limnology and oceanography,* **51**, 2428-2437.

Cáceres, C. E. (1998) Interspecific Variation in the Abundance, Production, and Emergence of *Daphnia* Diapausing Eggs. *Ecology,* **79**, 1699-1710.

Clegg, J. S. (1997) Embryos of *Artemia fansciscana* survive four years of continous anoxia: the case for complete metabolic rate depression. *The Journal of Experimental Biology,* **200**, 467-475.

De Meester, L., Gomez, A. and Simon, J. (2004) Evolutionary and ecological genetics of cyclical parthenogens. In: A. Moya and E. Font (eds) *Evolution from molecules to ecosystems.* Oxford university press, pp. 122-134.

De Meester, L. and Jager, H. (1993) Hatching of *Daphnia* sexual eggs. I. Intraspecific differences in the hatching responses of D. magna eggs. *Freshwater biology,* **30**, 219-226.

Decaestecker, E., De Meester, L. and Mergeay, J. (2009) Cyclical parthenogenesis in *Daphnia*: sexual versus asexual reproduction. In: I. Schön, K. Martens and P. Dijk (eds) *Lost Sex.* Springer, pp. 295-316.

Denlinger, D. L. (2002) Regulation of diapause. *Annual Review of Entomology,* **47**, 93-122.

Frisch, D., Morton, P. K., Chowdhury, P. R., Culver, B. W., Colbourne, J. K., Weider, L. J. and Jeyasingh, P. D. (2014) A millennial scale chronicle of evolutionary responses to cultural eutrophication in *Daphnia*. *Ecology letters,* **17**, 360-368.

Gliwicz, Z. M. and Guisande, C. (1992) Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia,* **91**, 463-467.

Goulden, C. E. and Place, A. R. (1990) Fatty acid synthesis and accumulation rates in daphniids. *Journal of Experimental Zoology,* **256**, 168-178.

Guillard, R. (1975) Cultures of phytoplankton for feeding of marine invertebrates. In: W.L. Smith and M.H. Chanley (eds) *Culture of marine invertebrate animals.* Plenum Press New York, pp. 29 - 60.

Hairston, N. G., Van Brunt, R. A., Kearns, C. M. and Engstrom, D. R. (1995) Age and Survivorship of Diapausing Eggs in a Sediment Egg Bank. *Ecology,* **76**, 1706-1711.

Hartwich, M., Martin-Creuzburg, D. Rothhaupt, K.O., Wacker, A. (2012) Oligotrophication of a large, deep lake alters food quantity and quality constraints at the primary producer–consumer interface. *Oikos,* **121**, 1702-1712.

Hazel, J. R. and Williams, E. E. (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in lipid research,* **29**, 167-227.

Heckmann, L., Sibly, R., Timmermans, M. and Callaghan, A. (2008) Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. *Frontiers in Zoology,* **5**, 1-11.

Kainz, M., Arts, M. T. and Mazumder, A. (2004) Essential fatty acids in the planktonic food web and their ecological role for higher trophic levels. *Limnology and oceanography,* **49**, 1784-1793.

Kast-Hutcheson, K., Rider, C. V. and Leblanc, G. A. (2001) The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environmental Toxicology and Chemistry,* **20**, 502-509.

Leonard, A. E., Pereira, S. L., Sprecher, H. and Huang, Y.-S. (2004) Elongation of long-chain fatty acids. *Progress in lipid research,* **43**, 36-54.

Martin-Creuzburg, D., Sperfeld, E. and Wacker, A. (2009) Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids. *Proceedings of the Royal Society B: Biological Sciences,* **276**, 1805-1814.

Martin-Creuzburg, D., Wacker, A. and Basen, T. (2010) Interactions between limiting nutrients: Consequences for somatic and population growth of *Daphnia magna*. *Limnology and oceanography,* **55**, 2597-2607.

Martin-Creuzburg, D., Wacker, A. and Von Elert, E. (2005) Life history consequences of sterol availability in the aquatic keystone species *Daphnia*. *Oecologia,* **144**, 362-372.

Martin Creuzburg, D., Wacker, A., Ziese, C. and Kainz, M. (2012) Dietary lipid quality affects temperature-mediated reaction norms of a freshwater key herbivore. *Oecologia,* **168**, 901 - 912.

Masclaux, H., Bec, A., Kainz, M. J., Desvilettes, C., Jouve, L. and Bourdier, G. (2009) Combined effects of food quality and temperature on somatic growth and reproduction of two freshwater cladocerans. *Limnology and oceanography,* **54**, 1323-1332.

Muller-Navarra, D., Brett, M., Liston, A. and Goldman, C. (2000) A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature,* **403**, 74 - 77.

Muller-Navarra, D. C., Brett, M. T., Park, S., Chandra, S., Ballantyne, A. P., Zorita, E. and Goldman, C. R. (2004) Unsaturated fatty acid content in seston and tropho-dynamic coupling in lakes. *Nature,* **427**, 69-72.

Pauwels, K., Stoks, R., Verbiest, A. and De Meester, L. (2007) Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*. *Hydrobiologia,* **594**, 91-96.

Persson, J. and Vrede, T. (2006) Polyunsaturated fatty acids in zooplankton: variation due to taxonomy and trophic position. *Freshwater biology,* **51**, 887-900.

Peters, R. H. (1987) Metabolism in *Daphnia*. In: R. H. Peters and R. De Bernardi (eds) *Daphnia.* Vol. 45. Memorie dell'Instituto Italiano di Idrobiologia, pp. 193-243.

Provasoli, U. (1968) Media and prospects for the cultivation of marine algae. In: A. Watanabe and A. Hattori (eds) *Cultures and Collections of Algae.* Japan Society PIant Physiology, pp. 63-75.

Pruitt, N. L. (1990) Adaptations to temperature in the cellular membranes of crustacea: membrane structure and metabolism. *Journal of Thermal Biology,* **15**, 1-8.

Schlotz, N., Ebert, D. and Martin-Creuzburg, D. (2013) Dietary supply with polyunsaturated fatty acids and resulting maternal effects influence host - parasite interactions. *BMC Ecology,* **13**, 41.

Sommer, U., Gliwicz, Z. M., Lampert, W. and Duncan, A. (1986) The PEG-model of seasonal succession of planktonic events in fresh waters. *Archiv für Hydrobiologie,* **106**, 433-471.

Sperfeld, E. and Wacker, A. (2011) Temperature- and cholesterol-induced changes in eicosapentaenoic acid limitation of *Daphnia magna* determined by a promising method to estimate growth saturation thresholds. *Limnology and oceanography,* **56**, 1273-1284.

Stanley, D. (2006) Prostaglandins and other eicosanoids in insects: biological significance. *Annual Review Entomology,* **51**, 25-44.

Stibor, H. (2002) The Role of Yolk Protein Dynamics and Predator Kairomones for the Life History of *Daphnia magna*. *Ecology,* **83**, 362-369.

Stillwell, W. and Wassall, S. R. (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chemistry and Physics of Lipids,* **126**, 1-27.

Tessier, A. J., Henry, L. L. and Goulden, C. E. (1983) Starvation in Daphnia: Energy reserves and reproductive allocation. *Limnology and oceanography,* **28**, 667-676.

Valentine, R. C. and Valentine, D. L. (2004) Omega-3 fatty acids in cellular membranes: a unified concept. *Progress in lipid research,* **43,** 383-402.

Von Elert, E. (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnol Oceanogr,* **47**, 1764-1773.

Von Elert, E., Martin-Creuzburg, D. and Le Coz, J. R. (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London. Series B: Biological Sciences,* **270**, 1209-1214.

Wacker, A. and Martin-Creuzburg, D. (2007) Allocation of essential lipids in *Daphnia magna* during exposure to poor food quality. *Funct Ecol,* **21**, 738-747.

Walne, P. R. (1965) Experimental rearing of the larvae of *Ostrea edulis* L. in the laboratory. *Fishery Investment,* **20,** 1-23.

Zaffagnini, F. (1987) Reproduction in *Daphnia*. In: R. H. Peters and R. De Bernardi (eds) *Daphnia.* Vol. 45. Memorie dell'Instituto Italiano di Idrobiologia, pp. 245-284.

Zhu, G.-Y., Geuns, J. M. C., Dussert, S., Swennen, R. and Panis, B. (2006) Change in sugar, sterol and fatty acid composition in banana meristems caused by sucrose-induced acclimation and its effects on cryopreservation. *Physiologia Plantarum,* **128**, 80-94.

Appendix 1

Table 1: Results of MANOVA comparing the fatty acid composition of sexual and asexual *D. magna* eggs produced on *Nannochloropsis limnetica* and *Scenedesmus obliquus*, and of sexual and asexual eggs directly isolated from a natural lake. Concentrations of the different fatty acids (ng egg^{-1}) were the dependent variables and type of food (*Nannochloropsis limnetica*, *Scenedesmus obliquus*, lake seston) and type of egg (sexual vs. asexual eggs) were the independent variables. Data sets for neutral lipids and phospholipids were analysed separately.

Neutral lipid fraction				**Phospholipid fraction**			
Variables	Df	F value	P value	Variables	Df	F value	P value
Food	2	32.784	**1.55E-09**	Food	2	89.769	**6.13E-13**
Egg type	1	3.027	0.075	Egg type	1	25.478	**0.0001**
Food x egg type	2	5.666	**0.0004**	Food x egg type	2	15.099	**5.08E-07**

Table 2: Results of contrast analyses following the MANOVA (Table 1, Appendix 1) comparing the fatty acid composition of sexual and asexual *D. magna* eggs produced on *Nannochloropsis limnetica* and *Scenedesmus obliquus*, and of sexual and asexual eggs directly isolated from a natural lake. Upper part of the table contrasts both egg types for every food source; the lower part of the table contrasts the laboratory treatments with each other and both together against eggs isolated from the natural lake. Data on neutral lipid and phospholipid fractions were analysed separately.

Neutral lipid fraction				**Phospholipid fraction**			
Variables	Df	F value	P value	Variables	Df	F value	P value
Egg type under Scenedesmus	1	10.769	**0.005**	Egg type under Scenedesmus	1	14.718	**0.002**
Egg type under Nannochloropsis	1	3.814	**0.042**	Egg type under Nannochloropsis	1	81.713	**7.48E-06**
Egg type when isolated from lake	1	11.929	**0.005**	Egg type when isolated from lake	1	7.040	**0.008**
Nanno vs Scene	1	13.345	**0.002**	Nanno vs Scene	1	60.682	**1.39E-05**
Lake vs Lab	1	10.139	**0.003**	Lake vs Lab	1	17.311	**0.0005**

Table 3: Results of ANOVA comparing the total fatty acid concentrations of sexual and asexual *D. magna* eggs produced on *Nannochloropsis limnetica* and *Scenedesmus obliquus*, and of sexual and asexual eggs directly isolated from a natural lake. Concentrations of the total fatty acid contents (ng egg^{-1}) were the dependent variables and type of food (*Nannochloropsis limnetica*, *Scenedesmus obliquus*, lake seston) and type of egg (sexual vs. asexual eggs) were the independent variables. Data sets for neutral lipids and phospholipids were analysed separately.

Neutral lipid fraction				**Phospholipid fraction**			
Variables	Df	F value	P value	Variables	Df	F value	P value
Food	2	9.392	**0.002**	Food	2	2.474	**0.112**
Egg type	1	4.843	**0.041**	Egg type	1	38.088	**7.95E-06**
Food x egg type	2	3.6176	**0.048**	Food x egg type	2	5.897	**0.011**

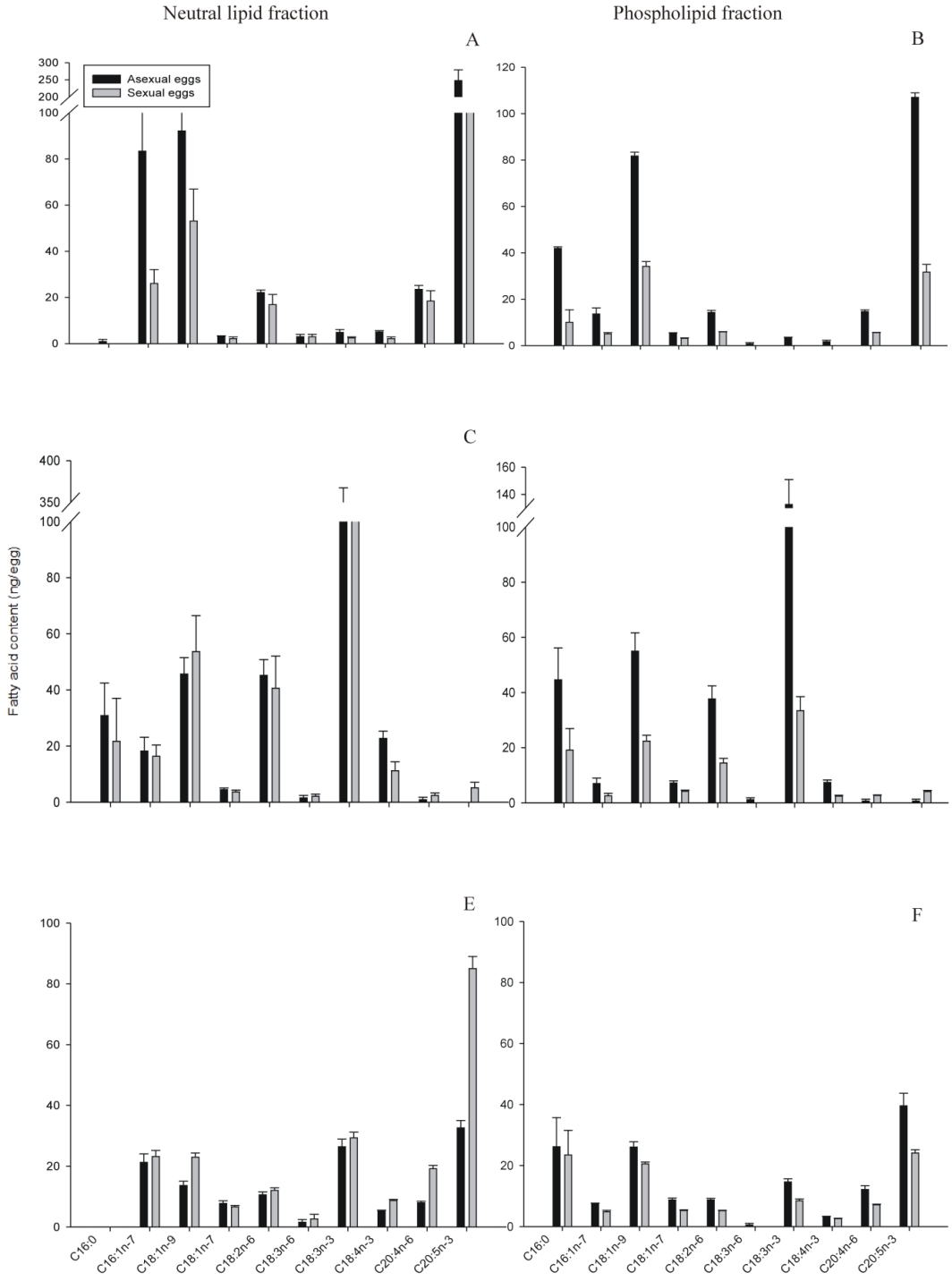

Figure 1: Fatty acid composition of sexual (grey bars) and asexual eggs (black bars) of *Daphnia magna* (ng egg^{-1}) produced on *Nannochloropsis limnetica* (A and B) and *Scenedesmus obliquus* (c and d), and of sexual and asexual eggs directly isolated from a natural pond (LRV) (E and F). The lipids were separated into a neutral lipid fraction (A, C, and E) and a phospholipid fraction (B, D, and F). Data are means of four replicates; error bars indicate one standard error.

Addendum

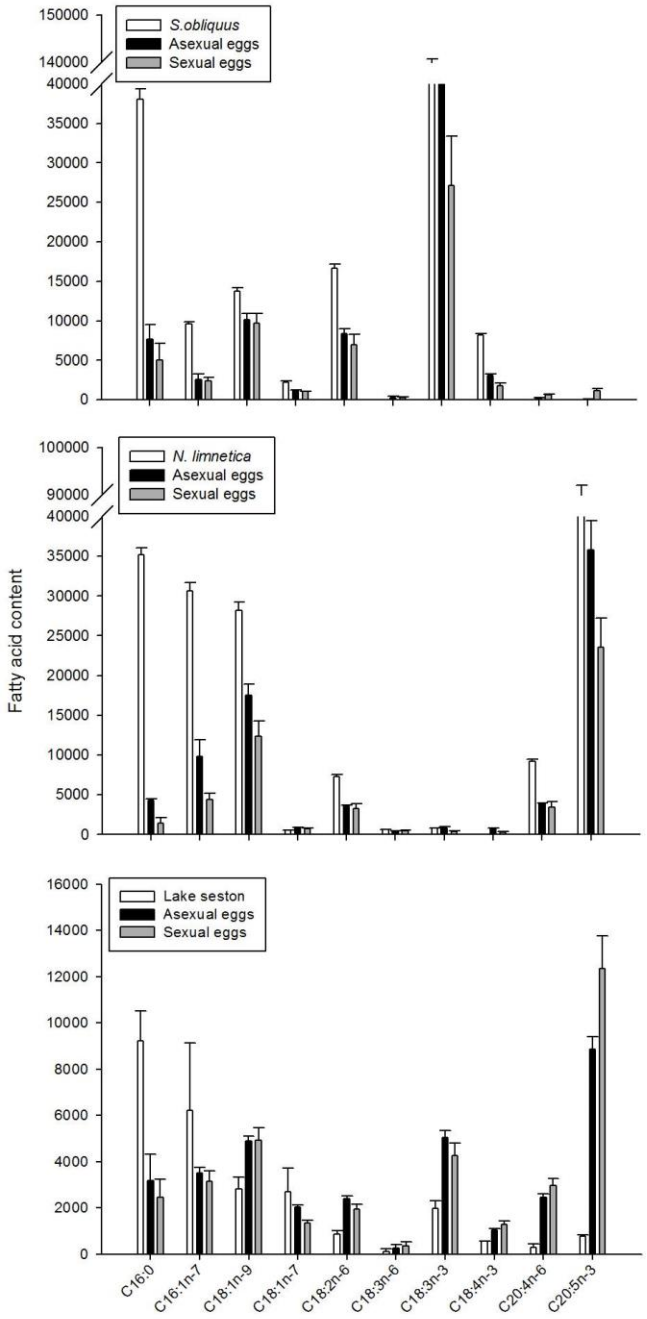

Figure 1: Fatty acid composition of food organisms (white bars) expressed in ng mg^{-1} carbon and of asexual (black bars) and sexual eggs (grey bars) expressed in ng mg^{-1} egg dry mass, illustrating the similar pattern found in the maternal food and the produced eggs. Data are means of four replicates; error bars indicate one standard error.

Chapter 2

Strong differences in trehalose levels between sexual and asexual eggs of the water flea *Daphnia magna*

ADINDA PUTMAN, EVELYNE VANVLASSELAER, BART PANIS, PATRICK VAN DIJCK AND LUC DE MEESTER

Abstract

Like many other aquatic organisms, the water flea *Daphnia magna* has to cope with fluctuating environmental conditions and challenges to colonize new habitats. The production of dormant eggs that are resistant to drying and freezing is important in helping them to overcome these problems. During favorable conditions, cyclic parthenogenetic *Daphnia* produce asexual eggs that develop immediately, whereas they switch to the production of sexual eggs when conditions start to deteriorate. These sexual eggs are dormant, can survive extreme environmental conditions and form a dormant egg bank where they can stay viable for decades. Resistance to drought and freezing requires special adaptations. Trehalose is an important compound for increasing desiccation tolerance in many anhydrobiotic organisms. We therefore compared sugar profiles of sexual and asexual eggs of *D. magna*. Trehalose accounts for 4.15% of the dry weight of sexual eggs, while asexual eggs only contain 0.006% trehalose, an almost 700 times difference. We tested whether we could increase the amount of trehalose in the asexual eggs by supplementation of the maternal food with trehalose containing liposomes. While supplementation lead to a tendency for higher levels of trehalose in the asexual eggs compared to control conditions, the difference was not significant and levels remained >200 times lower than those of sexual eggs. We also compared trehalose content in young and older dormant eggs and found no significant differences. Finally, we monitored concentrations of trehalose as the sexual egg develops, and found that the developing eggs start to metabolize trehalose at 18h of development.

1 Introduction

As desiccation presents a serious stress for all organisms, some have developed a strategy to cope with it. Several species can tolerate extreme desiccation by interrupting their life cycle at a certain stage and temporally enter a so-called anhydrobiotic state. This state is defined as an extremely dehydrated ametabolic state in which organisms retain the ability to resume life after rehydration (Alpert, 2006). Among a variety of species, ranging from unicellular organisms (Potts, 1994) to plants (Pampurova and Van Dijck, 2014), invertebrates having this ability include rotifers, nematodes, insect larvae, tardigrades, chironomid larvae, statoblasts of freshwater bryozoans and a range of aquatic crustaceans (Clegg, 1965; Madin and Crowe, 1975; Womersley and Smith, 1981; Watanabe et al., 2002; Tunnacliffe and Lapinski, 2003; Kikawada et al., 2005; Hengherr et al., 2008; Hengherr and Schill, 2011; Welnicz et al., 2011;).

Tolerance of severe dehydration is often associated with the presence or accumulation of the non-reducing disaccharide trehalose (Clegg, 2001), which is especially important for long term survival upon desiccation (Tapia and Koshland, 2014). In anhydrobiotic cysts of the crustacean *Artemia franciscana*, the nematode *Aphelencus avenae* and the chironomid larvae *Polypedilum vanderplankii*, the concentration of trehalose can reach levels as high as 15% of their dry weight (Clegg, 1965; Watanabe et al., 2002; Kikawada et al., 2005). Trehalose serves as an energy and carbon reserve, and during dehydration it is able to stabilize proteins in their native state, to preserve the integrity of membranes, and to protect from damage by oxygen radicals (Benaroudj et al. 2001; Elbein et al., 2003; Crowe, 2007). There are many postulated mechanisms of action for trehalose, but these are not mutually exclusive (Crowe et al., 2001; Sakurai et al., 2008). The water replacement hypothesis states that trehalose forms hydrogen bonds with macromolecules, replacing the water molecules during dehydration thereby stabilizing proteins and membranes (Crowe et al., 1987).Trehalose can also lower the phase transition temperature of the membrane by interacting with the polar head groups of the phospholipids, as is shown for the anhydrobiotic cysts of *Artemia* (Hontoria et al., 1998). A second hypothesizes suggests that the sugar trehalose has the capacity to form a biological glass that protects all macromolecules within its matrix (Sun and Leopold, 1997). Glassy states have not exclusively been found when high concentrations of trehalose are present. Several species of tardigrades and *Triops* accumulate only low amounts of trehalose (<1% dry weight) and still undergo vitrification (the process of forming a glass) during dehydration (Hengherr et al., 2008; Hengherr et al., 2011).

The invertebrate model organism *Daphnia magna* faces several challenges concerning desiccation. In their natural habitat they often encounter fluctuating environmental conditions and populations that inhabit temporary waters have to cope with a regular or occasional dry period. In addition, inland water bodies present a challenge with respect to the colonization of new habitats, as there are often no direct water connections between suitable habitats. Despite these challenges, many *Daphnia* species

are widely dispersed over a broad geographical range (Hebert, 1978) and are able to colonize even small isolated ponds. They cope with these problems of dispersal in time and space through their cyclic parthenogenetic reproduction cycle. *Daphnia* alternates the production of asexual eggs that develop immediately and sexual eggs that first go through a dormant stage (Brendonck and De Meester, 2003). These sexual eggs are encased in a protective envelope, the ephippium, and are deposited in the sediment where they form a dormant egg bank and can stay viable for decades (De Meester et al., 2004). After hatching, they reproduce asexually as long as the conditions are favorable, which allows for rapid population growth. By the end of the growing season, when the animals are exposed to deteriorating environmental conditions, *Daphnia* start again producing sexual, dormant eggs (De Meester et al., 2004; Hebert, 1978). Sexual eggs require special adaptations in order to be resistant to desiccation, extreme temperatures and digestion by animals during diapause and passive dispersal (Brendonck and De Meester, 2003; Radzikowski, 2013).

The resistance to desiccation and freezing during diapause requires special adaptations. Pauwels et al. (2007) reported that the dormant eggs of *Daphnia magna* contain more glycerol and heat shock proteins than asexual eggs. They both play a role in the protection of cell metabolism under stress conditions and both have also been found in anhydrobiotic cysts of *Artemia fransicana* (Clegg, 1997) and certain insects (Denlinger, 2002). Another compound important for desiccation tolerance of anhydrobiotic organisms and dormant stages is trehalose. Hengherr et al. (2011) reported trehalose in the dormant eggs of *D. magna, D. pulex* and referred to its role in the dehydration tolerance of these eggs. In the present study, we compare sugar concentrations in sexual and asexual eggs of *Daphnia magna* that were cultured under standardized laboratory conditions. Second, we assess whether trehalose levels in asexual eggs can be increased by feeding the *Daphnia* trehalose-loaded liposomes. Third, we compare trehalose content of young and older dormant eggs isolated from nature to quantify whether trehalose levels decline with age. Finally, we determine how long trehalose concentrations in sexual eggs stay high in developing embryos and when they start to metabolize trehalose, by quantifying trehalose content in sexual eggs at pre-set times after the eggs start developing.

2 Material and methods

2.1 Cultivation of algae

For all the experiments we used the green alga *Scenedesmus obliquus*, which is well-assimilated by *Daphnia* (Von Elert, 2002). Algae were cultivated in batch in aerated 10l vessels with illumination at 170 µmoles $m^{-2}s^{-1}$ at 18°C and harvested in the late-exponential growth phase. The culturing medium consisted of 10 ml l^{-1} of enriched seawater nutrients (Provasoli, 1968), 5 ml l^{-1} of Walne nutrients (Walne, 1965) and the vitamins B1, B12 and H. Food suspensions were prepared by concentrating the cells via centrifugation (4000rpm, 5min) followed by resuspension in tap water. Cell densities of the

food suspensions were counted with an Attune® acoustic focusing cytometer (Life technologies, Carlsbad, CA, USA). To analyse the sugar composition of the algal food source, 3 replicate samples of 50ml of a 1,000,000 cells ml^{-1} suspension of *S. obliquus* were freeze-dried and 1g dry weight was used for the analysis.

2.2 Sugar profiles of sexual and asexual eggs

To compare the biochemical composition between sexual and asexual eggs, we conducted an experiment in the laboratory to produce these eggs under experimental conditions that generate the same food quality. To obtain asexual eggs, per treatment five replicate 1L jars were set up for each of the 10 clones hatched from the sediment egg bank of 'Langerode vijver', a pond in Neerijse, Belgium (0°49'42.32"N, 4°38'21.49"O). In each jar, 15 individuals were cultured under standard conditions (20 ± 2°C and a photoperiod of 16L:8D). All cultures were fed with *S. obliquus* at an algal cell density of 150.000 cells ml^{-1}. Jars were cleaned every two days and food was renewed daily to keep algal concentration above the incipient limiting level. Females bearing their third clutch were dissected to collect asexual eggs in the first stage of daphnid embryonic development (according to Kast-Hutcheson et al. (2001)). From each of the five replicates, one sample of 100 eggs (10 eggs per clone) was collected and stored at -80°C.

We also induced sexual reproduction using the same clones. For this we reared 20 individuals per clone under standard conditions (20 ± 2°C, a photoperiod of 16L:8D and an algal cell density of 150.000 cells ml^{-1}). After they had released their second clutch, the densities were raised to 40 animals (four per clone) in a one liter jar to induce crowding. The feeding level was raised to 250.000 cells ml^{1}. This was done in five replicates. The photoperiod was switched between long-day (16L:8D) during five days and short-day (8L:16D) photoperiod during two days, as this stimulates sexual reproduction in *Daphnia* (De Meester and Jager, 1993). Once a week half the medium was renewed and all dormant eggs were collected. For every replicate, dormant eggs were stored in Eppendorf tubes in the dark at 4°C for at least one month. After storage, these laboratory-derived ephippia were decapsulated and five replicate samples consisting of 100 decapsulated sexual eggs each were collected for the sugar analysis and stored at -80°C. All samples were freeze-dried and the dry weight was determined.

Samples were homogenized in 200 µl of ethanol 80%. After 15 min of incubation at room temperature they were centrifuged for 10 min at 14000 rpm. The supernatans was collected and vacuum-dried (AES2010, Savant). Afterwards the samples were resuspended in 200µl ultrapure Milli-Q water. To purify the sugars, the samples were added to a Dowex column (1:2 v/v 50WX8:1X8; Sigma-Aldrich). 1.2 ml of ultrapure Milli-Q water was used as an eluent, of which 500 µl was transferred to a HPLC vial for the AS50 autosampler (Dionex). Samples were analysed using a Carbopac PA-100 column

(Dionex), ED50 electrochemical detector (Dionex) and eluted with 100 mM NaOH using a GS50 gradient pump (Dionex). Trehalose and Glucose standards were determined in parallel to calculate the sugar concentrations in the samples.

Using the package car of the statistical software R (version 3.0.2) the effects of egg type (sexual and asexual eggs) on the concentration of individual sugars was tested with a one-way analysis of variance (ANOVA).

2.3 Sugar profile of asexual eggs after trehalose supplementation

We compared sugar profiles of asexual eggs from mothers that were supplemented in different amounts with trehalose in their food. We used four different trehalose supplementation treatments that represent factorial combinations of liposomes containing different concentrations of trehalose (2.5 mg ml^{-1} (L1) and 5 mg ml^{-1} (L2)) and being provided at two different concentrations (densities of 0.25 µl ml^{-1} (low) and 0.5 µl ml^{-1} (high)). The fifth treatment was a control in which no trehalose containing liposomes were added. For each treatment, three replicates of each time ten *Daphnia* were reared individually in 200 ml jars (replicated 3 times) under the same conditions as described above for the asexual eggs. For each replicate one sample of 100 asexual eggs in the first stage of daphnid embryonic development (according to Kast-Hutcheson et al. (2001)) was collected. In total, 15 samples (5 treatments x 3 replicates of 100 eggs) were freeze-dried and stored at -80°C.

Algae were supplemented with trehalose containing liposomes, using a protocol described in Martin-Creuzburg et al. (2009). The liposomes bilayer consisted of 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and 1-palmitoyl-2-oleoyl-phosphatidylcholin (POPC; Sigma-Aldrich). Trehalose containing liposomes were prepared by adding 2.66 ml of trehalose stock solution to 6 mg POPG and 14 mg POPC dissolved in ethanol. Two different stock solutions were used containing trehalose concentrations of 2.5 mg ml^{-1} or 5 mg ml^{-1} ethanol. The resulting solutions were dried using a speed vac, dissolved in 12 ml buffer (20 mmol l^{-1} NaPi, 150 mmol l^{-1} NaCl, pH 7.0) and incubated on a rotary shaker (100 rpm) for 30 min. Subsequently, the liposome suspensions were sonicated in an ultrasonic bath. Excess free trehalose was removed by washing the liposomes in fresh buffer using an ultra-speed centrifuge (150 000 g, 90 min, 4°C). Afterwards the liposomes were resuspended in 20 ml of the liposome buffer. Prior to the addition of liposomes to the experimental beakers, the liposome stock suspensions were sonicated again (2 min). The liposome stock suspensions contained approximately $1X10^6$ liposomes ml^{-1} with a mean diameter of 6.6 µm. For the analysis of the sugar composition of these liposomes 3 replicate samples of 1 ml of the stock solution were used.

Chemical analysis was carried out as explained above.

For data-analysis, we used the package car of the statistical software R (version 3.0.2) and carried out a two-way ANOVA to test for differences between the effects of the food treatments (n=4) and a one-way ANOVA to test for the difference between the control conditions and the trehalose supplemented conditions on the concentration of individual sugars.

2.4 Sugar concentrations of young and old sexual eggs during diapause and early development

Several sediment cores, with a diameter of 15 cm and a maximum length of 20 cm, were taken from shallow lake 'Langerodevijver'. The cores were cut into sediment slices of 1 cm and stored in the dark at 4°C until further processing. "Young" and "old" ephippia were collected from the upper (1-8 cm, hatching rate: 70 - 100%) and lower (11-18 cm, hatching rate: < 40%) layers of the cores, respectively. The cores were not dated but with an average sedimentation rate of 0.3 – 1 cm per year in this type of eutrophic shallow systems, the dormant eggs in the lower section (11-18 cm) of the core are expected to be older than 15 years and likely more than 20 years old (Decaestecker et al. 2004). The young and old dormant eggs were decapsulated and four biological replicates of 100 young/old eggs were collected. Excess water was removed from the samples before freezing in liquid nitrogen and storage at -80°C. During all steps of sample acquisition precautions were taken to preserve the dormant state of the eggs. The sediment was stored in the dark at 4°C and the ephippia were collected and decapsulated on ice and illuminated only by monochromatic red light (λ = 620–750 nm).

Chemical analyses were performed using the same protocol as outlined above.

Using the package car of the statistical software R (version 3.0.2) the effect of egg type (old and young sexual eggs) on the concentrations of individual sugars was tested with a one-way ANOVA.

To study changes in trehalose content during dormancy termination and early development, 1600 young dormant eggs from the upper (1-8 cm, hatching rate: 70 – 100%) layers of the sediment core were exposed to standard hatching stimuli (20 ± 1°C, high light intensity, long day photoperiod: 16L/8D) (De Meester and Jager, 1993) for different periods of time (0, 2, 8 or 18 hours; a maximum exposure time of 18 hours was chosen because the eggs do not show morphological signs of differentiation until that time). The dormant eggs were decapsulated before exposure and the experiment was carried out in four biological replicates of 100 eggs per treatment. After exposure the eggs were collected, excess water was removed, the samples were frozen in liquid nitrogen and stored at -80°C.

Chemical analyses were again performed as outlined above.

The effect of time of development of sexual eggs (0h-2h-8h-18h) on the individual sugar concentrations was tested with a one-way ANOVA using the package car of the statistical software R (version 3.0.2). Differences between specific development times were tested with Tukey HSD posthoc tests.

3 Results

3.1 Sugar profile of sexual and asexual eggs

Both in the algae and egg samples, only two sugars were detected: glucose and trehalose. One gram of freeze-dried *Scenedesmus obliquus* contained 0.3 mmol trehalose and 1.69 mmol glucose.

In our experiment, in which sexual and asexual eggs were produced by the same clones reared on the same food, there is no significant difference between the glucose concentrations of sexual and asexual eggs of *D. magna* ($p = 0.1392$). Sexual eggs, however, contain higher concentrations of trehalose than asexual eggs (analysis per mg dry weight, $p = 4.882E^{-07}$; Figure 1). When the data are expressed as the amounts of sugars per egg, the differences are equally strong ($p_{glucose} = 0.7145$, $p_{trehlaose} = 1.836E^{-07}$; Figure 1).

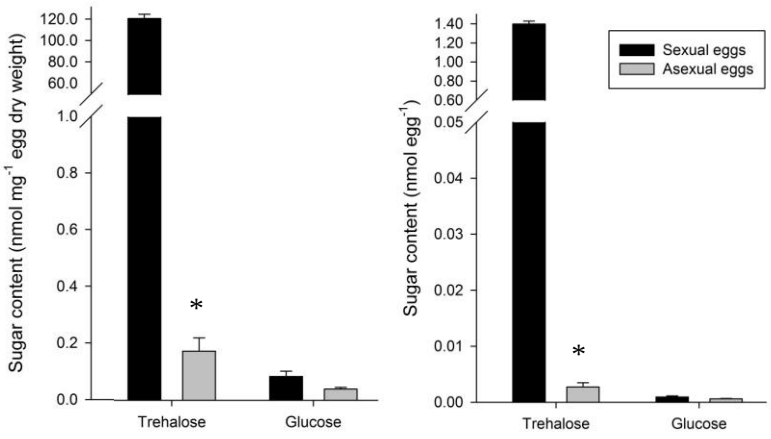

Figure 1: Sugar composition of sexual (black bars) and asexual eggs (grey bars) of *Daphnia magna* (Left panel: nmol mg^{-1} egg dry weight; right panel: nmol egg^{-1}) produced on *Scenedesmus obiquus*. Data are means of five replicates; error bars indicate one standard error. * indicates significant differences within one sugar type ($p < 0.05$, ANOVA).

3.2 Sugar profile of asexual eggs after trehalose supplementation

The liposomes used in this experiment contained 0.043 mg ml^{-1} (L1) and 0.12 mg ml^{-1} (L2) trehalose. Supplementing the food given to the *Daphnia* with trehalose containing liposomes resulted in a

tendency for increased trehalose concentrations in the eggs (Figure 2). The difference is, however, not significant ($p = 0.1475$; also not for glucose: $p = 0.1704$). There are no significant differences in trehalose concentrations of the eggs among the four liposome treatments ($p_{lipo\ food\ conc} = 0.9544$, $p_{lipo\ type} = 0.8290$).

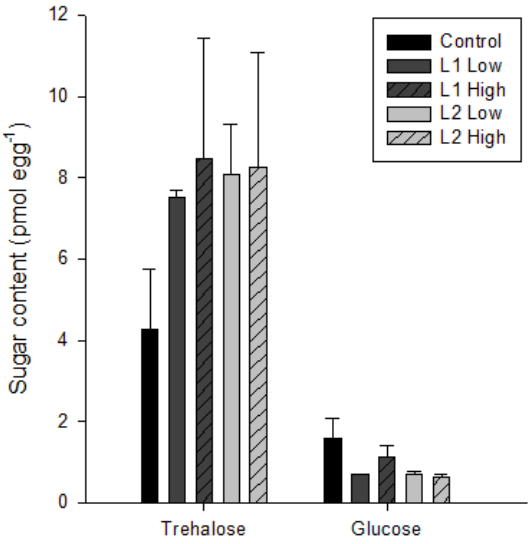

Figure 2: Sugar composition of asexual eggs of *Daphnia magna* (nmol egg^{-1}) produced on *Scenedesmus obliquus* (control) or on *S. obliquus* supplemented with liposomes containing 2.5 mg ml^{-1} (L1) or 5 mg ml^{-1} (L2) trehalose. The liposomes were fed to the *Daphnia* at a low (0.25 µl ml^{-1}) or high (0.5 µl ml^{-1}) concentration. Data are means of three replicates; error bars indicate one standard error. There are no significant differences among the treatments ($p < 0.05$, ANOVA).

3.3 Sugars of young and old sexual eggs during diapause and early development

We found no significant differences in sugar composition between old and young resting eggs (Figure 3; $p_{glucose} = 0.1271$, $p_{trehlaose} = 0.7711$).

For glucose the time of development has no influence on the concentration ($p = 0.7708$), but the concentration of trehalose significantly decreases at 18h of development (Table 1, Figure 3).

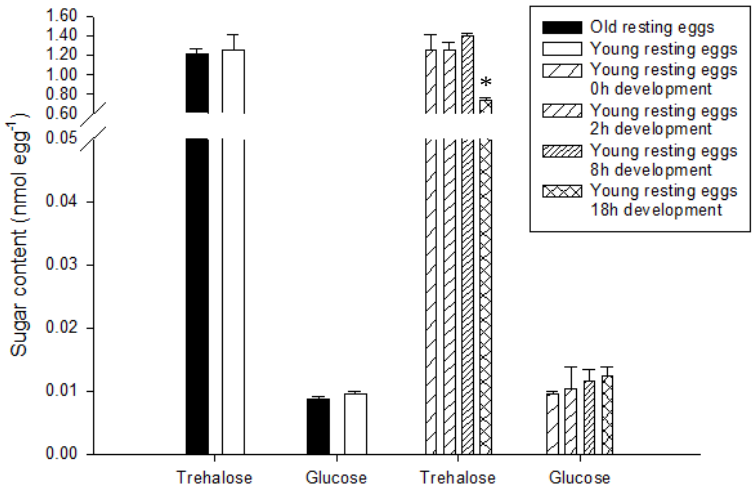

Figure 3: Trehalose concentration in old (black bars) and young (white bars) sexual eggs of *Daphnia magna* (nmol egg^{-1}) directly isolated from a natural pond. Hatched bars represent young eggs at different times of development. Data are means of five replicates; error bars indicate one standard error. * indicates significant differences within one sugar type ($p < 0.05$, ANOVA, followed by Tukey HSD posthoc test).

Table 1: Results of one-way ANOVA of the effect of time of development (0-2-8-18h) on the trehalose concentration in sexual eggs of *Daphnia magna* followed by a Tukey HSD posthoc test.

A) ANOVA

Variable	Df	F value	P value
Time of development	3	10.882	**0.001**

B) Tukey HSD Post hoc test

Time of development	0h	2h	8h	18h
0h	***	***	***	***
2h	1	***	***	***
8h	0.6875	0.68747	***	***
18h	**0.00636**	**0.00618**	**0.00115**	***

For this experiment we used sexual eggs directly derived from a natural pond, while in the previous experiment (section 3.1) we determined sugar composition of sexual eggs reared in the laboratory. To see if these environmental conditions have an influence on the sugar content of the eggs per unit mass we compared sugar concentrations of eggs from the laboratory and the field (Figure 4). We found that there is no difference in the trehalose concentration of eggs generated in the lab and those isolated

from the natural dormant egg bank (p = 0.3438). These two categories of eggs do, however, differ in their glucose concentrations ($p = 5.538E^{-08}$).

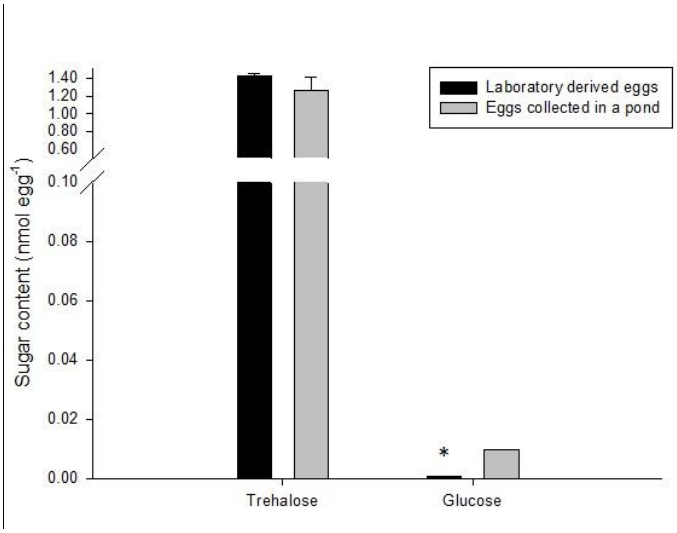

Figure 4: Sugar composition of sexual eggs of *Daphnia magna* generated under standard laboratory conditions with *Scenedesmus obiquus* as food (black bars) and collected directly from a pond (grey bars) (nmol egg^{-1}). Data are means of four replicates; error bars indicate one standard error. * indicates significant differences within one sugar type ($p < 0.05$, ANOVA).

4 Discussion

Sexual eggs are used as a vector for dispersal in time and space, so they often encounter stressful environments and must be able to survive for long periods of time (Hairston et al., 1995; Brendonck and De Meester, 2003). The results of earlier studies (Abrusan et al., 2007; Putman et al., 2015 (Chapter 1)) reported differences in the fatty acid composition between dormant and parthenogenetic eggs that are consistent with the differences in tolerance to drought and freezing of these two types of eggs. *D. magna* maintain a certain concentration of polyunsaturated fatty acids (PUFA) in their sexual eggs even when these are produced on a PUFA deficient diet. Especially the concentrations of eicosapentaenoic acid and arachidonic acid are higher in sexual eggs than in asexual eggs. Long chain PUFA are known to be able to maintain the integrity and fluidity of cell membranes (Pruitt, 1990) and are crucial for acclimatization to cold temperatures (Hazel and Williams, 1990; Masclaux et al., 2009). Additionally, Pauwels et al. (2007) reported that sexual eggs contain more glycerol and heat shock proteins than asexual eggs. They both fulfil a protective role in the cell during stress conditions and are also found in cysts of *Artemia fransicana* (Clegg et al., 1997). In accordance with these findings, our results in addition show that there are also substantial differences in the sugar composition of sexual and asexual eggs of *Daphnia magna*. Sexual eggs contain almost 700 times more trehalose per mg dry weight then asexual eggs produced under the same laboratory conditions. This seems to be an adaptive strategy to increase biochemical components improving resistance of dormant eggs, as the

concentrations of trehalose in parthenogenetic eggs remained several hundred times lower than those of sexual eggs irrespective of the amount of trehalose supplied by the food. Also similar amounts of trehalose were allocated to sexual eggs in nature as under standardized good food quality laboratory conditions.

The concentrations of trehalose we found in the resting eggs of *Daphnia magna* are slightly higher than those detected by Hengherr et al. (2011), but they are still low compared to anhydrobiotic cysts of *Artemia franciscana*, *Aphelencus avenae* and *Polypedilum vanderplankii* (Clegg, 1965; Watanabe et al., 2002; Kikawada et al., 2005). Also at relatively low concentrations, trehalose can play a valuable role in the ability of eggs or even adult organisms to survive desiccation. Comparable trehalose concentrations as the ones reported by us have also been found in anhydrobiotic organisms like monogonont rotifers and several tardigrade species (Caprioli, 2004; Hengherr et al., 2008) and in resting stages of *Triops* and freshwater Bryozoans (Hengerr et al., 2011; Hengherr and Schill, 2011). Low concentrations of this non-reducing disaccharide can still promote the formation of a glass, but it was shown by Hengherr et al. (2011) that for *D. pulex* there are no signs of a vitreous state during desiccation in resting eggs, so we can rule out the vitrification hypothesis (Hengerr et al., 2011; Hengherr and Schill, 2011). Yet there are some others ways in which trehalose can promote survival under drought and freezing conditions. Stabilization of membranes and proteins can be achieved by the formation of hydrogen bounds between the hydroxyl group of trehalose and the positively charged ends of proteins and phospholipids (Elbein, 2003). A synergism with other stress proteins, like Hsp 26 and Hsp 104, can also help to protect cells under stressful conditions (Elliott et al., 1996; Crowe et al., 2001; Viner and Clegg, 2001). Trehalose also has an important function as an energy storage molecule (Elbein et al., 2003). In accordance with the results for *A. franciscana* and *C. mucedo* the amount of trehalose declines during the early development of the resting stages, while during dormancy the level of trehalose remains constant, indicating that the developing organism uses trehalose as an early energy source (Clegg, 1965; Hengherr and Schill, 2011).

We conclude that the dormant eggs of *D. magna* have a much higher concentration of trehalose than the subitaneously developing parthenogenetic eggs, in line with the higher resistance to drying and freezing of the former. These differences in concentrations are very pronounced (x700) and are not very sensitive to food supply (no differences among dormant eggs isolated from nature and produced in the laboratory, no significant effect of trehalose supplementation during culture on trehalose levels of parthenogenetic eggs). Soon after development is initiated, the sexual eggs also start to metabolize the trehalose. This evidence adds to the biochemical properties that make sexual eggs more resistant, and which include higher concentrations of specific polyunsaturated fatty acids (Abrusan et al., 2007; Putman et al, 2015 (Chapter 1)) and glycerol and heat shock proteins (Pauwels et al., 2007).

Acknowledgements

AP enjoys a PhD fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). This work was financially supported by KU Leuven Research Fund grant PF2010/07. We thank Ben Souffriau and Nelson Avonce for technical assistance.

References

Abrusan, G., Fink, P. and Lampert, W. (2007) Biochemical limitation of resting egg production in *Daphnia*. *Limnology and oceanography,* **52,** 1724-1728.

Alpert, P. (2006) Constraints of tolerance: why are desiccation-tolerant organisms so small or rare? *Journal of Experimental Biology,* **209,** 1575-1584.

Benaroudj, N., Lee, D. H. and Goldberg, A. L. (2001) Trehalose Accumulation during Cellular Stress Protects Cells and Cellular Proteins from Damage by Oxygen Radicals. *Journal of Biological Chemistry,* **276,** 24261-24267.

Brendonck, L. and De Meester, L. (2003) Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia,* **491,** 65-84.

Caprioli, M., Krabbe Katholm, A., Melone, G., Ramløv, H., Ricci, C. and Santo, N. (2004) Trehalose in desiccated rotifers: a comparison between a bdelloid and a monogonont species. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **139,** 527-532.

Clegg, J. S. (1965) The origin of threhalose and its significance during the formation of encysted dormant embryos of Artmia salina. *Comparative Biochemistry and Physiology,* **14,** 135-143.

Clegg, J. S. (1997) Embryos of *Artemia fansciscana* survive four years of continous anoxia: the case for complete metabolic rate depression. *The Journal of Experimental Biology,* **200,** 467-475.

Clegg, J. S. (2001) Cryptobiosis — a peculiar state of biological organization. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **128,** 613-624.

Crowe, J. (2007) Trehalose As a "Chemical Chaperone". In: P. Csermely and L. Vígh (eds) *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks.* Vol. 594. Springer New York, pp. 143-158.

Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Aurell Wistrom, C. (1987) Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochemical Journal,* **242,** 1-10.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Tsvetkova, N., Wolkers, W. and Tablin, F. (2001) The Trehalose Myth Revisited: Introduction to a Symposium on Stabilization of Cells in the Dry State. *Cryobiology,* **43,** 89-105.

De Meester, L., Gomez, A. and Simon, J. (2004) Evolutionary and ecological genetics of cyclical parthenogens. In: A. Moya and E. Font (eds) *Evolution from molecules to ecosystems.* Oxford university press, pp. 122-134.

De Meester, L. and Jager, H. (1993) Hatching of *Daphnia* sexual eggs. I. Intraspecific differences in the hatching responses of D. magna eggs. *Freshwater biology,* **30,** 219-226.

Decaestecker, E., Lefever, C., De Meester, L. and Ebert, D. (2004) Haunted by the past: Evidence for dormant stage banks of microparasites and epibionts of Daphnia. *Limnology and oceanography,* **49,** 1355-1364.

Denlinger, D. L. (2002) Regulation of diapause. *Annual Review of Entomology,* **47,** 93-122.

Elbein, A. D., Pan, Y. T., Pastuszak, I. and Carroll, D. (2003) New insights on trehalose: a multifunctional molecule. *Glycobiology,* **13,** 17R-27R.

Hairston, N. G., Van Brunt, R. A., Kearns, C. M. and Engstrom, D. R. (1995) Age and Survivorship of Diapausing Eggs in a Sediment Egg Bank. *Ecology,* **76,** 1706-1711.

Hazel, J. R. and Williams, E. E. (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in lipid research,* **29,** 167-227.

Hebert, P. D. N. (1978) The population biology of *Daphnia* (Crustacea, Daphnidae). *Biological Reviews,* **53,** 387-426.

Hengherr, S., Brümmer, F. and Schill, R. O. (2008) Anhydrobiosis in tardigrades and its effects on longevity traits. *Journal of Zoology,* **275,** 216-220.

Hengherr, S., Heyer, A. G., Brümmer, F. and Schill, R. O. (2011) Trehalose and Vitreous States: Desiccation Tolerance of Dormant Stages of the Crustaceans Triops and Daphnia. *Physiological and Biochemical Zoology,* **84,** 147-153.

Hengherr, S. and Schill, R. O. (2011) Dormant stages in freshwater bryozoans—An adaptation to transcend environmental constraints. *Journal of Insect Physiology,* **57,** 595-601.

Hontoria, F., Crowe, J., Crowe, L. and Amat, F. (1998) Trehalose prevents imbitional damage in anhydrobiotic cysts of Artemia by depressing the phase transition temperature in lipids (with 7 figures). *Ergebnisse der limnologie,* **52,** 451-462.

Kast-Hutcheson, K., Rider, C. V. and Leblanc, G. A. (2001) The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environmental Toxicology and Chemistry,* **20,** 502-509.

Kikawada, T., Minakawa, N., Watanabe, M. and Okuda, T. (2005) Factors Inducing Successful Anhydrobiosis in the African Chironomid Polypedilum vanderplanki: Significance of the Larval Tubular Nest. *Integrative and Comparative Biology,* **45,** 710-714.

Madin, K. a. C. and Crowe, J. H. (1975) Anhydrobiosis in nematodes: Carbohydrate and lipid metabolism during dehydration. *Journal of Experimental Zoology,* **193,** 335-342.

Martin-Creuzburg, D., Sperfeld, E. and Wacker, A. (2009) Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids. *Proceedings of the Royal Society B: Biological Sciences,* **276,** 1805-1814.

Masclaux, H., Bec, A., Kainz, M. J., Desvilettes, C., Jouve, L. and Bourdier, G. (2009) Combined effects of food quality and temperature on somatic growth and reproduction of two freshwater cladocerans. *Limnology and oceanography,* **54,** 1323-1332.

Pampurova, S. and Van Dijck, P. (2014) The desiccation tolerant secrets of Selaginella lepidophylla: What we have learned so far? *Plant Physiology and Biochemistry,* **80,** 285-290.

Pauwels, K., Stoks, R., Verbiest, A. and De Meester, L. (2007) Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*. *Hydrobiologia,* **594,** 91-96.

Potts, M. (1994) Desiccation tolerance of prokaryotes. *Microbiological Reviews,* **58,** 755-805.

Provasoli, U. (1968) Media and prospects for the cultivation of marine algae. In: A. Watanabe and A. Hattori (eds) *Cultures and Collections of Algae.* Japan Society PIant Physiology, pp. 63-75.

Pruitt, N. L. (1990) Adaptations to temperature in the cellular membranes of crustacea: membrane structure and metabolism. *Journal of Thermal Biology,* **15,** 1-8.

Putman, A., Martin-Creuzburg, D., De Meester, L. and Panis, B. (2015) A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality. *Journal of Plankton Research,* **DOI:10.1093/plankt/fbv043**.

Radzikowski, J. (2013) Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *Journal of Plankton Research,* **35,** 707-732.

Sakurai, M., Furuki, T., Akao, K.-I., Tanaka, D., Nakahara, Y., Kikawada, T., Watanabe, M. and Okuda, T. (2008) Vitrification is essential for anhydrobiosis in an African chironomid, Polypedilum vanderplanki. *Proceedings of the National Academy of Sciences,* **105,** 5093-5098.

Tapia, H. and Koshland, Douglas e. (2014) Trehalose Is a Versatile and Long-Lived Chaperone for Desiccation Tolerance. *Current Biology,* **24,** 2758-2766.

Tunnacliffe, A. and Lapinski, J. (2003) Resurrecting Van Leeuwenhoek's rotifers: a reappraisal of the role of disaccharides in anhydrobiosis. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* **358,** 1755-1771.

Viner, R. I. and Clegg, J. S. (2001) Influence of trehalose on the molecular chaperone activity of p26, a small heat shock/α-crystallin protein. *Cell Stress & Chaperones,* **6,** 126-135.

Von Elert, E. (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography,* **47,** 1764 - 1773.

Walne, P. R. (1965) Experimental rearing of the larvae of *Ostrea edulis* L. in the laboratory. *Fishery Investment,* **20,** 1-23.

Watanabe, M., Kikawada, T., Minagawa, N., Yukuhiro, F. and Okuda, T. (2002) Mechanism allowing an insect to survive complete dehydration and extreme temperatures. *Journal of Experimental Biology,* **205,** 2799-2802.

Wełnicz, W., Grohme, M. A., Kaczmarek, Ł., Schill, R. O. and Frohme, M. (2011) Anhydrobiosis in tardigrades—The last decade. *Journal of Insect Physiology,* **57,** 577-583.

Womersley, C. and Smith, L. (1981) Anhydrobiosis in nematodes—I. The role of glycerol myo-inositol and trehalose during desiccation. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* **70,** 579-586.

Addendum: plasticity of sugar content under different food conditions

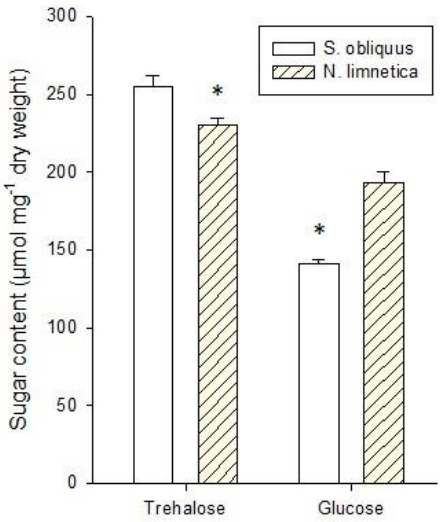

Figure 1: Sugar composition of algae *Scenedesmus obliquus* (open bars) and *Nannochloropsis limnetica* (hatched bars). Data are means of three replicates; error bars indicate one standard error. * indicates significant differences within one sugar type ($p < 0.05$, ANOVA).

Table 1: Results of MANOVA (A) and ANOVA (B and C) of the differences in sugar composition between the algae *Scenedesmus obliquus* and *Nannochloropsis limnetica* used as food organisms to culture *Daphnia magna*.

A) MANOVA

Variable	Df	Approx F	P-value
Food organisms	1	84.759	**0.002293**

B) ANOVA Trehalose

Variable	Df	F-value	P-value
Food organisms	1	9.2535	**0.03833**

B) ANOVA Glucose

Variable	Df	F-value	P-value
Food organisms	1	46.997	**0.002375**

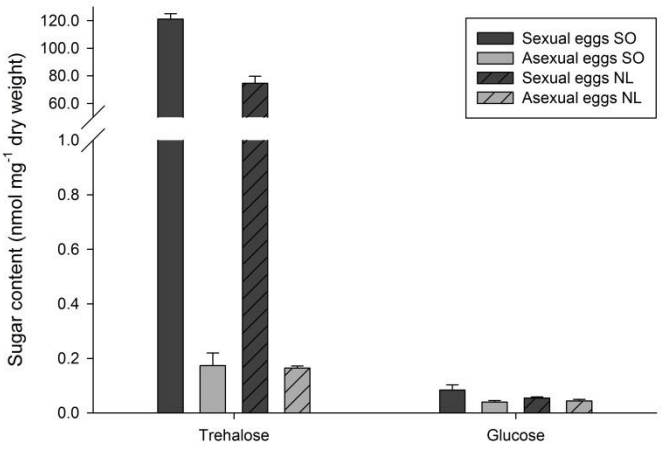

Figure 2: Sugar composition of sexual (dark grey bars) and asexual eggs (light grey bars) of *Daphnia magna* produced on *Scenedesmus obiquus* (open bars) or *Nannochloropsis limnetica* (hatched bars). Data are means of five replicates; error bars indicate one standard error.

Table 2: Results of MANOVA comparing the sugar composition of sexual and asexual eggs of *D. magna* (eggtype) produced on *Scenedesmus obliquus* or *Nannochloropsis limnetica* (foodtype).

Variable	Df	Approx F	P-value
Foodtype	1	2.282	0.1364
Eggtype	1	157.453	**8.566*E-11**
Foodtype:Eggtype	1	1.414	0.2738

Chapter 3

A comparison of polyamine levels between sexual and asexual eggs of *Daphnia magna* under different food regimes

ADINDA PUTMAN, BART PANIS, WIM VAN DEN ENDE AND LUC DE MEESTER

Abstract

Like many other organisms, the water flea *Daphnia* is characterized by a cyclic parthenogenetic reproduction cycle, in which phases of asexual reproduction are alternated with phases of sexual reproduction. Asexual eggs are produced under favorable conditions, develop immediately and enable fast population growth. In contrast, sexual reproduction takes place when conditions deteriorate and results in dormant eggs that can stay viable for decades. To achieve this long term survival, sexual eggs are able to cope with various stressors, such as drought and frost. This resistance requires special adaptations in terms of biochemistry. Polyamines easily bind to negatively charged macromolecules, such as DNA, RNA, phospholipids and proteins, enabling them to fulfill important functions in the cell metabolism during early development and to stabilize these structures under stress conditions. Along with other protective molecules, such as glycerol, trehalose and heat shock proteins, polyamines can enable eggs to survive harmful conditions encountered by sexual eggs during dormancy. We therefor compared the polyamine content of sexual and asexual eggs of *Daphnia magna* and checked for plasticity of this treat under different food conditions. Although the concentrations change with the food regimes, asexual eggs always contained higher amounts of diaminopropane and lower amounts of putrescine and spermidine per unit biomass in comparison with sexual eggs. Due to this high concentration of diaminopropane, one asexual egg contains a higher amount of polyamines compared to one sexual egg, however when data are expressed per mg dry weight there is nog difference in total polyamine concentration between the two egg types.

1 Introduction

Many organisms exhibit a facultative sexual life cycle, allowing them to combine the advantages of sexual and asexual reproduction (Schön et al., 2009). A well-documented, broadly studied facultative sexual species is the cladoceran *Daphnia magna* (De Meester et al., 2004; Miner et al., 2012). *D. magna* is a cyclic parthenogen that reproduces parthenogenetically during favourable conditions. Sexual eggs are produced when conditions deteriorate by the end of the growing season. The resulting fertilized eggs are encapsulated in a protective envelope, the ephippium, and are deposited in the sediment where they form a dormant egg bank. Generally, resting eggs are resistant to a multitude of stressors, such as desiccation, extreme temperatures and digestions by animals (Madin and Crowe et al., 1975; Clegg, 2005; Hengherr and Schill, 2011; Radzikowksi, 2013). This striking resistance allows them to survive harmful environmental conditions and disperse in space and time (Brendonck and De Meester, 2003). It has been shown that offspring from dormant eggs and subitaneous eggs have different life history strategies (Arbaciauskas, 1998; Càceres, 1998; Arbaciauskas and Lampert, 2003). These differences in both resistance to drought and freezing and in life history traits of hatchlings are likely to be a reflection of a distinct biochemical content of the eggs.

The striking resistance of dormant eggs to drought and freezing requires specific morphological and physiological adaptations, which may require different resource allocation strategies by the mother. For example, the disaccharide trehalose is an important factor in cold and drought tolerance of many anhydrobiotic organisms (Clegg,1965; 2001; Hengherr et al., 2011) and in previous work we have shown that trehalose content is much higher in sexual eggs compared to asexual eggs of *Daphnia magna* (Putman et al., subm. (Chapter 2)). Trehalose has a wide variety of functions, ranging from energy and carbon reserve to stabilizing proteins in their native state and preserving the integrity of membranes (Crowe et al. 2001; Elbein et al., 2003; Crowe, 2007). Two other biochemical components that have been found to play an important role in the protection of cell metabolism under stress conditions in anhydrobiotic cysts of *Artemia franciscana* (Clegg et al., 1997) and certain insects (Denlinger, 2002; Lencioni, 2004; Steinberg et al, 2012) are glycerol and heat shock proteins. In line with the expectations, Pauwels et al. (2007) reported that sexual, dormant eggs of *Daphnia magna* contain higher levels of glycerol and heat shock proteins than asexual, subitaneous eggs. The integrity and fluidity of the cell membrane is considered to be another crucial factor in cold and drought tolerance. Cell membranes are mainly composed of phospholipids, and the fluidity of the membrane is thus strongly dependent on the composition of the phospholipids and, more particularly, on the polyunsaturated fatty acids forming the phospholipid hydrophobic tails (Hazel and Williams, 1990; Pruitt, 1990; Masclaux et al., 2009). It has been reported that the fatty acid content differs between sexual and asexual eggs of *Daphnia* (Abrusan et al., 2007). Although asexual eggs contain a higher amount of phospholipids compared to sexual eggs, sexual eggs seem to require a certain minimum

amount of polyunsaturated fatty acids. Indeed, sexual eggs contain higher concentrations of long chain polyunsaturated fatty acids than asexual eggs, even under conditions where the diet lacked these polyunsaturated fatty acids (Abrusan et al., 2007; Putman et al., 2015 (Chapter 1)).

In plants, polyamine metabolism is also strongly involved in the resistance to abiotic stress, such as osmotic stress, pollutants, drought stress and cold acclimatisation (Kushad and Yelensky, 1987; Seki et al., 2007; Groppa and Benavides, 2008; Alcàzar et al., 2011). For instance, an increase of the putriscine level is correlated with higher survival of banana meristems after cryopreservation, and thus exposure to extreme freezing temperatures (Ramon et al., 2002).Polyamines are small polycations that are ubiquitously present in living cells (Pegg and McCann, 1982). The most common polyamines are putrescine (PUT), spermidine (SPD) and spermine (SPM). Among invertebrates (including the crustaceans) additional widely distributed polyamines have been described, including nor-spermidine (NSPD), nor-spermine (NSPM) and diaminopropane (DAP) (Stillway and Walle, 1977; Zappia et al., 1978; Hamana et al., 1991a; Hamana et al., 1991b). Polyamines easily interact with negatively charged cell structures and polymers such as DNA, RNA, phospholipids and proteins. In this way, polyamines aid to preserve the physiological integrity of these macromolecules under stress conditions. In addition, polyamines are involved in numerous crucial cellular functions such as cell division, cell growth, apoptosis, ion transport and protein synthesis (Pegg and McCann, 1982; Tabor and Tabor, 1984; Schuber, 1989; Igarashi and Kashiwagi, 2010). It has also been shown that polyamines are important for embryonic growth and differentiation in some arthropods (Callaerts et al., 1992; Watts et al., 1994).

Despite the widely documented positive influence of polyamines on stress resistance in plants, studies on animal taxa are rather scarce and for invertebrates limited to reports of changes in polyamine concentrations and their cellular functions during development and differentiation. In this study, we compare the polyamine content of sexual and asexual eggs of the water flea *Daphnia magna*. We expect that the highly resistant sexual egg contains higher concentrations of polyamines than the much less resistant asexual egg. Polyamines in dormant eggs might contribute to the maintenance of the integrity of cellular macromolecules and cellular structures. We also explore the plasticity in polyamine concentrations in both egg types when cultured under different food conditions, as it is known that the biochemical composition of *Daphnia* eggs is highly plastic, and it is expected that maternal resource allocation strategies and their plasticity might differ between the different egg types (Gliwicz and Guisande, 1992; Stibor, 2002; Wacker and Martin-Creuzburg, 2007; Putman et al., 2015 (Chapter 1)).

2 Material and methods

2.1 Cultivation and preparation of the food

For our experiments, we used the green alga *Scenedesmus obliquus* (SO) and the eustigmatophyte *Nannochloropsis limnetica* (NL) as food sources. These are both well-assimilated by Daphnia, but they differ qualitatively, amongst others in terms of long-chain polyunsaturated fatty acids (SO: Von Elert, 2002; NL: Martin-Creuzburg et al., 2009; Putman et al., 2015 (Chapter 1)).

Algae were cultivated in batch in aerated 10 liter vessels with constant illumination at 168.33 µmoles $m^{-2}s^{-1}$ at 18°C and harvested in the late-exponential growth phase. *S. obliquus* (SAG 276-3a) was grown in a medium consisting of 10 ml l^{-1} of enriched seawater (ES) nutrients (Provasoli, 1968), 5 ml l^{-1} of Walne nutrients (Walne, 1965) and the vitamins B1, B12 and H dissolved in dechlorinated tap water. *N. limnetica* (SAG 18.99) was grown in modified Woods Hole (WC) medium with the vitamins B1, B12 and H (Guillard, 1975). Food suspensions were prepared by concentrating the cells via centrifugation (4000 rpm, 5 min) followed by resuspension in tap water. Cell densities of the food suspensions were counted with an Attune® acoustic focusing cytometer (Life technologies, Carlsbad, CA, USA).

To analyze the polyamine composition of the algal food source, 3 replicate samples of 50ml *S. obliquus* and *N. limnetica* were freeze-dried and 1g dry weight of each were used for the analysis.

2.2 Egg collection

To compare the polyamine content of both types of eggs, we conducted a laboratory experiment to generate sexual and asexual eggs from females cultured under the same conditions only differing in their food source. We hatched 10 individuals from dormant eggs extracted from the dormant egg bank of the *D. magna* population in 'Langerode vijver', a pond in Neerijse, Belgium (0°49'42.32"N, 4°38'21.49"E). These animals were hatched by exposing the dormant eggs to hatching stimuli, i.e. a relatively high temperature (20°C), a long-day photoperiod (16L:8D) and fresh medium (tap water dechlorinated and aged for 24h) (De Meester and Jager, 1993). As these individuals hatched from sexual eggs, they constitute 10 different genotypes, i.e. 10 different clonal lineages. Hatched lineages were reared separately in 0.5 liter jars (density: 20 individuals of a single clonal lineage per liter) filled with aged tap water (aerated for 24h prior to use) under standardized conditions (20 ± 2 °C and a photoperiod of 16L:8D) for several generations. All cultures were fed 150.000 cells ml^{-1} of the green alga *Scenedesmus obliquus*, which corresponds to approximately 2.5 mg C l^{-1}. After this preconditioning phase, the second clutch of a new generation was subjected to the different experimental conditions.

To obtain asexual eggs, five replicate 1 liter jars per clone (with 10 clones in total), with 15 individuals of a single clone in each jar, were cultured under standard conditions (20 ± 2 °C and a photoperiod of 16L:8D) under two different food treatments. Cultures were either fed with *S. obliquus* or with *N. limnetica*, both at an algal cell density of 150.000 cells ml^{-1}. In both food treatments, jars were cleaned every two days and food was renewed daily to keep algal concentration above the incipient limiting level, the critical concentration which corresponds to the minimal food concentration above which feeding rates stay constant. Females bearing their third clutch were dissected to collect asexual eggs in the first stage of embryonic development (identified following Kast-Hutcheson et al., 2001). From each replicate, one sample of 150 eggs (i.e. 15 eggs per clone) was collected and stored at -80°C. In total, ten samples (five replicates x two food treatments) were collected.

We induced sexual reproduction using the same clones and food treatments as described above. Cultures were started by inoculating 20 neonates of a given clone under standard conditions (20 ± 2 °C, a photoperiod of 16L:8D and an algal cell density of 150.000 cells ml^{-1}). After the clones released second clutch, four individuals of each clone were put together in a 1 liter jar (replicated 5 times) and feeding level was raised to 250.000 cells ml^{-1} replenished every day. The photoperiod was switched between long-day (16L:8D) photoperiod during five days and short-day (8L:16D) photoperiod during two days, as this has been shown to stimulate sexual reproduction in *Daphnia* (De Meester and Jager, 1993). Once a week half the medium was renewed and all dormant eggs were collected. No hatching of dormant eggs occurred in these cultures, as a period of cold or drought is needed to break diapause of the dormant eggs. For every replicate, dormant eggs were stored in Eppendorf tubes in the dark at 4°C for at least one month. After storage, these laboratory-derived ephippia were decapsulated (i.e. the protective envelope was removed) and five replicate samples consisting of 150 sexual eggs were collected for the polyamine analysis for each treatment. The ten samples were stored at -80°C.

In total, twenty samples (i.e. sexual or asexual eggs x *S. obliquus* or *N. limnetica* x five replicates per treatment) were freeze-dried and weighted (dry mass) before polyamine analysis.

2.3 Polyamine analysis

For the analysis of the polyamines we modified the protocol described by Walter and Geuns (1987). All freeze-dried samples (20 egg samples and 6 algae samples) were first homogenized in 250 µl 4% perchloric acid containing 2 mg l^{-1} 1.7-diaminoheptane-2-HCl as an internal standard. After vortexing, samples were incubated at 4°C for one hour to extract the polyamines, after which the samples were centrifuged for 1 min at 13000 rpm and 50 µl of supernatant was collected. One hundred microliter 0.4M borate:NaOH buffer (pH 11) and 100 µl dansyl chloride solution (7 mg ml^{-1} acetone) were added. The solution was vortexed and heated to 60°C for 15 min in the dark. Then 0.6 ml toluene was added and the mixture was vortexed and centrifuged. The upper phase was transferred to a new tube

and 0.6 ml hexane was added. For purification of the polyamines, the samples were applied to a silica column that contained 250 mg silica gel 60 (Sigma-Aldrich). Before the samples were added, the columns were first rinsed with 250 µl toluene followed by 250 µl toluene/triethylamine (10:3 v/v). The samples were eluted with two times 0.3 ml ethylacetate. The eluent was evaporated in a vacuum centrifuge and the residue was dissolved in 250 µl methanol, of which 10 µl was injected in the HPLC. The high-perfomance liquid chromatographer (Shimadzu, Kyoto, Japan) was equipped with a fluorescence detector (RF-10A XL, exc.: 340mm, em.: 510 mm) and a column of 10 cm x 3 mm inner diameter containing 5 µm octadecyl-silica. The solvent and the column were heated to 50°C and the solvent flowed at a speed of 1.5 ml min^{-1}. The total analysis time per sample was 10 min, of which during the first two minutes the solvent consisted of 58% acetonitrile; from min 2 to 7.5 min this increased gradually from 58% to 91% followed by rinsing with 58% acetonitrile from 7.6 min to 10 min.

2.4 Data analysis

The absolute amount of each polyamine was normalized to the egg dry mass and egg number. Using the packages car and multcomp of the statistical software R version 3.0.2 (The R Foundation for Statistical Computing) the effects of the food source (*S. obliquus* and *N. limnetica*) and egg type (sexual and asexual eggs) on the concentrations of polyamines in eggs and algae were tested separately with a multivariate analysis of variance (MANOVA). For each polyamine, we also performed a separate analysis of variance (ANOVA) with the same variables.

3 Results

S. obliquus and *N. limnetica* differ qualitatively in their polyamine content ($p < 0.001$): *S. obliquus* contains much more putriscine ($p < 0.001$), while *N. limnetica* contains more spermidine ($p < 0.001$) (Figure 1). While in the algae we only detected the polyamines putriscine and spermidine, analyses of the egg samples also revealed diaminopropane at relatively high concentrations, spermidine was only detected at low concentrations in egg samples (Figure 2).

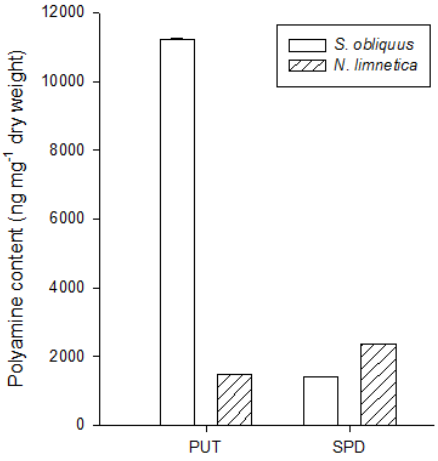

Figure 1: Polyamine content of S*cenedesmus obliquus* (open bars) and *Nannochloropsis limnetica* (hatched bars) expressed in nmol mg^{-1} dry weight. Bars represent the mean of three replicate samples; error bars indicate one standard error. PUT = Putrescine; SPD= spermidine

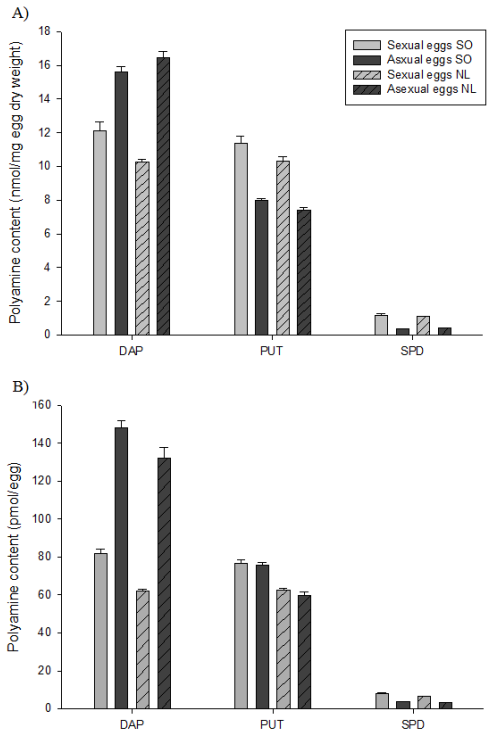

Figure 2: Polyamine content of sexual (light gray bars) and asexual eggs (dark gray bars) of *D. magna* that were cultured on *S. obliquus* (open bars) and *N. limnetica* (hatched bars). Data are expressed in nmol mg^{-1} dry weight in panel A and in pmol egg^{-1} in panel B. Bars represent means of five replicate samples; error bars indicate one standard error. DAP = Diaminoporpane; PUT = Putrescine; SPD = spermidine

The complete polyamine composition per mg egg (measured as dry weight) shows a significant food x egg type interaction (p=0.0006). Separate analysis on the individual polyamines revealed that this

interaction is only significant for DAP (p=0.003). Levels of DAP in sexual eggs are higher when eggs were produced on a diet of *S. obliquus* compared to a diet of *N. limnetica,* while the opposite pattern is observed for asexual eggs. When we analyze the polyamine composition expressed as polyamines per egg, the food x egg type interaction is not significant (Table 1).

Table 1: Results of MANOVA (for all polyamines) and ANOVA (for separate polyamines) comparing the polyamine content of sexual and asexual eggs (factor 'egg type') of *D. magna* when cultured on different diets (*N. limnetica* vs. *S. obliquus*) (factor 'food'). Concentrations of the different polyamines were the dependent variables and type of food and egg type (sexual vs. asexual eggs) were the independent variables.

Polyamines per mg egg dry weight				**Polyamines per egg**			
Variables	Df	F value	P value	Variables	Df	F value	P value
All polyamines				*All polyamines*			
Food	1	4.21	**0.03**	Food	1	32.53	**1.45E-06**
Egg type	1	755.77	**1.03E-15**	Egg type	1	763.17	**9.60E-16**
Food x egg type	1	10.99	**0.0006**	Food x egg type	1	2.44	0.11
DAP				*DAP*			
Food	1	1.77	0.20	Food	1	24.74	**0.0001**
Egg type	1	160.18	**9.48E-10**	Egg type	1	363.78	**1.99E-12**
Food x egg type	1	11.97	**0.003**	Food x egg type	1	0.27	0.61
PUT				*PUT*			
Food	1	8.09	**0.01**	Food	1	68.89	**3.43E-07**
Egg type	1	130.42	**4.20E-09**	Egg type	1	1.19	0.29
Food x egg type	1	0.71	0.41	Food x egg type	1	0.22	0.64
SPD				*SPD*			
Food	1	0.43	0.52	Food	1	7.78	**0.01**
Egg type	1	281.40	**1.41E-11**	Egg type	1	152.79	**1.34E-09**
Food x egg type	1	1.66	0.22	Food x egg type	1	2.91	0.11

There is a highly significant effect of egg type for all polyamines together and for each of the three polyamines separately on their concentrations per mg dry weight. For diaminopropane, the concentrations per mg dry weight in asexual eggs is higher than in sexual eggs, while putriscine and spermidine occur in higher concentrations per mg dry weight in the sexual than in the asexual eggs (Table 1, Figure 2a). Polyamine content of the food source only influenced the content of putriscine when measured per mg egg dry weight. In contrast, when we analyze the concentrations per egg, egg type is highly significant for DAP and SPD but not for PUT, while the effect of food type is highly significant for all three polyamines (Figure 2b). This difference in PUT level can simply be explained by the fact that sexual eggs (6.4 µg) are lighter than asexual eggs (8.8 µg).

The different proportions of PUT in sexual and asexual eggs, due to their weight discrepancies, also influence the effect of the egg type on the total concentration of polyamines per egg (Table 2). When data are expressed per mg there is no significant difference between the egg types ($p = 0.15278$), while there is a significant difference when data are expressed per egg ($p = 3.055*E^{-09}$). One asexual egg contains more polyamines than one sexual eggs, mostly due to a higher level of DAP (Figure 2). Additionally, eggs produced under a diet of *S. obliquus* contain more polyamines per egg than eggs produces under a *N. limnetica* diet ($p = 1.057*E^{-05}$). For the data expressed per mg dry weight we found that asexual eggs contained more polyamines than sexual eggs when produced under a maternal diet of *N. limnetica*, the opposite was found when both egg types where mothers were fed *S. obliquus* ($p = 0.02362$).

Table 2: Results of ANOVA comparing the total polyamine concentration of sexual and asexual eggs (factor 'egg type') of *D. magna* when cultured on different diets (*N. limnetica* vs. *S. obliquus*) (factor 'food'). Concentrations of the different polyamines were the dependent variables and type of food and egg type (sexual vs. asexual eggs) were the independent variables.

Polyamines per mg egg dry weight				**Polyamines per egg**			
Variables	Df	F value	P value	Variables	Df	F value	P value
Total concentration				*Total concentration*			
Food	1	4.1993	0.05721	Food	1	39.6905	**1.06E-05**
Egg type	1	2.2536	0.15278	Egg type	1	136.332	**3.06E-09**
Food x egg type	1	6.2552	**0.02362**	Food x egg type	1	0.0808	0.7799

4 Discussion

Sexual and asexual eggs of the water flea *Daphnia magna* have very different ecological functions. Sexual eggs need to be resistant to various stressors, as they mainly function as a vector for dispersal in space and time. In contrast, asexual eggs do not need the biochemically costly resistance as they develop immediately in the favorable environment provided by the maternal brood pouch. To acquire enhanced resistance, sexual eggs contain multiple stress protectant molecules, including higher concentrations of trehalose, glycerol, heat shock proteins and polyunsaturated fatty acids than asexual eggs (Abrusan et al., 2007; Pauwels et al., 2007; Putman et al., 2015 (Chapter 1); Putman et al., subm. (Chapter 2)). Here we add that sexual eggs also differ from asexual eggs by having a higher concentration standardized by mg dry weight) of putriscine and spermidine but a lower concentration of diaminopropane.

The polyamines spermidine and its precursor putriscine are among the most abundant polyamines in eukaryotic cells and exert many essential functions in the cellular metabolism and stress protection (Pegg and McCann, 1982; Tabor and Tabor, 1984; Groppa and Benavides, 2008; Alcàzar et al., 2011). Increased levels of polyamines contribute to the survival of plants under conditions of drought and low or freezing temperatures (Kushad and Yelenosky, 1987; Ramon et al., 2002; Seki et al., 2007; Cuevas et al., 2008). In accordance with these previous studies, we hypothesized that the polyamine content of sexual eggs would be higher than polyamine content of asexual eggs and we found that levels of spermidine and putriscine were higher per mg dry weight in sexual eggs as compared to asexual eggs. It is well established that they fulfill their role during cell growth and proliferation by interacting with and stabilizing negatively charged macromolecules. However, their functions during stress responses remain open for debate. One of the hypotheses is that their binding properties to membranes enable them to maintain their fluidity and prevent them from leakage (Smith, 1985; Zheliaskova et al., 2000). Another hypothesis is that they have an antioxidant effect, combining their ability to act as a radical scavenger and to inhibit lipid peroxidation (Kitada et al., 1979; Bors et al., 1989; Ha et al., 1998).

It has been shown that, also for arthropods, polyamines are important cellular components during early differentiation and embryonic growth (Callaerts et al., 1992; Pegg and McCann, 1982; Tabor and Tabor, 1984; Watts et al., 1994). Hatchlings from sexual eggs have a higher juvenile growth rate, have a larger body size at maturity and mature earlier compared to asexually produced juveniles (Arbaciauskas, 1998; Arbaciauskas and Lampert, 2003). This is likely achieved by a higher metabolic rate of sexual as compared to asexual offspring, and so this may be an alternative reason for the higher levels of putrescine and spermidine in sexual compared to asexual eggs.

In contrast with putrescine and spermidine, diaminopropane concentrations are higher in asexual than in sexual eggs. Diaminopropane is a metabolite produced during the oxidation of the polyamines spermidine and spermine (Stillway and Walle, 1977; Smith, 1989). Probably, their high concentrations in asexual eggs reflect high metabolic activity associated with the start of development. This is in accordance with the results found in seeds of *Cucumus savitis*, where it was shown that catabolism of spermidine can account for a raise in the amount of diaminopropane during early developmental stages (Flayeh et al., 1984). Besides a catabolite of spermidine, diaminopropane is also a precursor for β-alanine, nor-spermidine and nor-spermine, which play an important role in the stress tolerance of plants (Cona et al., 2006; Hamana et al., 1984). Nor-spermidine and nor-spermine have been shown to be common polyamines in Crustacea and other invertebrates (Hamana et al., 1989; Haman et al., 1991; Zappia et al., 1978). We did, however, not detect these polyamines in our samples.

Maternal food conditions affected the polyamine content of the eggs. Although the differences are small, the higher putrescine content of *S. obliquus* compared to *N. limnetica* is reflected in the egg samples (when data are expressed per mg dry weight). This is consistent with earlier studies reporting

that the biochemical composition of the maternal diet is of great influence for the biochemical composition of the eggs (Wacker and Martin-Creuzburg, 2007; Schlotz et al., 2013; Putman et al., 2015 (Chapter 1)). The allocation levels per egg for all three polyamines are higher when the mothers were cultured with *S. obliquus* as their food source. Even though *S. obliquus* contains an overload of putriscine (up to eight times more) and less spermidine as compared to *N. limnetica*, all clones invested higher levels of putriscine and spermidine in sexual eggs regardless of the polyamine content of their food source, so our data clearly indicate the importance of polyamines for sexual eggs.

Allocation of polyamines to eggs of *D. magna* is mostly dependent on the type of egg (dormant versus subitaneous) and only minor on the maternal diet. In general, the sexual, stress resistant dormant eggs contain higher concentrations of polyamines than asexual eggs, except for the metabolite diaminopropane. Our results strongly suggest that polyamines, in addition to glycerol, heat shock proteins, trehalose and fatty acids, form another important piece in the puzzle of understanding the establishment of the striking resistance of sexual eggs to harsh environmental conditions, such as drought or cold temperatures.

Acknowledgements

AP enjoys a PhD fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). This work was financially supported by KU Leuven Research Fund grant PF2010/07. We thank Hilde Verlinden and Tom Struyf for technical assistance.

References

Abrusan, G., Fink, P. and Lampert, W. (2007) Biochemical limitation of resting egg production in *Daphnia*. *Limnology and oceanography*, **52,** 1724-1728.

Alcázar, R., Cuevas, J. C., Planas, J., Zarza, X., Bortolotti, C., Carrasco, P., Salinas, J., Tiburcio, A. F. and Altabella, T. (2011) Integration of polyamines in the cold acclimation response. *Plant Science*, **180,** 31-38.

Arbaciauskas, K. (1998) Life-history traits of exephippial and parthenogenetically derived daphnids: indicators of different life-history strategies. *Archiv für Hydrobiologie Special Issues Advances in Limnology*, **52,** 339-358.

Arbaciauskas, K. and Lampert, W. (2003) Seasonal adaptation of ex-ephippio and parthenogenetic offspring of *Daphnia magna*: differences in life history and physiology. *Functional Ecology*, **17,** 431-437.

Bors, W., Langebartels, C., Michel, C. and Sandermann Jr, H. (1989) Polyamines as radical scavengers and protectants against ozone damage. *Phytochemistry*, **28,** 1589-1595.

Brendonck, L. and De Meester, L. (2003) Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia*, **491,** 65-84.

Cáceres, C. E. (1998) Interspecific variation in the abundance, production, and emergence of Daphnia diapausing eggs. *Ecology*, **79,** 1699-1710.

Callaerts, P., Geuns, J. and De Loof, A. (1992) Polyamine changes during early development of Drosophila melanogaster. *Journal of Insect Physiology*, **38,** 751-758.

Clegg, J. S. (1965) The origin of threhalose and its significance during the formation of encysted dormant embryos of Artmia salina. *Comparative Biochemistry and Physiology*, **14,** 135-143.

Clegg, J. S. (1997) Embryos of *Artemia fansciscana* survive four years of continous anoxia: the case for complete metabolic rate depression. *The Journal of Experimental Biology*, **200,** 467-475.

Clegg, J. S. (2001) Cryptobiosis — a peculiar state of biological organization. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **128,** 613-624.

Clegg, J. S. (2005) Desiccation Tolerance in Encysted Embryos of the Animal Extremophile, Artemia. *Integrative and Comparative Biology*, **45,** 715-724.

Cona, A., Rea, G., Angelini, R., Federico, R. and Tavladoraki, P. (2006) Functions of amine oxidases in plant development and defence. *Trends in Plant Science*, **11,** 80-88.

Crowe, J. (2007) Trehalose As a "Chemical Chaperone". In: P. Csermely and L. Vígh (eds) *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks.* Vol. 594. Springer New York, pp. 143-158.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Tsvetkova, N., Wolkers, W. and Tablin, F. (2001) The Trehalose Myth Revisited: Introduction to a Symposium on Stabilization of Cells in the Dry State. *Cryobiology*, **43,** 89-105.

Cuevas, J. C., López-Cobollo, R., Alcázar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A. F. and Ferrando, A. (2008) Putrescine Is Involved in Arabidopsis Freezing

Tolerance and Cold Acclimation by Regulating Abscisic Acid Levels in Response to Low Temperature. *Plant Physiology,* **148,** 1094-1105.

De Meester, L., Gomez, A. and Simon, J. (2004) Evolutionary and ecological genetics of cyclical parthenogens. In: A. Moya and E. Font (eds) *Evolution from molecules to ecosystems.* Oxford university press, pp. 122-134.

De Meester, L. and Jager, H. (1993) Hatching of *Daphnia* sexual eggs. I. Intraspecific differences in the hatching responses of D. magna eggs. *Freshwater biology,* **30,** 219-226.

Denlinger, D. L. (2002) Regulation of diapause. *Annual Review of Entomology,* **47,** 93-122.

Elbein, A. D., Pan, Y. T., Pastuszak, I. and Carroll, D. (2003) New insights on trehalose: a multifunctional molecule. *Glycobiology,* **13,** 17R-27R.

Flayeh, K. a. M., Najafi, S. I., Al-Delymi, A. M. and Hajar, M. A. (1984) 1,3-Diaminopropane and spermidine in cucumis sativus (cucumber). *Phytochemistry,* **23,** 989-990.

Gliwicz, Z. M. and Guisande, C. (1992) Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia,* **91,** 463-467.

Groppa, M. D. and Benavides, M. P. (2008) Polyamines and abiotic stress: recent advances. *Amino Acids,* **34,** 35-45.

Guillard, R. (1975) Cultures of phytoplankton for feeding of marine invertebrates. In: W.L. Smith and M.H. Chanley (eds) *Culture of marine invertebrate animals.* Plenum Press New York, pp. 29 - 60.

Ha, H. C., Sirisoma, N. S., Kuppusamy, P., Zweier, J. L., Woster, P. M. and Casero, R. A. (1998) The natural polyamine spermine functions directly as a free radical scavenger. *Proceedings of the National Academy of Sciences,* **95,** 11140-11145.

Hamana, K., Matsuzaki, S. and Inoue, K. (1984) Changes in Polyamine Levels in Various Organs of Bombyx mori during Its Life Cycle. *Journal of Biochemistry,* **95,** 1803-1809.

Hamana, K., Niitsu, M., Samejima, K. and Matsuzaki, S. (1991a) Novel polyamines in insects and spiders. *Comparative Biochemistry and Physiology - Part B: Biochemistry and Molecular Biology,* **100,** 399-402.

Hamana, K., Niitsu, M., Samejima, K. and Matsuzaki, S. (1991b) Novel tretaamines, pentaamines and hexaamines in sea urchin, sea cumcumber, sea squirt and bivalves. *Comparative Biochemistry Physiology - Part B: Biochemistry and molecular biology,* **100,** 59-62.

Hamana, K., Suzuki, M., Wakabayashi, T. and Matsuzaki, S. (1989) Polyamine levels in the gonads, sperm and salivary gland of cricket, cockroach, fly and midge. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* **92,** 691-695.

Hazel, J. R. and Williams, E. E. (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in lipid research,* **29,** 167-227.

Hengherr, S., Heyer, A. G., Brümmer, F. and Schill, R. O. (2011) Trehalose and Vitreous States: Desiccation Tolerance of Dormant Stages of the CrustaceansTriopsandDaphnia. *Physiological and Biochemical Zoology,* **84,** 147-153.

Hengherr, S. and Schill, R. O. (2011) Dormant stages in freshwater bryozoans—An adaptation to transcend environmental constraints. *Journal of Insect Physiology,* **57,** 595-601.

Igarashi, K. and Kashiwagi, K. (2010) Modulation of cellular function by polyamines. *The International Journal of Biochemistry & Cell Biology,* **42,** 39-51.

Kast-Hutcheson, K., Rider, C. V. and Leblanc, G. A. (2001) The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environmental Toxicology and Chemistry,* **20,** 502-509.

Kitada, M., Igarashi, K., Hirose, S. and Kitagawa, H. (1979) Inhibition by polyamines of lipid peroxide formation in rat liver microsomes. *Biochemical and Biophysical Research Communications,* **87,** 388-394.

Kushad, M. M. and Yelenosky, G. (1987) Evaluation of Polyamine and Proline Levels during Low Temperature Acclimation of Citrus. *Plant Physiology,* **84,** 692-695.

Lencioni, V. (2004) Survival strategies of freshwater insects in cold environments. *2004,* 11.

Madin, K. a. C. and Crowe, J. H. (1975) Anhydrobiosis in nematodes: Carbohydrate and lipid metabolism during dehydration. *Journal of Experimental Zoology,* **193,** 335-342.

Martin-Creuzburg, D. and Elert, E. (2009) Good food versus bad food: the role of sterols and polyunsaturated fatty acids in determining growth and reproduction of *Daphnia magna*. *Aquatic Ecology,* **43,** 943-950.

Masclaux, H., Bec, A., Kainz, M. J., Desvilettes, C., Jouve, L. and Bourdier, G. (2009) Combined effects of food quality and temperature on somatic growth and reproduction of two freshwater cladocerans. *Limnology and oceanography,* **54,** 1323-1332.

Miner, B. E., De Meester, L., Pfrender, M. E., Lampert, W. and Hairston, N. G. (2012) Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society of London Biological sciences,* **279,** 1873-1882.

Pauwels, K., Stoks, R., Verbiest, A. and De Meester, L. (2007) Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*. *Hydrobiologia,* **594,** 91-96.

Pegg, A. E. and Mccann, P. P. (1982) Polyamine metabolism and function. *American Journal of Physiology,* **243,** 212-221.

Provasoli, U. (1968) Media and prospects for the cultivation of marine algae. In: A. Watanabe and A. Hattori (eds) *Cultures and Collections of Algae.* Japan Society PIant Physiology, pp. 63-75.

Pruitt, N. L. (1990) Adaptations to temperature in the cellular membranes of crustacea: membrane structure and metabolism. *Journal of Thermal Biology,* **15,** 1-8.

Putman, A., Martin-Creuzburg, D., De Meester, L. and Panis, B. (2015) A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality. *Journal of Plankton Research,* **DOI:10.1093/plankt/fbv043**.

Radzikowski, J. (2013) Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *Journal of Plankton Research,* **35,** 707-732.

Ramon, M., Geuns, J. M. C., Swennen, R. and Panis, B. (2002) Polyamines and fatty acids in sucrose precultured banana meristems and correlation with survival rate after cryopreservation. *Cryoletters,* **23,** 345-352.

Schlotz, N., Ebert, D. and Martin-Creuzburg, D. (2013) Dietary supply with polyunsaturated fatty acids and resulting maternal effects influence host - parasite interactions. *BMC Ecology*, **13,** 41.

Schön, I., Martens, K. and Dijk, P. (2009) *Lost Sex.* Vol. 1, Springer.

Schuber, F. (1989) Influence of polyamines on membrane functions. *Biochemical Journal,* **260,** 1-10.

Seki, M., Umezawa, T., Urano, K. and Shinozaki, K. (2007) Regulatory metabolic networks in drought stress responses. *Current Opinion in Plant Biology,* **10,** 296-302.

Smith, T. A. (1985) Polyamines. *Annual Review of Plant Physiology,* **36,** 117-143.

Steinberg, C. W. (2012) Heat Shock Proteins: The Minimal, but Universal, Stress Proteome*Stress Ecology.* Springer Netherlands, pp. 107-130.

Stibor, H. (2002) The Role of Yolk Protein Dynamics and Predator Kairomones for the Life History of *Daphnia magna*. *Ecology,* **83,** 362-369.

Stillway, L. W. and Walle, T. (1977) Identification of the unusual polyamines 3,3′-diaminodipropylamine and n,n′-bis(3-aminopropyl)-1,3-propanediamine in the white shrimp Penaeus setiferus. *Biochemical and Biophysical Research Communications,* **77,** 1103-1107.

Tabor, C. W. and Tabor, H. (1984) Polyamines. *Annual Review of Biochemistry,* **53,** 749-790.

Von Elert, E. (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology Oceanography,* **47,** 1764 - 1773.

Wacker, A. and Martin-Creuzburg, D. (2007) Allocation of essential lipids in Daphnia magna during exposure to poor food quality. *Functional Ecology,* **21,** 738-747.

Walne, P. R. (1965) Experimental rearing of the larvae of *Ostrea edulis* L. in the laboratory. *Fishery Investment,* **20,** 1-23.

Walter, H. J.-P. and Geuns, J. M. C. (1987) High Speed HPLC Analysis of Polyamines in Plant Tissues. *Plant Physiology,* **83,** 232-234.

Watts, S. A., Lee, K. J. and Cline, G. B. (1994) Elevated ornithine decarboxylase activity and polyamine levels during early development in the brine shrimp Artemia franciscana. *Journal of Experimental Zoology,* **270,** 426-431.

Zappia, V., Porta, R., Carteni-Farina, M., De Rosa, M. and Gambacorta, A. (1978) Polyamine distribution in eukaryotes: occurance of sym-nor-spermidine and sym-nor-spermine in arthropods. *FEBS Letters,* **94,** 161-165.

Zheliaskova, A., Naydenova, S. and Petrov, A. G. (2000) Interaction of phospholipid bilayers with polyamines of different length. *European Biophysics Journal,* **29,** 153-157.

Chapter 4

Egg fatty acid profiles and life history features of *Daphnia magna* clones surviving winter

ADINDA PUTMAN, DOMINIK MARTIN-CREUZBURG, BART PANIS AND LUC DE MEESTER

Abstract

The life cycle of the water flea *Daphnia magna* is characterized by an alternation of sexual and asexual reproduction. This mode of reproduction is an adaptation to the fluctuating environments in the water bodies these animals inhabit. Most *Daphnia* populations produce dormant eggs prior to the onset of winter in temperate zones and hatch from the dormant egg bank at the onset of the next growing season. Yet, in permanent habitats this strategy is most often accompanied by individuals that try to overwinter as parthenogenetic females. If successful, these genotypes have a head start and can increase their fitness substantially. We here tested whether these animals are a random subset of the population by comparing the life histories of their offspring with those of clones originating from the dormant egg bank at different temperatures (12-18-24 °C). Moreover, as overwintering females have been selected to cope with cold temperatures, we compared lipid composition of the parthenogenetic eggs of both types of genotypes produced under the same conditions as in the life history experiment. Our results show that there is no difference between the performance of actively overwintering clones and passively overwintering clones after two generations. However, when clones are cultured at 12°C for multiple generations, age at maturity and clutch release of actively overwintering clones is lower than that of passively overwintering clones. Although temperature did have a strong influence on the fatty acid composition, there were no differences in fatty acid composition between the two overwintering strategies.

1 Introduction

Daphnia are keystone species in a wide geographical distribution of aquatic habitats and are a model system in ecology and evolutionary biology (Hebert, 1987; Lampert, 2006; Miner et al., 2012). One of the major reasons for their success is their cyclic parthenogenetic reproduction cycle. They alternate between the production of asexual eggs that develop immediately and the production of sexual eggs, which first go through a dormant state prior to development (Brendonck and De Meester, 2004). This life cycle allows them to combine the advantages of a fast population growth rate during periods of asexual reproduction with the advantages of sexual recombination (Decaestecker et al., 2009). In temperate zones, sexual reproduction occurs before the onset of winter so that the populations can survive as resistant, dormant eggs. However, due to the combined mode of reproduction *Daphnia* have the opportunity to choose between two options to survive the harmful conditions of the winter, i.e. as a parthenogenetic female or as a dormant egg. Although parthenogenetic females are sometimes able to survive the low temperatures encountered during winter due to a reduced metabolism enhancing the longevity at low food conditions (Dawidowicz and Loose, 1992; Lampert et al., 2014), it involves some risks such as the occurrence of catastrophic events like oxygen depletion under ice can take place. The advantage of surviving as a parthenogenetic female is that they are already large and are able to start reproducing as soon as the conditions improve during spring, so that they have an important head start compared to females that hatch from dormant eggs. On the other hand, overwintering as a dormant embryo offers more security as these eggs are able to survive severe stress conditions due to their biochemical composition and their protective envelope, the ephippium (Radzikowski, 2013; Putman et al., subm. (Chapter 2)). Hatchlings from dormant eggs have a higher juvenile growth rate and are larger at maturation at high food conditions compared to hatchlings from asexual eggs (Arbaciauskas, 2003), but they still have a disadvantage compared to the overwintering female in terms of offspring contribution to the population. These two strategies are not mutually exclusive, as a female *Daphnia* can switch back to asexual reproduction after the production of dormant egg(s) (Zaffagnini, 1987). Earlier studies show that, at least in permanent ponds, most females apply this mixed strategy, trying to survive the winter as parthenogenetic females after the production of some dormant eggs as insurance (Lampert et al., 2010; 2012; Lampert et al., 2014).

The cold temperature experienced by *Daphnia* that try to overwinter as parthenogenetic females is likely to exert a strong selective pressure. According to the "temperature-size rule", body size decreases with increasing temperature (Atkinson and Sibly, 1997; Angilletta et al., 2004). The ability to survive in an environment subjected to fluctuating temperatures depends to a large extent on the physiological plasticity of the membrane composition (Farkas et al., 1984; Hazel and Williams, 1990). Winter-active and cold-acclimated ectotherms accumulate polyunsaturated fatty acids (PUFA) in their body tissue and eggs to maintain membrane fluidity and functions (Farkas et al., 1984; Pruitt, 1990;

Schlechtriem et al., 2006; Sperfeld and Wacker, 2012). As a result, the *Daphnia* that survive the winter season may be expected not to be a random sample of the population but may have been selected for large body size and altered biochemical components of their cell so as to cope with cold temperatures.

D. magna lives in highly fluctuating habitats and Van Doorslaer et al. (2010) showed that *Daphnia* can adapt to changing temperature conditions within one growing season. Additionally, Pajk et al. (2012) showed that intergenerational changes in temperature affect the fitness of the offspring. Maternal effects, transmission of information about environmental variability, are well known to strongly influence the quality of the offspring (Mousseau and Fox, 1998). In *Daphnia* it has been shown that predation pressure can affect offspring defense mechanisms (Agrawal et al., 1999) and egg biochemical composition (Stibor, 2002). Egg biochemical composition can also be influenced by the food quality and quantity (Gliwicz and Guisande, 1992; Wacker and Martin-Creuzburg, 2007; Putman et al., 2015). Even the mode of reproduction can be affected by maternal food composition and concentrations (La Montagne and Mc Cauley, 2001; Abrusan et al., 2007).

Recent studies have shown that clonal diversity drastically decreases during winter conditions and only some parthenogenetic females do survive the winter (Lampert et al., 2012; Lampert et al., 2014). In addition, Carvalho et al. (1987) showed that winter-collected animals have a different fitness profile than summer-collected clones. Cold acclimated females can alter the fatty acid composition of their body tissue and eggs (Schlechtriem et al., 2006; Sperfeld and Wacker, 2012). Comparing the offspring from clones that hatched from the dormant egg bank with clones that survived winter as active females enabled us to identify whether overwintering clones are a random subset of the population or rather have been selected for specific trait values. Secondly, this comparison is also directly relevant to assess the profitability of the two strategies of overwintering. Early in the growing season, offspring from dormant eggs and overwintering females directly compete with each other in building up the spring population. Small differences in the intrinsic rate of increase between clones with different overwintering strategies might thus impact population structure. Arbaciauskas (1998) has shown that the life history of individuals that hatch from dormant eggs are adapted for fast population growth. Here, we compare offspring of parthenogenetic eggs of the two alternate overwintering strategies for both life history traits (body size, intrinsic rate of population growth) and fatty acid composition of their eggs.

2 Material and methods

To compare the life history characteristics and fatty acid composition of *D. magna* clones which are able to survive the harsh environmental conditions during the winter season (hereafter called "overwintering population/clones") with those hatching in the spring (hereafter called "spring

hatchlings") we selected 10 clones from each population from 'Langerode vijver' (LRV), a pond in Neerijse, Belgium (0°49'42.32"N, 4°38'21.49"E). The clones of the overwintering population were sampled during winter of 2012-2013 (average winter temperature was 5°C during the day and 1°C during the night). They were screened for genetic distinctiveness using 12 microsatellite markers (multiplexes M01 and M03 (Jansen et al., 2011)) and ten distinct clones were selected. Clones of the spring hatching population were hatched from a sample of the dormant egg bank of LRV, collected at the same time as the overwintering population. The superficial layer (upper 5 cm) of the sediment was sampled, as for this shallow eutrophic lake this corresponds to the active egg bank of recent years (Càceres, 1998; Decaestecker et al., 2004). To obtain ephippia the sediment sample was sieved over first a 1 mm and then a 250 µm mesh-sized sieve. Ephippia were manually isolated from the sieved sediment fraction and decapsulated. Hatching (10 clones) was stimulated by exposing the dormant eggs to a relatively high temperature (20°C), a long-day photoperiod (16L:8D) and fresh medium (tap water dechlorinated and aged for 24h) (De Meester and Jager, 1993). Hatchlings were cultured as clonal lineages in the laboratory. Hatchlings are all genetically unique, as dormant eggs are sexually produced.

Animals of all clones (overwintering population and spring hatchlings) were reared with 4 individuals in a 210 ml jars filled with aged tap water (aerated for 24h prior to use) under standard conditions (20 ± 2 °C and a photoperiod of 16L:8D) for at least two generations to purge from prior environmental differences and maternal effects before the onset of the experiments. All cultures were fed 150.000 cells ml^{-1} of *S. obliquus*, which corresponds to approximately 2.5 mg C L^{-1}.

2.1 Life table experiment

For the life table experiment, four third-clutch juveniles (born within 12h) of all clones (overwintering population and spring hatchlings) were cultured individually in 210 ml jars filled with aged tap water and distributed over the three temperature treatments, i.e. 12°C, 18°C and 24°C. These animals (first generation) were monitored until the release of second clutch. Three of the second clutch neonates were divided over the same three temperatures, i.e. one neonate at 12°C, one at 18°C and one at 24°C. This second generation was also monitored until the release of their second clutch. In this way, we could compare life history traits among populations in relation to temperatures, and do so for animals cultured for one or for two generations on their temperature. Second, by transplanting the second generation animals across temperatures, we can also test for maternal effects (Lynch and Enis, 1983) All experimental animals were fed 150.000 cells ml^{-1} of *Nannochloropsis limnetica*, jars were cleaned and the medium was refreshed daily.

During the experiment we determined the following life history characteristics: age and size at maturity, age at release of first and second clutch, number of offspring in first and second clutch and

the size of the offspring. Performance 'r' was calculated iteratively for each individual based on the timing of reproduction (x) and the number of offspring (m_x) using the Euler-Lotka equation ($1 = \sum e^{-rx} l_x m_x$); this was only done for animals that survived until release of second clutch.

2.2 Fatty acid analysis

For the second part we again raised third-clutch juveniles (born within 12h) of all clones (selected and non-selected population) in 210 ml jars filled with aged tap water and distributed them over the three temperature treatments, i.e. 12°C, 18°C and 24°C. Every jar contained 2 animals and we had 8 animals per clone per temperature. Animals were fed 150.000 cells ml^{-1} of *Nannochloropsis limnetica* and jars were cleaned and medium was refreshed daily. The second clutch of the animals was collected and for the both populations 4 samples of 100 eggs (10 eggs per clone) per temperature. Samples were freeze-dried and stored at -80°C (2 populations x 3 temperatures x 4 replicates = 24 samples).

The fatty acid composition of the egg samples was analyzed as described in Putman et al. (2015; (Chapter 1)). Briefly, lipids were first extracted from the samples and separated into several classes, based on the charge of their head group, by column chromatography using a silica column. The first fraction contains the nonpolar head groups, such as sterols and glycolipids, and was eluted with 5.53 mL of $CHCl_3$. A second fraction contains the slightly more polar sphingolipids and glycolipids and was eluted with 2.67 mL of acetone, followed by 2.67 mL of acetone:MeOH:acetic acid (100:5:1). A third fraction contains the phospholipids which have a polar head group and was eluted by 4 mL of a MeOH:$CHCl_3$:H_2O mixture (100:50:40). In a second step, the fatty acid tails were transesterified with 4 mL of 3 N methanolic HCl (60°C, 15 min) and 100 µL of internal standard (20 µg mL^{-1} 17:0 ME and 25 µg mL^{-1} 23:0 ME) was added. After cooling down to room temperature, the fatty acid methyl esters (FAME) were further extracted and analyzed by gas chromatography (GC; Hewlett-Packard 6890™) equipped with a flame ionization detector (FID) and a DB-225 (J&W Scientific, 30 m × 0.25 mm inner diameter × 0.25 µm film) capillary column for analysis of the FAME. The FAME were identified by comparison of retention times with those of reference compounds (Sigma-Aldrich). Fatty acids were quantified by comparison with internal standards and by using multipoint standard calibration curves determined for each FAME from mixtures of known composition (Sigma-Aldrich). The absolute amount of each FAME was normalized to the egg dry mass and the egg number.

2.3 Cultivation and preparation of the food

During the preconditioning phase we used the green alga *Scenedesmus obliquus* (SAG 276-3a) and during the experiments we used the eustigmatophyte *Nannochloropsis limnetica* (SAG 18.99) to rear *Daphnia magna.* These two algae are characterised by highly distinct fatty acid profiles, i.e. *S. obliquus* lacks PUFA with more than 18 carbon atoms, while *N. limnetica* contains high

concentrations of C20-PUFA, especially eicosapentaenoic acid (Martin-Creuzburg et al., 2009; Putman et al. 2015; Von Elert, 2002). We chose *Nannochloropsis* for the experiment because we wanted to optimize the fatty acid profile of the *Daphnia*.

Algae were grown in batch cultures at 18°C in aerated 10 liter vessels with illumination at 170 µmol quanta m^{-2} s^{-1} and harvested in the late-exponential growth phase. *S. obliquus* was grown in a medium consisting of 10 ml L^{-1} of enriched seawater (ES) nutrients (Provasoli, 1968), 5 ml L^{-1} of Walne nutrients (Walne, 1965) and the vitamins B1, B12 and H dissolved in dechlorinated tap water. *N. limnetica* was grown in modified Woods Hole (WC) medium with the vitamins B1, B12 and H (Guillard, 1975). Food suspensions were prepared by concentrating the cells via centrifugation (2500g, 5 min) followed by resuspension in tap water. Cell densities of the food suspensions were counted with an Attune® acoustic focusing flow cytometer (Life technologies, Carlsbad, CA, USA).

2.4 Data analysis

The results of the life table experiments were analyzed using a general linear mixed model (GLM), with the life history characteristics as dependent variables, clone identity as a random factor and temperature and population (overwintering population and spring hatchlings) as fixed independent variables. For the second generation of the life table we added the temperature of the first generation as an extra fixed independent variable. To check for difference between traits of significant factors and their interactions Tukey-Kramer post-hoc tests were performed. These analysis were performed in SAS 9.3 (SAS institute Inc., 2002-2010).

Effects of population (overwintering population and spring hatchlings) and temperature on the fatty acid composition of the different lipid classes were tested using a multivariate analysis of variance (MANOVA). Total amounts of fatty acids and concentrations of single fatty acids were compared between populations and temperatures using two-way analyses of variance (ANOVA). Lipid classes were analyzed separately. In case of significant effects of temperature we ran a Tukey HSD post-hoc test to identify which temperatures lead to different FA concentrations. The statistical analyses were performed on concentration per unit dry mass as well as concentration per egg. These analyses were performed with the statistical software R (version 3.1.2; The R Foundation for Statistical Computing) using the packages car, multcomp and lme4.

3 Results

3.1 Life table experiment

For the first generation, performance ‘r’ is significantly lower for clones that overwintered compared to the spring hatched population, independent of the temperature they are raised on. Performance significantly increases with higher temperatures in both populations (Table 1, Figure 1).

Age at maturity, age at 1st clutch and at 2nd clutch increase with decreasing temperatures but there is no difference between the populations (Table 1, only that for age at release of first clutch is shown in Figure 1).

Clones from hatchlings in spring were larger at maturity than clones from the overwintering population. For both populations animals raised at 24°C were smallest at maturity (Table 1, Figure 1). There was a significant difference between the length of animals grown at 24°C and 18°C ($p < 0.001$) and animals grown at 24°C compared to 12°C ($p = 0.035$), while there was no significant difference between the size of animals cultured at 12°C and 18°C ($p = 0.451$).

The cumulative number of offspring produced during 1st and 2nd clutch was lower for clones from the overwintering population than from the spring hatched population. According to the linear model, the number of offspring is also influenced by the temperature the animals are cultured on (Table 1, Figure 1). Post-hoc tests revealed that there is a significant effect of temperature on the number of offspring for the spring hatched population but not for the overwintering population. Clones from the spring hatched population raised at 24°C produced significantly less neonates than clones from the same population raised at 12°C ($p = 0.0138$).

Temperature had no significant influence on the size of the juveniles of 1st and 2nd clutch. Neonates were always larger in the spring hatched population than in the overwintering population (Table 1).

In the second generation of the life table we found a strong significant influence of the second generation temperature (TempF2) on performance and a significant two-way interaction with the temperature of the first generation, but no effect of the population (Table 2). There was no significant difference between the performances of animals raised at the same temperature in their second generation (Table 3), all clones had the highest performance when raised at 24°C in their second generation, intermediate performance when raised at 18°C and lowest performance at 12°C (TK post-hoc, all $P < 0.0001$; Figure 1).

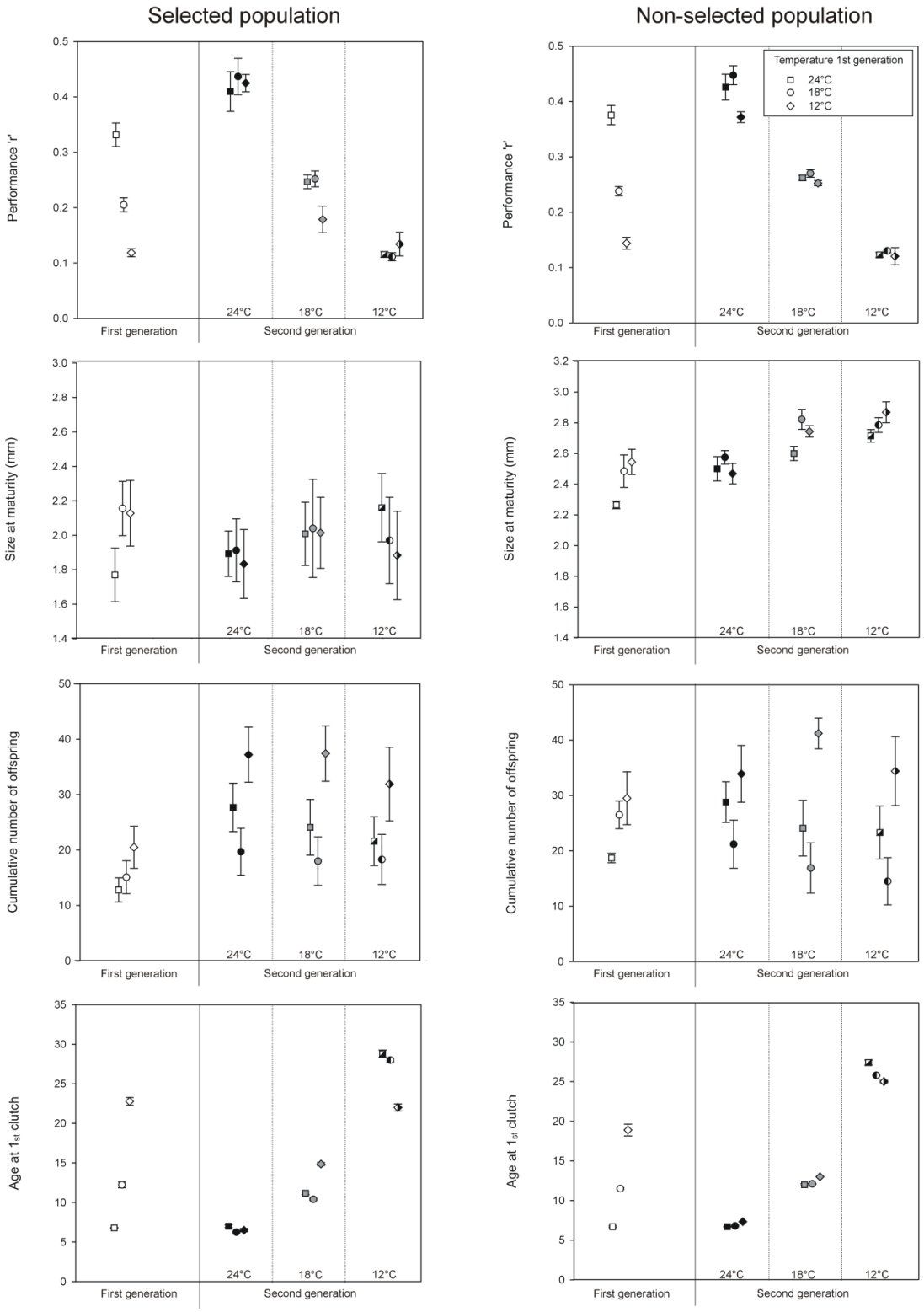

Figure 1: Life history values of a overwintering population (left part) and spring hatchlings (right part) of *D. magna* cultured under different temperatures for two generations. Symbols represent the temperature encountered during the first generation and the fillings of the symbols represent the temperature during the second generation. Data are means of 10 clones and error bars indicate one standard error.

Table 1: Results of the first generation life table analyzed with a general linear mixed models testing for the effects of temperature (Temp), population (Pop) and their interaction on performance 'r', age at maturity, 1st clutch and 2nd clutch, size at maturity, cumulative number of offspring (1st and 2nd clutch) and the size of the juveniles of 1st and 2nd clutch. Clone was included as a random factor in all models. Significant differences indicated in bold ($p < 0.05$).

	Performance 'r'			Age at maturity			Size at maturity			Cumulative number offspring		
	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value
Temperature	2	148.0756	**<2 E-16**	2	245.7012	**<2 E-16**	2	8.0488	**0.0014**	2	5.8459	**0.0065**
Population	1	6.3967	**0.0212**	1	0.3818	0.5444	1	7.192	**0.0152**	1	6.8314	**0.0177**
Temp:Pop	2	0.2238	0.8006	2	1.5045	0.2363	2	0.7517	0.4791	2	0.4075	0.6685
	Age at 1st clutch			Age at 2nd clutch			Size juveniles 1st clutch			Size juveniles 2nd clutch		
	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value
Temperature	2	229.9612	**<2 E-16**	2	167.4636	**<2 E-16**	2	2.4572	0.1013	2	2.3699	0.1125
Population	1	0.6415	0.4338	1	0.5616	0.4636	1	9.9749	**0.0055**	1	8.4101	**0.0096**
Temp:Pop	2	0.2919	0.7487	2	1.977	0.1558	2	2.5621	0.0924	2	1.3603	0.2731

Table 2: Results of the second generation life table analyzed with a general linear mixed model testing for the effects of the temperature of the first generation (TempF1), temperature of the second generation (Temp F2), population (Pop) and their interactions on performance 'r', age at maturity, 1st clutch and 2nd clutch, size at maturity, cumulative number of offspring (1st and 2nd clutch) and the size of the juveniles of 1st and 2nd clutch. Clone was included as a random factor in all models. Significant differences indicated in bold ($p < 0.05$).

	Performance 'r'			Age at maturity			Size at maturity			Cumulative number offspring		
	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value
Temp F1	2	1.07	0.3463	2	0.86	0.4269	2	1.42	0.2468	2	0.43	0.6502
Temp F2	2	437.33	**<0.0001**	2	815.25	**<0.0001**	2	11.49	**<0.0001**	2	23.99	**<0.0001**
Pop	1	0.14	0.7134	1	2.48	0.1184	1	295.01	**<0.0001**	1	18.52	**<0.0001**
Temp F1:TempF2	4	3.65	**0.0084**	4	2.29	0.0652	4	0.8	0.5253	4	0.41	0.7997
Temp F1:Pop	2	2.02	0.1383	2	1.39	0.2546	2	0.62	0.5426	2	0.6	0.5506
Temp F2:Pop	2	2.08	0.1304	2	1.15	0.3209	2	1.83	0.1662	2	2.6	0.0796
Temp F1:TempF2:Pop	4	2.2	0.0751	4	4.1	**0.0041**	4	0.53	0.7122	4	0.39	0.818
	Age at 1st clutch			Age at 2nd clutch			Size juveniles 1st clutch			Size juveniles 2nd clutch		
	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value
Temp F1	2	1.54	0.2207	2	1.98	0.1448	2	0.56	0.5704	2	2.06	0.1335
Temp F2	2	929.47	**<0.0001**	2	1748.75	**<0.0001**	2	0.41	0.6617	2	1.27	0.2864
Pop	1	0	0.9666	1	0.98	0.325	1	75.23	**<0.0001**	1	46.1	**<0.0001**
Temp F1:TempF2	4	15.15	**<0.0001**	4	22.1	**<0.0001**	4	1.39	0.2428	4	1.36	0.2549
Temp F1:Pop	2	1	0.3734	2	2.79	0.0673	2	1.05	0.3527	2	0.08	0.919
Temp F2:Pop	2	0.14	0.8709	2	0.54	0.5829	2	0.71	0.4962	2	0.25	0.7819
Temp F1:TempF2:Pop	4	5.7	**0.0004**	4	8.01	**<0.0001**	4	1.39	0.2442	4	1.75	0.1477

Table 3: Results of a Tukey-Kramer post-hoc on the significant effect of the interaction between the first and second generation temperature on the performance, analyzed by a general linear mixed model. We show P-values for all combinations within the same second generation temperature.

	TempF1-TempF2 vs. TempF1-TempF2		P-value
F2 12°C	12-12	18-12	0.6064
	12-12	24-12	0.2605
	18-12	24-12	0.9999
F2 18°C	12-18	18-18	0.4011
	12-18	24-18	0.7646
	18-18	24-18	0.9984
F2 24°C	12-24	18-24	0.8239
	12-24	24-24	0.9995
	18-24	24-24	0.933

Age at maturity is significantly affected by temperature of the second generation and the three-way interaction between first and second generation temperature and population (Table 2). Age at 1st and 2nd clutch likewise show a significant three-way interaction between temperature of the 1st and 2nd generation and population (Table 2). The lower the temperature the longer it takes to mature and release clutches and, comparable with the performance, this is independent of the temperature of the maternal generation. TK post–hoc test revealed that there are only significant differences between the populations for clones that were cultured at 12°C for two consecutive generations ($P_{Age\ at\ maturity}$ = 0.0057, $P_{Age\ at\ 1st\ clutch}$ = 0.0114, $P_{Age\ at\ 2nd\ clutch}$ = *0.0592*), clones from the overwintering population needed less time to mature or release brood.

Size at maturity is only influenced by population and temperature of the second generation (Table 2). Similar to our observation for the first generation, clones that overwintered as a parthenogenetic female are always smaller at maturity, independent of the culturing temperature in both generations, and animals raised at 24°C in their second generation tend to be smaller than those raised at 12°C or 18°C in their second generation (Figure 1).

The same pattern was found for the cumulative number of offspring (Table 2, Figure 1): the number of offspring is always lower for the overwintering population and clones at 24°C produce less offspring, in both populations (Figure 2).

Juveniles of the overwintering population are always smaller than those of the spring hatched population, independent of the F1 and F2 temperature (Table 2).

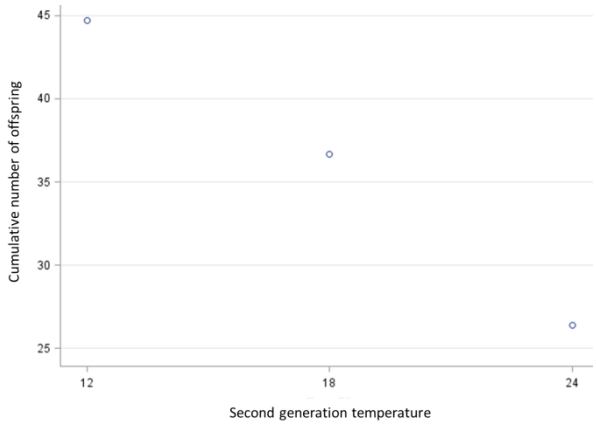

Figure 2: Tukey-Kramer LS means comparisons of the second generation temperatures in the general linear model for cumulative number of offspring.

3.2 Fatty acid analysis

In none of the samples we observed fatty acids in the second fraction, which is supposed to contain the fatty acids of sphingolipids and glycolipids (in accordance with Putman et al., 2015).

The fatty acid compositions of the neutral lipid fraction (fraction 1, Figure 3) and the phospholipid fraction (fraction 3) are affected by temperature (MANOVA, Table 4) but not by population.

Targeted analyses for each fatty acid separately did show tendencies for differences (Table 6, $p < 0.05$). However, differences disappeared after correction for multiple testing. Temperature only affected the total concentration of fatty acids derived of phospholipids, with more fatty acids with decreasing temperature particularly for the overwintering population (Table 5 and 6). Only a few fatty acids were influenced by temperature (Table 6). Especially EPA (C20:5 n-3) strongly increases with decreasing temperature, while ALA (C18:3 n-3) increases with increasing temperature (Figure 3).

All results mentioned above are based on the data expressed per mg dry weight. Analysis of the data per egg gave almost identical results.

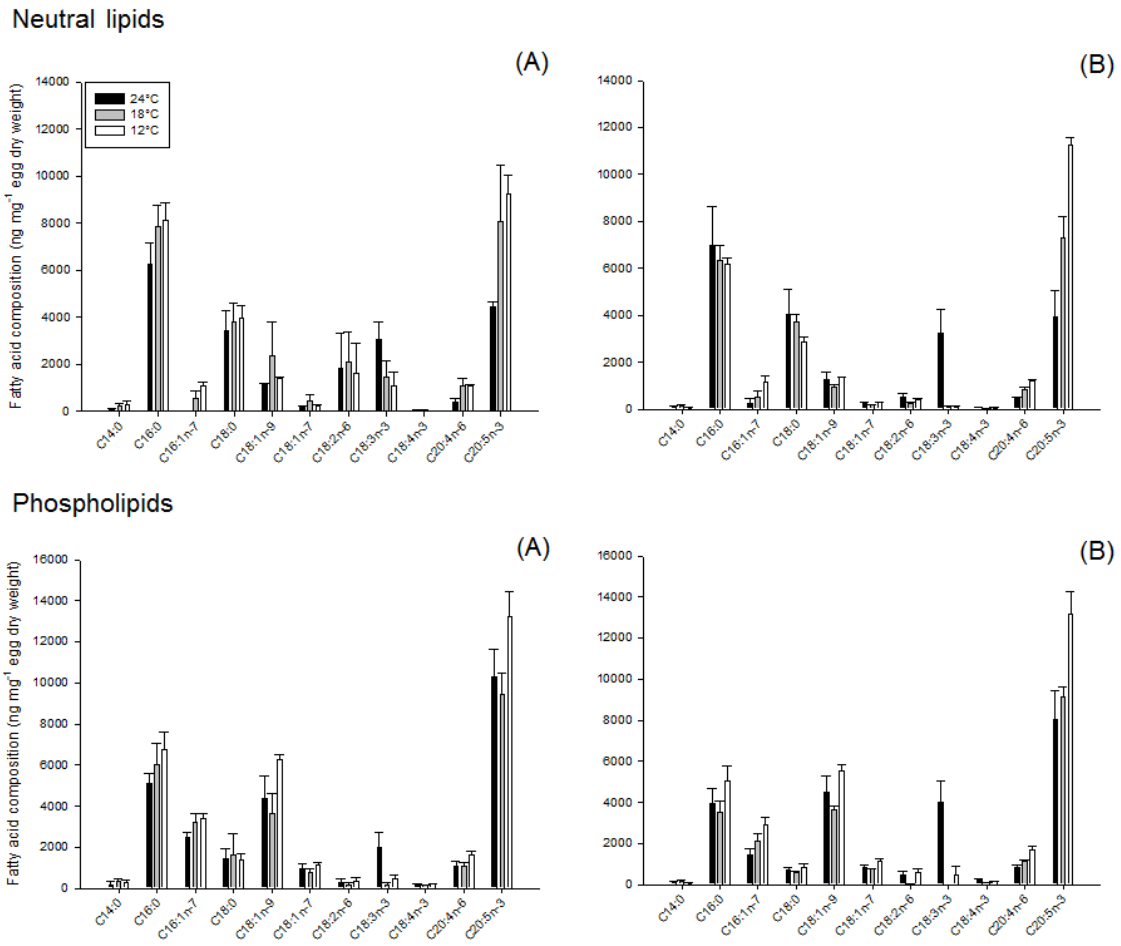

Figure 3: Fatty acid composition (µg mg^{-1} dry weight) of the neutral lipid and phospholipid fractions of asexual eggs of a selected (A) and non-selected (B) population of *D. magna* produced under different temperatures. Data are means of 4 replicates and error bars indicate one standard error.

Table 4: Results of MANOVA testing for the effects of temperature (Temp), population (Pop) and their interaction on the fatty acid composition (ng mg^{-1} dry weight) of asexual eggs of *D. magna*. Datasets for neutral lipids and phospholipids were analyzed separately. Significant differences indicated in bold ($p > 0.05$).

	Neutral lipids			*Phospholipids*		
	Df	Approx F	P-value	Df	Approx F	P-value
Temperature	2	2.9230	**0.0122**	2	2.2826	**0.0400**
Population	1	1.7208	0.2255	1	2.2802	0.1259
Temp:Pop	2	0.5697	0.8953	2	2.0745	0.0602

Table 5: Fatty acid composition of asexual eggs of a overwintering and spring hatched population of *D. magna* produced under different temperatures. The total concentration and the concentration of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) (µg mg^{-1} dry weight) and the percentages of essential fatty acids are given. Data are means of four replicates.

Neutral lipids

	Overwintering population			Spring hatched population		
	24°C	18°C	12°C	24°C	18°C	12°C
∑ FA	20.85 +/- 3.14	27.94 +/- 5.22	28.07 +/- 1.98	21.04 +/- 5.37	20.36 +/- 1.47	24.98 +/- 0.85
SFA	9.74 +/- 1.82	11.87 +/- 1.82	12.38 +/- 1.45	11.12 +/- 2.74	10.17 +/- 1.03	9.07 +/- 0.56
MUFA	1.34 +/- 0.11	3.32 +/- 2.03	2.68 +/- 0.34	1.72 +/- 0.60	1.65 +/- 0.40	2.85 +/- 0.30
(n-3) PUFA	7.52 +/- 0.98	9.56 +/- 3.15	10.30 +/- 1.41	7.23 +/- 2.14	7.41 +/- 0.95	11.40 +/- 0.38
(n-6) PUFA	2.25 +/- 1.62	3.18 +/- 1.61	2.71 +/- 1.33	0.97 +/-0.26	1.13 +/- 0.14	1.66 +/- 0.07
C18:2 n-6	8.85%	24.21%	5.84%	2.47%	3.45%	1.69%
C18:3 n-3	14.73%	13.47%	3.81%	15.47%	3.41%	0.33%
C20:4 n-6	1.96%	6.67%	3.81%	2.14%	6.21%	4.96%
C20:5 n-3	21.21%	46.15%	32.84%	18.58%	60.79%	45.12%
C22:6 n-3	nd	nd	nd	nd	nd	nd

Phospholipids

	Overwintering population			Spring hatched population		
	24°C	18°C	12°C	24°C	18°C	12°C
∑ FA	28.35+/-2.45	26.56+/-2.06	35.11+/-0.64	25.18+/-4.78	21.19+/-1.65	31.59+/-2.79
SFA	6.74 +/- 1.13	7.95 +/- 2.18	8.39 +/- 1.31	4.80 +/- 0.91	4.27 +/- 0.60	8.39 +/- 1.31
MUFA	7.75 +/- 1.63	7.59 +/- 1.57	10.85 +/- 0.52	6.81 +/- 1.67	6.57 +/- 0.56	9.56 +/- 0.79
(n-3) PUFA	12.46 +/- 2.12	9.76 +/- 1.17	13.88 +/- 1.40	12.28 +/- 2.47	9.22 +/- 0.53	13.76 +/- 1.56
(n-6) PUFA	1.40 +/- 0.40	1.25 +/- 0.28	1.99 +/- 0.33	1.29 +/- 0.33	1.13 +/- 0.08	2.29 +/-0.37
C18:2 n-6	1.01%	5.07%	0.98%	1.86%	0.43%	1.86%
C18:3 n-3	6.95%	7.51%	1.33%	15.85%	0.00%	1.45%
C20:4 n-6	3.91%	8.39%	4.69%	3.25%	4.27%	5.40%
C20:5 n-3	36.29%	48.86%	37.63%	31.96%	31.86%	41.57%
C22:6 n-3	nd	nd	nd	nd	nd	nd

Table 6: Results of ANOVA testing for the effects of temperature (Temp), population (Pop) and their interaction on the fatty acid composition (ng mg^{-1} dry weight) of asexual eggs of *D. magna*. Datasets for neutral lipids and phospholipids were analyzed separately. Significant differences after Holm-Bonferroni corrections indicated in bold ($p < 0.05$).

		Neutral lipids			*Phospholipids*		
Fatty acids	Variables	Df	F value	P value	Df	F value	P value
C14:0	Temp	2	0.8442	0.4462	2	0.0503	0.6132
	Pop	1	2.0462	0.1697	1	4.4948	0.0482
	Temp:Pop	2	1.1236	0.3469	2	0.3655	0.6989
C16:0	Temp	2	0.1967	0.8232	2	1.9183	0.1757
	Pop	1	1.4411	0.2455	1	8.2821	0.0100
	Temp:Pop	2	1.7770	0.3306	2	0.3765	0.6915
C16:1	Temp	2	8.3501	**0.0027**	2	6.5360	0.0073
	Pop	1	0.2676	0.6113	1	10.2891	0.0049
	Temp:Pop	2	0.1451	0.8659	2	0.4024	0.6746
C18:0	Temp	2	0.1518	0.8602	2	0.0029	0.9972
	Pop	1	0.0997	0.7558	1	3.5077	0.0774
	Temp:Pop	2	0.7589	0.4826	2	0.1397	0.8705
C18:1n-9	Temp	2	0.3045	0.7412	2	5.2166	0.0163
	Pop	1	0.8766	0.3615	1	0.1180	0.7352
	Temp:Pop	2	1.0015	0.3869	2	0.2412	0.7882
C18:1n-7	Temp	2	0.3430	0.7142	2	4.1587	0.0329
	Pop	1	0.0740	0.7886	1	0.0525	0.8213
	Temp:Pop	2	1.0159	0.3819	2	0.0975	0.9076
C18:2n-6	Temp	2	0.0183	0.9819	2	2.9709	0.0767
	Pop	1	3.5454	0.0760	1	0.4064	0.5318
	Temp:Pop	2	0.0604	0.9417	2	1.0113	0.3835
C18:3n-3	Temp	2	10.6807	**0.0009**	2	15.5971	**0.0001**
	Pop	1	1.9993	0.1744	1	1.8060	0.1957
	Temp:Pop	2	0.8301	0.4520	2	2.3223	0.1267
C18:4n-3	Temp	2	0.4040	0.6736	2	7.7239	**0.0038**
	Pop	1	0.8032	0.3820	1	0.9332	0.3468
	Temp:Pop	2	1.5162	0.2463	2	2.5243	0.1081
C20:4n-6	Temp	2	10.3493	**0.0010**	2	11.4525	**0.0006**
	Pop	1	0.0005	0.9823	1	0.2184	0.0646
	Temp:Pop	2	0.7223	0.4992	2	0.7938	0.4673
C20:5n-3	Temp	2	12.8432	**0.0003**	2	8.1246	**0.0031**
	Pop	1	0.0683	0.7967	1	0.9124	0.3521
	Temp:Pop	2	0.8393	0.4482	2	0.5473	0.5278
Total	Temp	2	1.2936	0.2986	2	6.4157	0.0078
	Pop	1	1.5080	0.2353	1	3.2947	0.0862
	Temp:Pop	2	0.6268	0.5456	2	0.0949	0.9099

4 Discussion

The water flea *Daphnia magna* inhabits aquatic environments that are variable in both biotic and abiotic environmental conditions, such as temperature, drought, food and predation. Although many female *Daphnia* try to actively survive the winter, only few of them succeed as clonal diversity drops drastically during winter (Lampert et al., 2014). To avoid the negative impact of winter conditions, *Daphnia* starts the production of dormant eggs when conditions deteriorate (Brendonck and De Meester, 2003). These dormant eggs are stress resistant and serve as kind of insurance against loss of the genetic diversity (Lampert et al., 2010). We observed that clones that were able to actively survive the winter as females did differ in their life history traits from clones that survived the winter as dormant eggs. Performance was lower for actively overwintering clones in the first generation, mostly due to a lower number of offspring. In the second generation number of offspring is still lower for overwintering populations but this difference is less pronounced, whereby performance is no longer different between both populations. Although performance was comparable, age at maturity and age at clutch release did differ between both populations if they were cultured at 12°C for two generations. Juveniles from actively overwintering clones are faster than juveniles from the spring hatched population.

One of the most important biotic factors fluctuating in time and space is temperature. These variations in temperature have implications on the life history and physiology of many animals, including *Daphnia*. *Daphnia* grow faster at warmer temperatures, consequently being smaller at maturity and having a lower number of offspring at higher temperatures when food is non-limiting (Gliwicz and Boavida, 1996; Atkinson and Sibly, 1997; Angilletta, 2004). There are several studies confirming this "temperature-size rule" (Giebelhausen and Lampert, 2001; Masclaux et al., 2009; Sperfeld and Wacker, 2009). Accordingly, we found a strong positive correlation between the performance and culturing temperature in both generations. For all clones used in this study, best performance temperature is at 24°C, consistent with the results of Mitchell and Lampert (2000) and Mitchell et al. (2004). Yet, optimal temperature seems to be clone specific as some other studies observed a decline in growth rate with increasing temperatures above 20°C (Giebelhausen and Lampert, 2001; Masclaux et al., 2009).

Pjak et al. (2012) showed that not only the culturing temperature of the experimental generation affects the life history of the animals, but also the temperature of the maternal generation. In contrast, we found that there is no difference in life history between clones cultured at the same temperature in the second generation, even if they were cultured at other temperatures in the previous generation. In Pjak et al. (2012) fitness was highest for 15°C-acclimated animals raised at 20°C, while we only observed that at 12°C the age at maturity of the cold-acclimated animals actively surviving winter is lower than those animals acclimated at 18°C or 24°C.

Besides the effect on life history, temperature also affects the biochemical composition of body tissue and eggs of *Daphnia*. Crustacea exposed to low temperatures accumulate polyunsaturated fatty acids (Farkas et al., 1984; Schlechtriem et al., 2006; Sperfeld and Wacker, 2012). Our results confirm these results. Eggs produced under colder temperatures contained more unsaturated fatty acids, especially EPA. The concentration of ALA was an exception and decreases with decreasing temperature. It is already known that *Daphnia* can produce EPA by converting the precursor ALA (Farkas te al., 1984; Goulden and Place, 1990; Schlechtriem et al., 2006). Moreover, Schlechtriem et al. (2006) suggested that the increased proportions of unsaturation may be due to a cold induced expression of delta-9-desaturase as has been found for Carp (Tiku et al., 1996). This increased degree of unsaturation, most pronounced in phospholipids, plays an important role in the adaptation to cold because it allows the animals to maintain membrane fluidity and functioning (Hazel and Williams, 1990; Pruitt, 1990). In contrast with Sperfeld and Wacker (2012) who only found differences between temperatures in the concentrations of fatty acids of body tissue and not for eggs, the fatty acid composition of our egg samples did differ between temperature regimes. As expected, differences were more pronounced for the phospholipid fraction than for the neutral lipids. Sperfeld and Wacker (2012) did not separate the total lipid fraction into phospholipids and neutral lipids.

We conclude that there are some important differences between passively and actively overwintering clones of *D. magna* concerning their life histories. Temperature had a strong influence on both the life history of female *Daphnia* and the fatty acid composition of the eggs and clones from the actively overwintering population and the spring hatching population were influenced differently by the temperatures. In general performance of all clones was lower at lower temperature, and performance of clones selected to survive winter as a parthenogenetic female was lower than of spring hatching clones in the first generation. Additionally, actively overwintering clones seem to be adapted to reproduce faster at cold temperatures. Our results also suggest that clones from overwintering populations invest more in EPA compared to ALA in the phospholipids of the eggs than clones that survive winter as dormant stages.

Acknowledgements

AP enjoyed a PhD fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) and the work was financially supported by KU Leuven Research Fund project PF/2010/007. We thank P. Merkel for technical assistance and M. Schepens for help during the experiments.

References

Abrusan, G., Fink, P. and Lampert, W. (2007) Biochemical limitation of resting egg production in *Daphnia*. *Limnology and oceanography,* **52,** 1724-1728.

Agrawal, A. A., Laforsch, C. and Tollrian, R. (1999) Transgenerational induction of defences in animals and plants. *Nature,* **401,** 60-63.

Angilletta, M. J., Steury, T. D. and Sears, M. W. (2004) Temperature, Growth Rate, and Body Size in Ectotherms: Fitting Pieces of a Life-History Puzzle. *Integrative and Comparative Biology,* **44,** 498-509.

Arbaciauskas, K. (1998) Life-history traits of exephippial and parthenogenetically derived daphnids: indicators of different life-history strategies. *Archiv für Hydrobiologie Special Issues Advances in Limnology,* **52,** 339-358.

Arbaciauskas, K. and Lampert, W. (2003) Seasonal adaptation of ex-ephippio and parthenogenetic offspring of *Daphnia magna*: differences in life history and physiology. *Functional Ecology,* **17,** 431-437.

Atkinson, D. and Sibly, R. M. (1997) Why are organisms usually bigger in colder environments? Making sense of a life history puzzle. *Trends in Ecology & Evolution,* **12,** 235-239.

Brendonck, L. and De Meester, L. (2003) Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia,* **491,** 65-84.

Cáceres, C. E. (1998) Interspecific Variation in the Abundance, Production, and Emergence of *Daphnia* Diapausing Eggs. *Ecology,* **79,** 1699-1710.

Carpenter, S. R., Kitchell, J. F., Hodgson , J. R., Cochran, P. A., Elser, J. J., Elser, M. M., Lodge, D. M., Kretchmer, D. and He, X. (1987) Regulation of lake primary production by food web structure. *Ecology,* **68,** 1863-1876.

Carvalhoa, G. R. and Crisp, D. J. (1987) The clonal ecology of *Daphnia magna* (Crustacea: cladocera). *Journal of Animal Ecology,* **56,** 453-468.

Dawidowicz, P. and Loose, C. J. (1992) Metabolic costs during predator-induced dielvertical migration of Daphnia. *Limnology and oceanography,* **37,** 1589-1595.

De Meester, L., Gomez, A. and Simon, J. (2004) Evolutionary and ecological genetics of cyclical parthenogens. In: A. Moya and E. Font (eds) *Evolution from molecules to ecosystems.* Oxford university press, pp. 122-134.

De Meester, L. and Jager, H. (1993) Hatching of *Daphnia* sexual eggs. I. Intraspecific differences in the hatching responses of D. magna eggs. *Freshwater biology,* **30,** 219-226.

Decaestecker, E., De Meester, L. and Mergeay, J. (2009) Cyclical Parthenogenesis in Daphnia: Sexual Versus Asexual Reproduction. In: I. Schön, K. Martens and P. Dijk (eds) *Lost Sex.* Springer Netherlands, pp. 295-316.

Decaestecker, E., Lefever, C., De Meester, L. and Ebert, D. (2004) Haunted by the past: Evidence for dormant stage banks of microparasites and epibionts of Daphnia. *Limnology and oceanography,* **49,** 1355-1364.

Farkas, T., Nemecz, G. and Csengeri, I. (1984) Differential response of lipid metabolism and membrane physical state by an actively and passively overwintering planktonic crustacean. *Lipids,* **19,** 436-442.

Giebelhausen, B. and Lampert, W. (2001) Temperature reaction norms of Daphnia magna: the effect of food concentration. *Freshwater biology,* **46,** 281-289.

Gliwicz, Z. M. and Boavida, M. J. (1996) Clutch size and body size at first reproduction in Daphnia pulicaria at different levels of food and predation. *Journal of Plankton Research,* **18,** 863-880.

Gliwicz, Z. M. and Guisande, C. (1992) Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. *Oecologia,* **91,** 463-467.

Goulden, C. E. and Place, A. R. (1990) Fatty acid synthesis and accumulation rates in daphniids. *Journal of Experimental Zoology,* **256,** 168-178.

Guillard, R. (1975) Cultures of phytoplankton for feeding of marine invertebrates. *Culture of marine invertebrate animals,* 29 - 60.

Hazel, J. R. and Williams, E. E. (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in lipid research,* **29,** 167-227.

Hebert, P. D. N. (1978) The population biology of *Daphnia* (Crustacea, Daphnidae). *Biological Reviews,* **53,** 387-426.

Lamontagne, J. M. and Mccauley, E. (2001) Maternal effects in Daphnia: what mothers are telling their offspring and do they listen? *Ecology letters,* **4,** 64-71.

Lampert, K. P., Regmi, B. P., Wathne, I. and Larsson, P. (2014) Clonal diversity and turnover in an overwintering Daphnia pulex population, and the effect of fish predation. *Freshwater biology,* **59,** 1735-1743.

Lampert, W. (2006) Daphnia: model herbivore, predator and prey. *Polish Journal of Ecology,* **54,** 607 - 620.

Lampert, W., Lampert, K. P. and Larsson, P. (2012) Coexisting overwintering strategies in *Daphnia pulex*: Clonal differences in sexual reproduction. *Fundamental and Applied Limnology / Archiv für Hydrobiologie,* **179,** 281-291.

Lampert, W., Lampert, K. P. and Larssona, P. (2010) Coexisting overwintering strategies in Daphnia pulex: A test of genetic differences and growth responses. *Limnology and oceanography,* **55,** 1893-1900.

Lynch, M. and Ennis, R. (1983) Resource availibility, maternal effects and longevity. *Experimental gerontology,* **18,** 147-165.

Martin-Creuzburg, D., Sperfeld, E. and Wacker, A. (2009) Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids. *Proceedings of the Royal Society B: Biological Sciences,* **276,** 1805-1814.

Masclaux, H., Bec, A., Kainz, M. J., Desvilettes, C., Jouve, L. and Bourdier, G. (2009) Combined effects of food quality and temperature on somatic growth and reproduction of two freshwater cladocerans. *Limnology and oceanography,* **54,** 1323-1332.

Miner, B. E., De Meester, L., Pfrender, M. E., Lampert, W. and Hairston, N. G. (2012) Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society of London Biological sciences,* **279,** 1873-1882.

Mitchell and Lampert (2000) Temperature adaptation in a geographically widespread zooplankter, Daphnia magna. *Journal of Evolutionary Biology,* **13,** 371-382.

Mitchell, E. S., Halves, J. and Lampert, W. (2004) Coexistence of similar genotypes of Daphnia magna in intermittent populations: response to thermal stress. *Oikos,* **106,** 469-478.

Mousseau, T. A. and Fox, C. W. The adaptive significance of maternal effects. *Trends in Ecology & Evolution,* **13,** 403-407.

Pjak, F., Von Elert, E. and Fink, P. (2012) Interaction of changes in food quality and temperature reveals maternal effects on fitness parameters of a keystone aquatic herbivore. *Limnology and oceanography,* **57,** 281-292.

Provasoli, U. (1968) Media and prospects for the cultivation of marine algae. In: A. Watanabe and A. Hattori (eds) *Cultures and Collections of Algae.* Japan Society PIant Physiology, pp. 63-75.

Pruitt, N. L. (1990) Adaptations to temperature in the cellular membranes of crustacea: membrane structure and metabolism. *Journal of Thermal Biology,* **15,** 1-8.

Putman, A., Martin-Creuzburg, D., De Meester, L. and Panis, B. (2015) A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality. *Journal of Plankton Research,* **DOI:10.1093/plankt/fbv043**.

Radzikowski, J. (2013) Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *Journal of Plankton Research,* **35,** 707-732.

Schlechtriem, C. (2006) Effect of temperature on the fatty acid composition and temporal trajectories of fatty acids in fasting Daphnia pulex (Crustacea, Cladocera). *Lipids,* **41,** 397.

Sperfeld, E., Martin-Creuzburg, D. and Wacker, A. (2012) Multiple resource limitation theory applied to herbivorous consumers: Liebig's minimum rule vs. interactive co-limitation. *Ecology letters,* **15,** 142-150.

Sperfeld, E. and Wacker, A. (2009) Effects of temperature and dietary sterol availability on growth and cholesterol allocation of the aquatic keystone species Daphnia. *Journal of Experimental Biology,* **212,** 3051-3059.

Sperfeld, E. and Wacker, A. (2012) Temperature affects the limitation of Daphnia magna by eicosapentaenoic acid, and the fatty acid composition of body tissue and eggs. *Freshwater biology,* **57,** 497-508.

Stibor, H. (2002) The Role of Yolk Protein Dynamics and Predator Kairomones for the Life History of *Daphnia magna*. *Ecology,* **83,** 362-369.

Tiku, P. E., Gracey, A. Y., Macartney, A. I., Beynon, R. J. and Cossins, A. R. (1996) Cold-Induced Expression of Δ9-Desaturase in Carp by Transcriptional and Posttranslational Mechanisms. *Science,* **271,** 815-818.

Van Doorslaer, W., Stoks, R., Swillen, I., Feuchtmayr, H., Atkinson, D., Moss, B. and De Meester, L. (2010) Experimental thermal microevolution in community-embedded Daphnia populations. *Climate research,* **43,** 81.

Von Elert, E. (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography*, **47,** 1764 - 1773.

Wacker, A. and Martin-Creuzburg, D. (2007) Allocation of essential lipids in Daphnia magna during exposure to poor food quality. *Functional Ecology*, **21,** 738-747.

Walne, P. R. (1965) Experimental rearing of the larvae of *Ostrea edulis* L. in the laboratory. *Fishery Investment,* **20,** 1-23.

Zaffagnini, F. (1987) Reproduction in *Daphnia*. In: R. H. Peters and R. De Bernardi (eds) *Daphnia.* Vol. 45. Memorie dell'Instituto Italiano di Idrobiologia, pp. 245-284.

Part II

Chapter 5

Towards the eternal life for *Daphnia* clones: Cryopreservation of asexual eggs of *D. magna*

ADINDA PUTMAN, BART PANIS, NIELS DESMET AND LUC DE MEESTER

Abstract

For the important model organism *Daphnia* it would be a very big step forward if the clonal lineages used in many research applications could be safely stored by cryopreservation. Asexual eggs are very sensitive to drought and freezing temperatures, mainly due to their high water content. This is a common feature among embryos of aquatic organisms and it is the main reason why there are only limited numbers of established cryopreservation protocols for these embryos. To obtain surviving eggs after cryopreservation it is thus essential to drastically reduce this water content without affecting viability. In this study we present a step by step development of a cryopreservation protocol for asexual eggs of *D. magna*. A two-step pretreatment of the eggs and freezing by droplet vitrification led to successful cryopreservation of these eggs, although at low numbers. The pretreatment consisted of a glycerol loading followed by osmotic dehydration in a vitrification solution. We also showed that intracellular trehalose has a beneficial effect on the survival of cryopreserved asexual eggs.

1 Introduction

The water flea *Daphnia* is a keystone species in aquatic ecosystems with a wide geographical distribution (Hebert, 1978; Carpenter et al., 1987; Lampert, 2006) and an important model organism in ecological and evolutionary biological research (Lampert, 2011; Seda and Petrusek, 2011). Recent publication of the complete *Daphnia pulex* genome (Colbourne et al., 2011) generates even more opportunities (Tautz, 2011; Miner et al., 2012). Moreover, *Daphnia*, especially *Daphnia magna*, are the most widely used invertebrates in ecotoxicological studies (Walker, 2014). One of the biggest advantages of *Daphnia* is their parthenogenetic reproduction cycle, alternating the production of subitaneous asexual eggs and dormant sexual eggs (Hebert, 1978; Benzie, 2005). Dormant eggs are stress resistant, consequently able to stay viable for long periods of time and forming some kind of archive in the sediment (Hairston et al., 1995; Brendonck and De Meester, 2003; De Meester et al., 2004).

Though, most of the applications use well-characterized clonal lines generated through asexual reproduction. This enables researchers to work with large numbers of genetically identical individuals and to disentangle genotypic interactions and phenotypic plasticity (De Meester et al., 2004; Tollrian and Leese, 2010; Simon et al., 2011). However, one major disadvantage to the use of these clonal lineages, is that they are maintained by continuous culturing, which is laborious and time and space consuming. Additionally, there is a risk of contamination, diseases or accidental loss. Cryopreservation of the genetic lines would be a good and safe alternative. Preservation of biological tissue at sub-zero temperatures as low as -196°C can be regarded as the ultimate storage method as all metabolic processes are stopped at these ultra-low temperatures (Hodgson, 1994; Mazur et al., 2008). Most cells and organisms, however, do not survive exposure to freezing temperatures, and particularly the extreme low temperatures used for cryopreservation are not tolerated. A cryopreservation protocol therefore always involves some steps preparing the tissue and preventing freeze damage. Avoiding the formation of destructive ice crystals is essential for survival after cryopreservation (Pegg, 1987; Mazur, 2004).

In general, eggs of aquatic organisms have high water contents and they are consequently very sensitive to low temperatures. In contrast to the numerous protocols for successful cryopreservation of sperm, there are only limited numbers of successful cryopreservation protocols of aquatic invertebrate eggs (Gwo et al., 2000; Zhang, 2004). Reports of successful cryopreservation include embryos of the bivalves Pacific oyster, Blue mussel and Greenshell mussel (Lin and Chao, 2000; Paredes et al., 2012; 2013), embryos of sea uchrin (Bellas and Paredes, 2011) and embryos of the rotifer *Branchionus plicatilis* (Toledo and Kurokura, 1990). Although many attempts, also for fish embryos there are no successful cryopreservation protocols yet (Zhang, 2004; Mauger et al., 2006; Tsai et al., 2009).

In this paper we describe a stepwise approach towards successful cryopreservation of asexual *Daphnia magna* eggs. We have chosen to use vitrification as freezing method, as it is currently considered to be the most suitable technique for cryopreservation of embryos (Kulus and Zalewska, 2014; Fahy and Wowk, 2015). Vitrification aims to cryopreserve without the formation of ice crystals, via ultra-rapid cooling and high intracellular viscosity (Fahy et al., 1984). Sufficient dehydration can be achieved by air-drying, freeze-dehydration or the application of cryoprotectants. Penetrating cryoprotectants are used to increase intracellular solute concentrations and non-penetrating cryoprotectants for osmotic dehydration. Asexual eggs are very sensitive to air-drying and cryopreservation and this in contrast to dormant eggs, which are able to survive cryopreservation (i.e. temperatures of -196°C) after air-drying for 24h (Putman et al., unpubl. data (see Box III Introduction). The drought and freeze resistance of dormant eggs is partly due to the presence of natural cryoprotectants, including glycerol and trehalose (Pauwels et al., 2007, Putman et al., subm. (Chapter 2)). As these compatible solutes tend to be less toxic then other cryoprotectants (Fuller, 2004) we will use those for the development of an appropriate cryopreservation protocol for the asexual eggs. Additionally, we will add either ethylene glycol or methanol to the vitrification solution as they are both widely used cryoprotectants in vitrification protocols (Ali and Shelton, 1993; Elsen et al., 2007; Mullen et al., 2008; Mullen and Fahy, 2011; 2012).

2 Material and methods

2.1 Egg collection

The asexual eggs of *Daphnia magna* used in all experiments were derived from a laboratory culture of a clone hatched form the sediment egg bank of 'Langerode vijver', a pond in Neerijse, Belgium (0°49'42.32"N, 4°38'21.49"O). Animals were cultured in 0.5 L jars, with a density of 20 individuals per liter, in dechlorinated tap water (aerated for 24h prior to use) at 20°C and a photoperiod of 16h light and 8h dark (further referred to as standard conditions). Jars were cleaned and water was renewed every two days and all animals were fed daily with 150.000 cells ml^{-1} of *Scenedesumus obliquus*.

To collect asexual eggs females bearing their third clutch were dissected and eggs were gently blown out of the brood pouch with water. All eggs used were in the first stage of development according to Kast-Hutcheson et al. (2001), characterized by minimal cell differentiation. Before the start of the experiments eggs were visually checked for possible damage resulting from previous handlings.

2.2 Glycerol loading

2.2.1 Toxicity of glycerol

In order to determine the toxicity of glycerol to asexual eggs of *D. magna*, eggs were exposed to different concentrations (0% (control), 10%, 20% and 30%) for different periods of time (15 min, 30 min and 60 min) in a full factorial design. Eggs from different clutches were randomly distributed among the treatments and per treatment 50 eggs were tested.

Eggs in 50 µl of dechlorinated tap water were transferred with a glass pipet into 450 µl of the glycerol solution. After completion of the exposure time, eggs were rinsed twice in dechlorinated tap water and transferred to fresh dechlorinated tap water and placed under standard conditions to allow hatching. Hatching was checked daily for 10 days.

Results were analyzed with a generalized linear mixed model (GLMM) with binomial error distribution and a logit-link function using the package lme4 of the software R (version 3.1.2). To check for differences between the treatments and the control conditions, Dunnett post-hoc tests were performed following a simplified general linear model on the same data.

2.2.2 Measurements of changes of egg volume due to glycerol exposure

For every treatment of the previous experiment with a survival rate above 50% the volume changes caused by the exposure to glycerol were checked. Per treatment 50 eggs were measured before and after exposure to the glycerol treatment. Pictures of the eggs were taken with an Olympus CKX41 inverted microscope equipped with a digital camera (Olympus colour view III) and measurements were performed on these pictures using the imaging software Cell^P (Olympus soft imaging solutions).

The obtained results proved to be not normally distributed so the effect of the glycerol treatment was analyzed with a Kruskal Wallis test using the statistical software R (version 3.1.2).

2.2.3 Glycerol content of eggs

Additionally to the volume measurements also the concentration of glycerol inside the eggs was measured of the treatments of the first experiment with an average survival above 50%. For this again 50 eggs per treatment were used. They were isolated, exposed to the different glycerol treatments, rinsed twice in dechlorinated tap water and transferred to an Eppendorf tube with a minimal amount of water. To stop metabolism and possible breakdown of glycerol, samples were flash frozen in liquid nitrogen and stored at -80°C prior to analysis.

Eggs were homogenized in 25 µl PBS-buffer (0.15 M NaCl, 10 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.4) and ultrasonified for 3 min. Next, samples were centrifuged for 15 min at 13000 rpm and 10 µl of the supernatant was transferred to a new Eppendorf tube and diluted 40 times. Of this solution 2.4 µl was transferred to a 384-well plate. Then, 38.4 µl Free Glycerol Reagent (FGR; Sima Aldrich) was added and the plate was incubated for 10 min at 37°C. The initial absorbance, corresponding to the concentration of free glycerol, was measured at 540 nm with a spectrophotometer (Infinit M200, Tecan, Suisse). Subsequently, 9.6 µl Triglyceride Reagent (TgR; Sigma Aldrich) was added, plate was incubated again for 10 min at 37°C and final absorbance was measured at 540nm quantifying the amount of triglycerides. TgR is a lipase catalyzing the hydrolysis of triglycerides into glycerol and fatty acids. The difference between the initial (free glycerol) and the final absorbance (triglycerides) corresponds to the amount of glycerol that was bound. To convert the absorbance's to concentrations a calibration curve (0, 0.005, 0.01, 0.025, 0.05 and 0.1 µg glycerol ml^{-1}) ran along with the samples.

The effect of the different treatments on the amount of glycerol able to penetrate in the cell was tested performing a one-way analysis of variance (ANOVA), followed by a Tukey HSD post-hoc test to check for difference among the treatments with the packages car and multcomp of statistical software R (version 3.1.2).

2.3 Osmotic dehydration

2.3.1 Vitrifying capacity of different solutions

The behavior of several mixtures of cryoprotectants when plunged into liquid nitrogen was tested. This to determine a vitrification solution that vitrifies and does not show cold crystallization with lowest possible total solute concentration. For this, mixtures of two compositions varying in the concentrations of the individual cryoprotectants were tested:

Mixture 1:

- Glycerol: 10 - 15 - 20 %
- Ethylene glycol (EG): 15 – 20 – 25 – 30 – 35 – 40 %
- Trehalose: 0.1 – 0.25 – 0.5 M

Mixture 2:

- Glycerol: 10 - 15 - 20 %
- Methanol: 15 – 20 – 25 – 30 – 35 – 40 %
- Trehalose: 0.1 – 0.25 – 0.5 M

Of each solution 2 ml was pipetted into a cryotube, which was submerged in liquid nitrogen for 30 sec and thawed in a water bath of 35 - 40°C. Both during cooling and warming samples were visually

inspected for crystallization, the formation of ice crystals changes the color of the solution from transparent to snow white.

2.3.2 Toxicity test of vitrifying solutions

The toxicity of the three solutions of both mixtures with the lowest total concentrations that were still vitrifying and did not cold crystallize was tested. The sensitivity of the eggs towards the vitrification solutions was tested twice, first on eggs that did not receive any previous treatment and then on eggs that were preloaded with glycerol. For every treatment 50 eggs were exposed to the solutions, randomized over the different clutches.

Eggs were exposed to the different solutions for 5, 10, 20 or 30 min and rinsed twice after exposure. Two control conditions were added, eggs which did not undergo any treatment and eggs that were only loaded with glycerol. Exposure to the vitrification solutions was done at 0°C because at this temperature glycerol and ethylene glycol are considered not able to penetrate the cell membrane. Exposed eggs were placed in a controlled environment and hatching was monitored for 10 days.

Processing of the results was done with a GLMM with binomial error distribution and a logit-link function using the package lme4 of the software R (version 3.1.2). Datasets for the two mixtures were analyzed separately.

2.3.3 Measurement of the egg volume during osmotic dehydration

To quantify the osmotic dehydration of the eggs as a consequence of exposure to the vitrification solutions with or without previous glycerol loading the volume reduction of the eggs was determined. This was done for the treatments of the previous experiment with the highest survival (five for mixture 1 and four for mixture 2). The procedure and statistical analysis was the same as in section 2.2.2. Datasets for the two mixtures were analyzed separately.

2.4 Droplet vitrification

We used the technique of droplet vitrification, meaning that the eggs were placed in a small amount (10µl) of the vitrification solution on an aluminum strip and directly plunged in liquid nitrogen. The selected vitrification solutions resulting in the highest survival and degree of dehydration in combination with the best glycerol pretreatment were used as preparative steps for cryopreservation. In all previous experiments eggs were treated individually, but in the freezing experiments eggs were pooled per ten (ten eggs represent one replicate). In total three freezing experiments were performed (Figure 1). In the first experiment, a vitrification solution of mixture 1 (VS3 containing glycerol, EG and trehalose) was used, while in experiment 2 and 3 a vitrification solution of mixture 2 (VS 4 containing glycerol, methanol and trehalose) was used. The eggs of experiment 1 and 2 were not

manipulated before the start of the vitrification protocol. Eggs used in experiment 3 were produced under different conditions and their trehalose and polyunsaturated fatty acid (PUFA) content was increased through a manipulation of the allocation strategy of *Daphnia*. Previous experiments showed that when females were fed algae with a higher content of C20 polyunsaturated fatty acids that they then allocate more of these polyunsaturated fatty acids to their eggs (Putman et al., 2015 (Chapter 1)) and that trehalose content of the eggs tended to increase with supplementation of the maternal food with trehalose containing liposomes (Putman et al., subm. (Chapter 2)). In all experiments we used 50 eggs for the control treatments (no freezing; * in figure 1) and 500 eggs for the droplet vitrification.

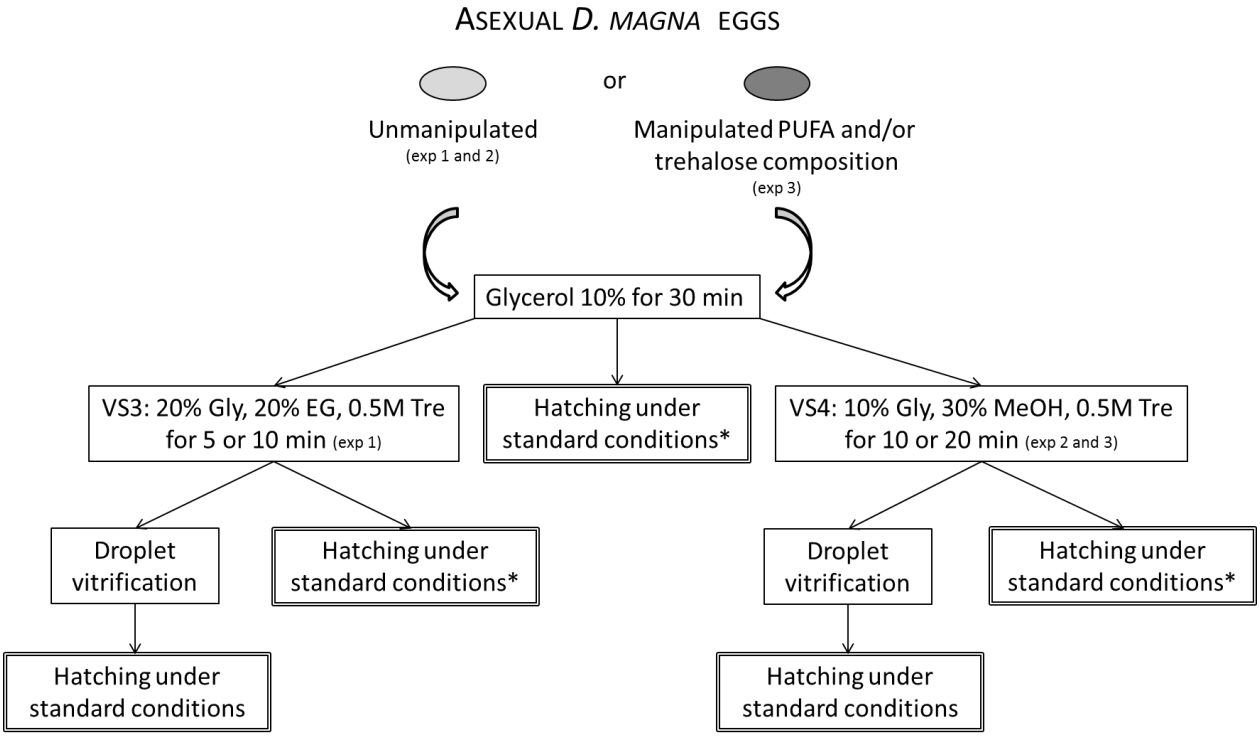

Figure 1: Overview of all steps used for cryopreservation of asexual *D. magna* eggs. Control treatments are indicated with an *.

3 Results

3.1 Glycerol loading

3.1.1 Toxicity of glycerol

In order to reduce mortality caused by the toxic effects of cryoprotectants different glycerol concentration and exposure times were tested. Both the concentration of the glycerol solution and the exposure time had a significant effect on the survival of the asexual eggs (Table 1), the higher the glycerol concentrations and the longer the exposure time the lower the survival rate (Figure 2). Additionally, also the clutch did have a significant effect on the survival of the eggs ($p = 2.025\ E^{-05}$).

The Dunnett post-hoc test revealed that survival of eggs exposed to 10% glycerol for 15 min did not significantly differed from survival in the control conditions (Figure 2, p = 0.3215) and exposure to 20% glycerol for 15 min was also marginal non-significant (p = 0.0565).

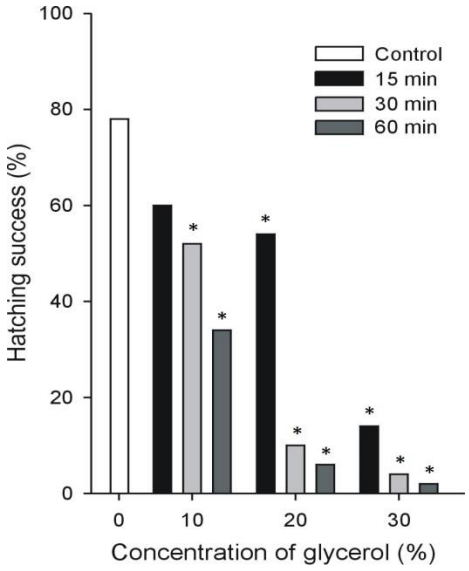

Figure 2: Effects of glycerol exposure on the percentage of hatching of asexual *D. magna* eggs. Eggs were exposed to glycerol solutions with different concentrations (0-10-20-30%) for different time periods (0-15-20-30 min). * indicates significant difference from the control conditions ($p < 0.05$, general linear model followed by Dunnett post-hoc test)

Table 1: Results of GLMM on the effect of glycerol exposure on the survival of asexual *D. magna* eggs. Number of hatchlings was the dependent variable and concentration of glycerol and exposure time were the independent variables, clutch number was added to the analysis as a random block factor.

Variables	Chi²	Df	P value
Concentration	37.4735	2	**7.29 E-09**
Exposure time	52.4108	2	**4.16 E-12**
Concentration:time	9.2008	4	0.05627

3.1.2 Measurements of changes of egg volume due to glycerol exposure

Treatments of the previous experiment with a survival rate above 50% were: exposure to 10% glycerol for 15 min and 30 min and exposure to 20% glycerol for 15 min. For these treatments volume changes during the exposure were checked. The average volume reduction was 19.24% and there was no significant difference between the treatments (Kruskal Wallis, p = 0.2002).

3.1.3 Glycerol content of eggs

For the same treatments as mentioned above (10% glycerol for 15 min and 30 min and 20% glycerol for 15 min) the amount of glycerol in the cells was measured. The amount of glycerol is significantly higher in eggs that were loaded with glycerol ($p = 0.0097$) and although not significant there is a trend of increasing intracellular glycerol with increasing exposure time and concentration of the glycerol solution (Figure 3).

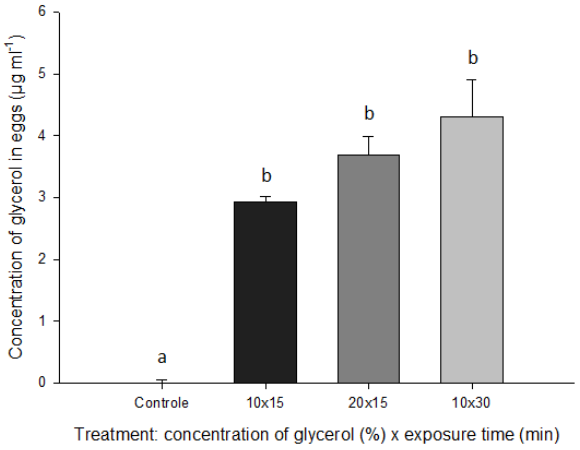

Figure 3: Concentration of glycerol penetrating in the asexual *D. magna* eggs during exposure to glycerol solution of varying concentrations (0-10-20%) for different exposure time (0-15-30 min). Data are means of three replicates; error bars indicate one standard error. Distinct letters indicate significant differences among the treatments ($p < 0.05$, ANOVA followed by Tukey HSD post-hoc test)

The percentage of glycerol was highest in asexual eggs exposed to a 10% glycerol solution for 30 min and 52% of the eggs exposed to this treatment are able to survive, so we will use this treatment as the first step of the egg pretreatment in the vitrification protocol.

3.2 Osmotic dehydration

3.2.1 Vitrifying capacity of different solutions

For each of the two mixtures three solutions were selected, varying in the composition of cryoprotectants (VS1-6, Figure 4).

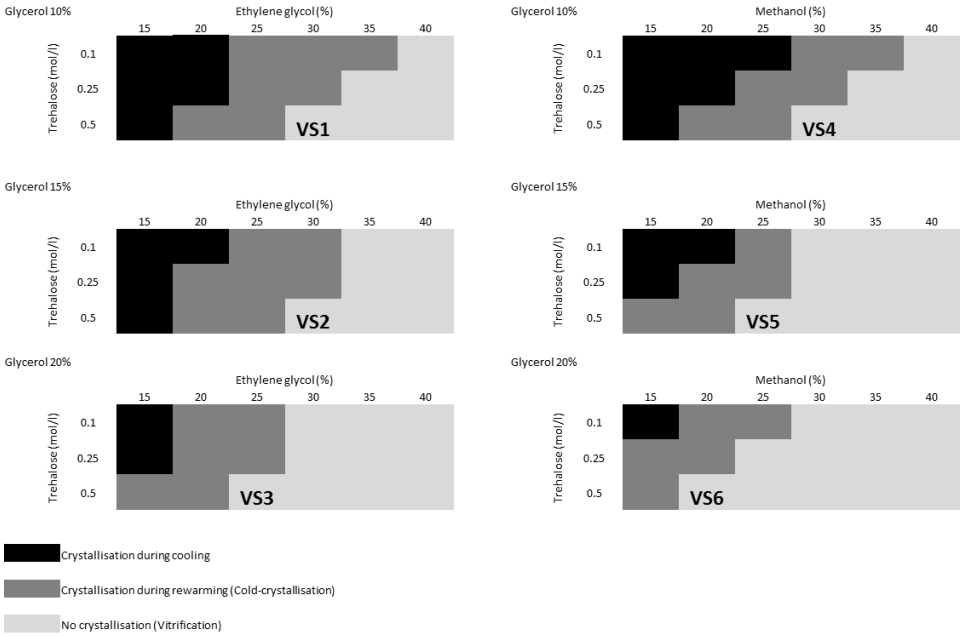

Figure 4: Vitrification capacities of mixture of cryoprotectants tested by submerging in liquid nitrogen (-196°C). Three categories were distinguished: mixture that crystallized during cooling (black boxes), mixtures that crystallized during warming (dark gray boxes) and mixtures that did not crystallize (light gray boxes).

Mixtures containing glycerol, EG and trehalose started to vitrify from 45% of solutes (glycerol and EG), while mixture consisting of glycerol, methanol and trehalose already vitrified from a solute concentration of 40% (glycerol and methanol).

3.2.2 Toxicity test of vitrifying solutions

In this experiment we tested the toxicity of the selected vitrification solutions to asexual eggs. Control conditions show the same survival rates as in previous experiments, i.e. about 80% for non-treated eggs and around 60% for eggs only loaded with glycerol (Figure 4). Results for the two mixtures were analyzed separately.

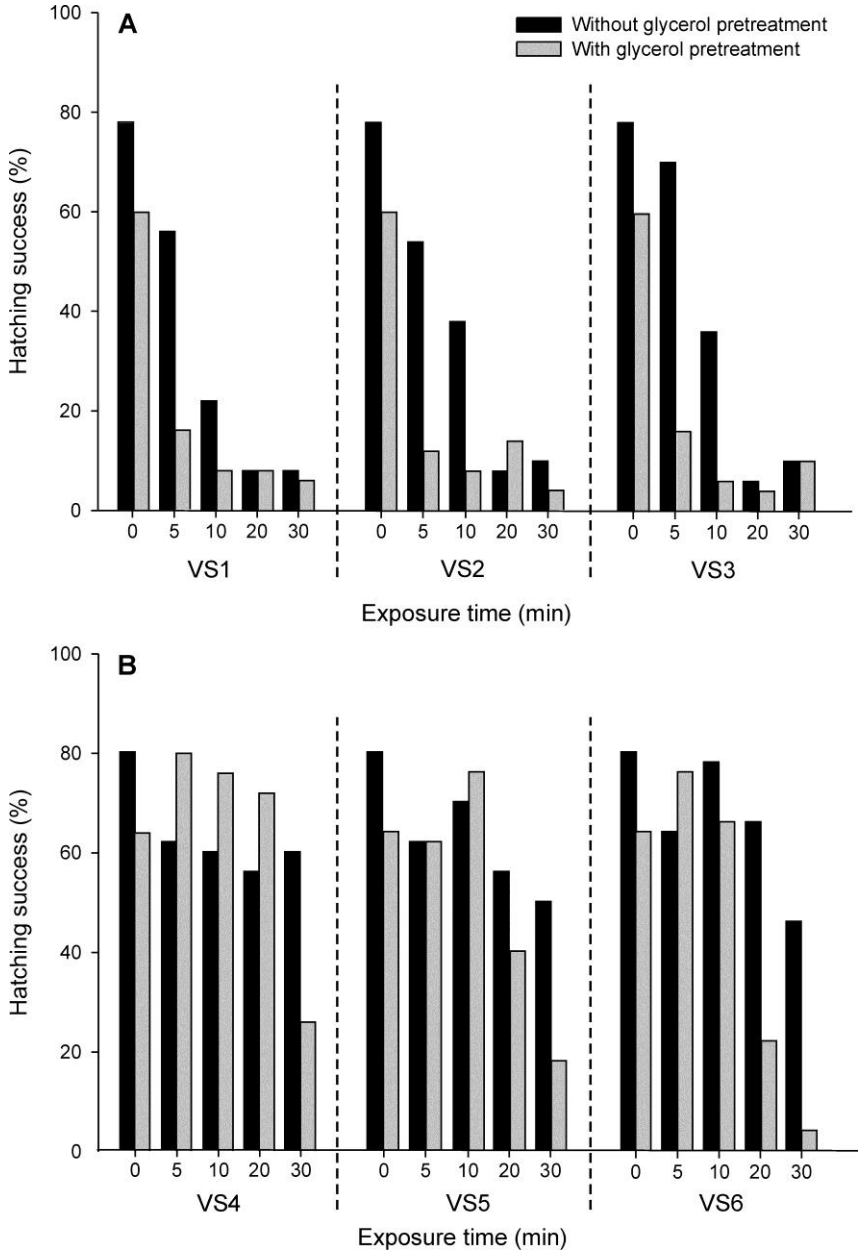

Figure 5: Effects of exposure to vitrification solutions on the percentage of hatching of asexual *D. magna* eggs. Eggs were exposed to vitrification solutions containing glycerol, ethylene glycol and trehalose (A) or vitrification solutions containing glycerol, methanol and trehalose (B) for different time periods (0-15-20-30 min).

Toxicity of vitrification solution 1-3 did not significantly differ from each other, but survival steeply decreased with exposure time (Table 2, Figure 5A). Surprisingly mortality was even higher if the vitrification treatment was combined with the glycerol pretreatment, while we assumed it would protect from VS toxicity (Table 2, Figure 5A).

In contrast, combination of the glycerol pretreatment and exposure to VS4-6 often led to higher survival then when eggs were only exposed to the vitrification solutions and the effect of exposure time differed for the distinct vitrification solutions (Table 2, Figure 5B).

Table 2: Results of GLMM on the effect of exposure to vitrification solutions on the survival of asexual *D. magna* eggs. Number of hatchlings was the dependent variable and composition of vitrification solution, exposure time and whether or not the eggs underwent a glycerol pretreatment step were the independent variables, clutch number was added to the analysis as a random block factor. Vitrification solutions of mixture 1 contained varying concentrations of glycerol, ethylene glycol and trehalose and vitrification solutions of mixture 2 contained glycerol, methanol and trehalose.

	Mixture 1				*Mixture 2*		
Variables	Chi²	Df	P value	Variables	Chi²	Df	P value
Vitrification solution	2.0298	2	0.3624	Vitrification solution	2.9503	2	0.22875
Exposure time	62.3706	3	**1.83 E-13**	Exposure time	85.4068	3	**<2.2 E-16**
Glycerol pretreatment	42.4317	1	**7.32 E-11**	Glycerol pretreatment	6.5562	1	**0.01045**
Vitr sol:time	6.2729	6	0.3933	Vitr sol:time	14.3202	6	**0.02626**
Vitr sol:Gly pretreat	1.6717	2	0.4335	Vitr sol:Gly pretreat	20.1849	2	**4.14 E-05**
Time:Gy pretreat	35.6892	3	8.71 E-08	Time:Gy pretreat	55.2277	3	**6.14 E-12**
Vitr sol:Gly:time	3.6685	6	0.7214	Vitr sol:Gly:time	9.0793	6	0.16917

3.2.3 Measurement of egg volume during osmotic dehydration

Subsequently, we also studied the volume change during the exposure to vitrification solutions of the treatments with the highest survival rates (Figure 5). Treatments selected for mixture 1 are exposure to VS1 for 5 min (56% survival), exposure to VS2 for 5 (54% survival) and 10 min (38% survival) and exposure to VS3 for 5 (70% survival) and 10 min (36% survival). Treatments selected for mixture 2 are exposure to VS4 for 10 (60% survival) and 20 min (56% survival), exposure to VS5 for 10 min (70% survival) and exposure to VS6 for 10 min (66% survival).

Results for the two mixtures were again analyzed separately. Survival did not significantly differ between the vitrification solutions of the same mixtures (Kruskal Wallis, $p_{mixture1} = 0.1218$, $p_{mixture2} = 0.1108$). However, applying the glycerol pretreatment did have a significant influence on the egg volume (Kruskal Wallis, $p_{mixture1\ and\ 2} < 0.0001$). Exposure to VS1-3 results in a volume reduction of the asexual eggs with about 20%, and in combination with glycerol loading lead to shrinkage of even 35% (Figure 6A). For VS4-6 the volume changes are less pronounced with 7% and 10%, for respectively exposure to the vitrification solutions without and with glycerol pretreatment (Figure 6B).

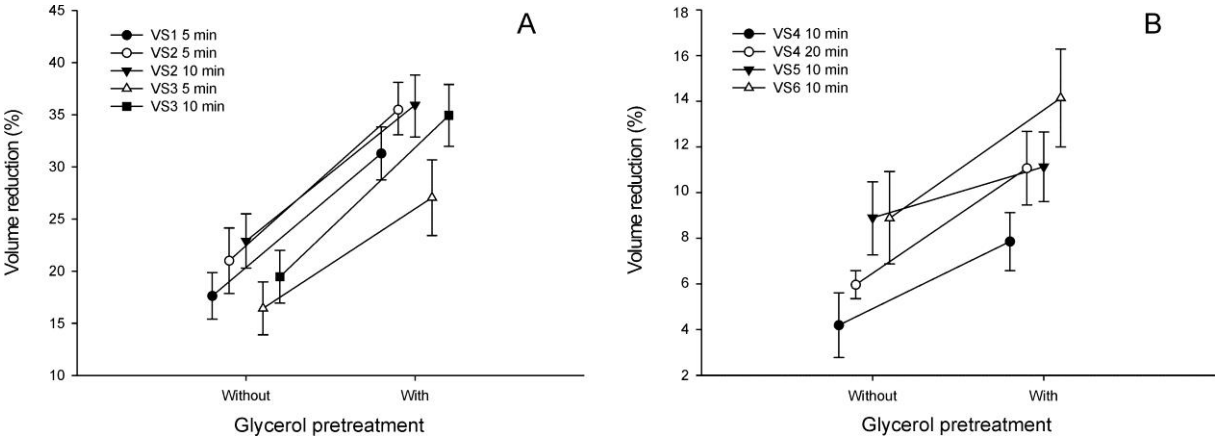

Figure 6: Effect of exposure to vitrification solution and/or glycerol on the volume of asexual eggs of *D. magna*. Data are means of fifty replicate eggs; error bars indicate one standard error.

3.3 Droplet vitrification

From previous experiments treatments causing lowest mortality were selected to use as pretreatments in the vitrification protocol and followed by the droplet vitrification (Figure 1).

Survival off all control conditions without freezing (* in figure 1) was similar to survival in the previous experiments (Figure 7). There were only survivors after freezing if eggs were pretreated in two steps, i.e. with both the glycerol loading and the osmotic dehydration in a vitrification solution. After osmotic dehydration with VS3 three of thousand eggs survived droplet vitrification, one after 5 min of exposure and two after 10 min of exposure (Figure 7). After osmotic dehydration with VS4 seven of thousand eggs survived droplet vitrification but only if there fatty acid and trehalose content was manipulated, six after 10 min of exposure and one after 20 min of exposure (Figure 7).

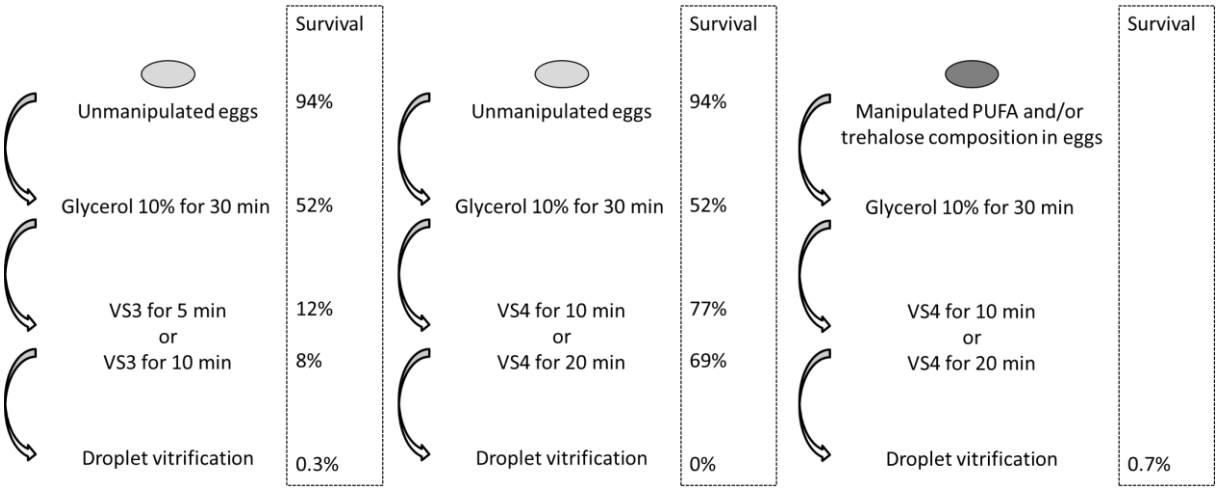

Figure 7: Overview of the survival rate (%) of a sexual *D. magna* eggs exposed to the multiple steps of the cryopreservation protocol.

4 Discussion

This is the first report on the survival of parthenogenetic eggs of *Daphnia* after exposure to liquid nitrogen. We show that asexual eggs of *Daphnia magna* are capable of surviving cryopreservation provided they are sufficiently dehydrated. The most successful protocol was the one in which eggs were dehydrated in two steps, through glycerol loading followed by osmotic dehydration in a vitrification solution. The safe preservation of clonal lines would give researchers the opportunity to work with genetically identical individuals in studies performed at different times and places, increasing comparability, reliability and repeatability.

The cryoprotectant glycerol is due to its hydroxyl groups able to stabilize membranes by the formation of hydrogen bonds with the polar phospholipid heads (Bryant et al., 2001). It is a small sugar alcohol able to penetrate the cell membrane at a slow rate at room temperature, thereby increasing the intracellular viscosity and kinetically inhibiting ice formation (Polge, 1949; Fuller, 2004; Muldrew et al., 2004). This has been confirmed by our results for the asexual *D. magna* eggs. The concentration of glycerol only increased to about 0.033% per egg during exposure to glycerol solutions of 10-20%, while at the same time the volume decreased with about 20% as a consequence of water loss. Glycerol is a compatible solute and thus naturally produced by many organisms to withstand drought and cold temperatures, including *Daphnia* concentrations (Eroglu, 2003; Fuller, 2004; Pauwels et al., 2007). The consequence is also that it is less toxic than other cryoprotectants at high concentration. Nevertheless, we still found an increasing mortality with increasing glycerol concentration and exposure time mainly due to osmotic toxicity. Concentrations higher than 20% glycerol are not tolerated well by the asexual eggs of *D. magna*.

To reduce the toxic effects of individual cryoprotectants we used a mixture of cryoprotectants, as we needed high concentrations to achieve a vitrified state of the biological tissue (or the formation of a glass) (Fahy et al., 1984; 2004; Muldrew at al., 2004). We have applied glycerol, ethylene glycol and trehalose in one mixture and in the other mixture ethylene glycol was substituted by methanol. Trehalose was added to both vitrification solutions because it is known to have beneficial effects on the vitrification capacity of a solution by lowering its vitrification temperature (Kuleshova et al., 1999). The disaccharide trehalose is a non-penetrating cryoprotectant and like glycerol it is a compatible solute also used by many invertebrates, including *Daphnia,* as protective molecule against stressful conditions encountered by their dormant eggs (Clegg, 2001; Hengherr et al., 2011; Putman et al., subm. (Chapter 2)). Ethylene glycol and methanol are commonly used cryoprotectants because of their low toxicity towards embryos at vitrifying concentrations (Ali and Shelton, 1993; Mullen et al., 2008). Ethylene glycol is a synthetic ice blocking agent able to penetrate membranes depending on the application temperature (Irdani et al., 2006). It is used in many successful cryopreservation protocols of mammalian embryos (Mullen and Fahy, 2011; 2012) and in the vitrification solution used to

cryopreserve embryos of the arthropod *Drosophila melanogaster* (Steponkus et al., 1990; Steponkus and Caldwell, 1993). Methanol is a fast penetrating cryoprotectant among others used for the vitrification of nematodes and fish spermatozoa (Galway and Curran, 1995; Zhang, 2004; Elsen et al., 2007). Osmotic dehydration was applied at 0°C, consequently glycerol and ethylene glycerol are no longer able to penetrate through the cell membrane. Consequently, mixture 1 only contains non-penetrating cryoprotectants and can only extract water from the eggs, while mixture 2 contains a combination of penetrating and non-penetrating cryoprotectants. This difference resulted in a clearly different volume reduction. The shrinkage of eggs exposed to mixture 1 was about 10% higher than when eggs were exposed to mixture 2, resulting in higher mortality upon exposure to mixture 1 due to osmotic toxicity.

Apart from the ability to lower the vitrification temperature of a solution, trehalose has many more functions, such as stabilizing proteins, phospholipids and other macromolecules (Crowe et al., 2001; Crowe 2007), but therefore trehalose has to be present inside the cell. As trehalose is not able to penetrate through a cell membrane this could be achieved using techniques like microinjection or electroporation (Eroglu et al., 2002; 2003; Shirakashi, 2002) or by inducing adaptive metabolism (Putman et al., subm. (Chapter 2)). The little increase from 0.006% to 0.011% trehalose per egg caused by trehalose supplementation of the maternal food led to some promising results.

We prove that cryopreservation of the asexual eggs of *Daphnia magna* is possible although there is still some optimization needed to increase the survival rate. Cryopreservation protocols of aquatic embryos are scarce and we believe that the techniques and approaches used in this study can contribute to the development of cryopreservation protocols for embryos of other species. We believe that a successful cryopreservation can be developed for most organisms provided a logic approach is followed. Starting with a study of the biochemical composition of the tissue to be preserved, as this can provide valuable information on which cryoprotectants to use, and then step by step optimizing all steps of the protocol.

Acknowledgements

AP enjoyed a PhD fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) and the work was financially supported by KU Leuven Research Fund project PF/2010/007. We thank S. Navis and M. Jansen for help during the experiments.

5 References

Ali, J. and Shelton, J. N. (1993) Design of vitrification solutions for the cryopreservation of embryos. *Journal of Reproduction and Fertility,* **99,** 471-477.

Bellas, J. and Paredes, E. (2011) Advances in the cryopreservation of sea-urchin embryos: Potential application in marine water quality assessment. *Cryobiology,* **62,** 174-180.

Benzie, J. A. (2005) The genus Daphnia (including Daphniopsis)(Anomopoda: Daphniidae). In: H. J. F. Dumont (ed) *Guide to the identification of the microinvertebrates of the continental waters of the world.* Ghent: Kenobi productions and Leiden: Backhuys, pp 376

Brendonck, L. and De Meester, L. (2003) Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia,* **491,** 65-84.

Bryant, G., Koster, K. L. and Wolfe, J. (2001) Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research,* **11,** 17-25.

Carpenter, S. R., Kitchell, J. F., Hodgson , J. R., Cochran, P. A., Elser, J. J., Elser, M. M., Lodge, D. M., Kretchmer, D. and He, X. (1987) Regulation of lake primary production by food web structure. *Ecology,* **68,** 1863-1876.

Clegg, J. S. (2001) Cryptobiosis — a peculiar state of biological organization. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **128,** 613-624.

Colbourne, J., Pfender, M., Gilbert, D., Thomas, W., Tucker, A., Oakley, T., Tokishita, S., Aerts, A., Arnold, G., Basu, M., Bauer, D., Caceres, C., Carmel, L., Casola, C., Choi, J., Detter, J., Dong, Q., Dusheyko, S., Eads, B., Frohlich, T., Geiler-Samerotte, K., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E., Kultz, D., Laforsch, C., Lindquist, E. and Lopez, J. (2011) The ecoresponsive genome of Daphnia pulex. *Science,* **331,** 555 - 561.

Crowe, J. (2007) Trehalose As a "Chemical Chaperone". In: P. Csermely and L. Vígh (eds) *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks.* Springer New York, pp. 143-158.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Tsvetkova, N., Wolkers, W. and Tablin, F. (2001) The Trehalose Myth Revisited: Introduction to a Symposium on Stabilization of Cells in the Dry State. *Cryobiology,* **43,** 89-105.

De Meester, L., Gomez, A. and Simon, J. (2004) Evolutionary and ecological genetics of cyclical parthenogens. In: A. Moya and E. Font (eds) *Evolution from molecules to ecosystems.* Oxford university press, pp. 122-134.

Elsen, A., Vallterra, S. F., Van Wauwe, T., Thuy, T. T. T., Swennen, R., De Waele, D. and Panis, B. (2007) Cryopreservation of Radopholus similis, a tropical plant-parasitic nematode. *Cryobiology,* **55,** 148-157.

Eroglu, A., Lawitts, J. A., Toner, M. and Toth, T. L. (2003) Quantitative microinjection of trehalose into mouse oocytes and zygotes, and its effect on development. *Cryobiology,* **46,** 121-134.

Eroglu, A., Toner, M. and Toth, T. L. (2002) Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. *Fertility and sterility,* **77,** 152-158.

Fahy, G. and Wowk, B. (2015) Principles of Cryopreservation by Vitrification. In: W. F. Wolkers and H. Oldenhof (eds) *Cryopreservation and Freeze-Drying Protocols.* Springer New York, pp. 21-82.

Fahy, G. M., Macfarlane, D. R., Angeli, C. A. and Meryman, H. T. (1984) Vitrification as an Approach to Cryopreservation'. *Cryobiology,* **21,** 407-426.

Fahy, G. M., Wowk, B., Wu, J. and Paynter, S. (2004) Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology,* **48,** 22-35.

Fuller, B. J. (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryoletters,* **25,** 375-388.

Galway, N. J. and Curran, J. (1995) Cryopreservation of Pratylenchys spp. *Journal of Nematology,* **27,** 483-485.

Gwo, J. C. (2000) Cryopreservation of aquatic invertebrate semen: a review. *Aquaculture Research,* **31,** 259-271.

Hairston, N. G., Van Brunt, R. A., Kearns, C. M. and Engstrom, D. R. (1995) Age and Survivorship of Diapausing Eggs in a Sediment Egg Bank. *Ecology,* **76,** 1706-1711.

Hebert, P. D. N. (1978) The population biology of *Daphnia* (Crustacea, Daphnidae). *Biological Reviews,* **53,** 387-426.

Hengherr, S., Heyer, A. G., Brümmer, F. and Schill, R. O. (2011) Trehalose and Vitreous States: Desiccation Tolerance of Dormant Stages of the CrustaceansTriopsandDaphnia. *Physiological and Biochemical Zoology,* **84,** 147-153.

Hodgson, J. (1994) The Culture of Cryopreservation. *Nat Biotech,* **12,** 82-82.

Irdani, T., Carletti, B., Ambrogioni, L. and Roversi, P. F. (2006) Rapid-cooling and storage of plant nematodes at −140°C. *Cryobiology,* **52,** 319-322.

Jaromir, S. and Petrusek, A. (2011) Daphnia as a model organism in limnology and aquatic biology: introductory remarks. *Journal of limnology,* **70,** 337-344.

Kast-Hutcheson, K., Rider, C. V. and Leblanc, G. A. (2001) The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environmental Toxicology and Chemistry,* **20,** 502-509.

Kuleshova, L. L., Macfarlane, D. R., Trounson, A. O. and Shaw, J. M. (1999) Sugars Exert a Major Influence on the Vitrification Properties of Ethylene Glycol-Based Solutions and Have Low Toxicity to Embryos and Oocytes. *Cryobiology,* **38,** 119-130.

Kulus, D. and Zalewska, M. (2014) Cryopreservation as a tool used in long-term storage of ornamental species – A review. *Scientia Horticulturae,* **168,** 88-107.

Lampert, W. (2006) Daphnia: Model herbivore, predator and prey. *Polish journal of ecology,* **54,** 607-620.

Lampert, W. (2011) Daphnia: development of a model organism in ecology and evolution. International Ecology Institute Oldendorf/Luhe.

Lin, T. T. and Chao, N. H. (2000) Cryopreservation of eggs and embryos of shellfish. In: T. R. Tiersch and P. Mazik (eds) *Cryopreservation in Aquatic Species.* World Aquaculture Society Baton Rouge, Louisiana, USA, pp. 240-250.

Mauger, P. E., Le Bail, P. Y. and Labbé, C. (2006) Cryobanking of fish somatic cells: Optimizations of fin explant culture and fin cell cryopreservation. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **144,** 29-37.

Mazur, P. (2004) Principles of Cryobiology. In: B. J. Fuller, N. Lane and E. E. Benson (eds) *Life in the frozen state.* CRC Press LLC, pp. 3-65.

Mazur, P., Leibo, S. P. and Seidel, G. E. (2008) Cryopreservation of the Germplasm of Animals Used in Biological and Medical Research: Importance, Impact, Status, and Future Directions. *Biology of Reproduction,* **78,** 2-12.

Miner, B. E., De Meester, L., Pfrender, M. E., Lampert, W. and Hairston, N. G. (2012) Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society of London Biological sciences,* **279,** 1873-1882.

Muldrew, K., Acker, J. P., Elliott, J. and Mcgann, L. E. (2004) The water to ice transition: implications for living cells. In: B. J. Fuller, N. Lane and E. E. Benson (eds) *Life in the frozen state.* CRC Press LLC, pp. 67-108.

Mullen, S. and Fahy, G. (2011) Fundamental aspects of vitrification as a method of reproductive cell, tissue, and organ cryopreservation. In: Donnez J. and Kim S. (eds) *Principles & practice of fertility preservatio.* Cambridge University Press, Cambridge, pp.145-163.

Mullen, S. F. and Fahy, G. M. (2012) A chronologic review of mature oocyte vitrification research in cattle, pigs, and sheep. *Theriogenology,* **78,** 1709-1719.

Mullen, S. F., Li, M., Li, Y., Chen, Z.-J. and Critser, J. K. (2008) Human oocyte vitrification: the permeability of metaphase II oocytes to water and ethylene glycol and the appliance toward vitrification. *Fertility and sterility,* **89,** 1812-1825.

Paredes, E., Adams, S. L., Tervit, H. R., Smith, J. F., Mcgowan, L. T., Gale, S. L., Morrish, J. R. and Watts, E. (2012) Cryopreservation of Greenshell™ mussel (Perna canaliculus) trochophore larvae. *Cryobiology,* **65,** 256-262.

Paredes, E., Bellas, J. and Adams, S. L. (2013) Comparative cryopreservation study of trochophore larvae from two species of bivalves: Pacific oyster (Crassostrea gigas) and Blue mussel (Mytilus galloprovincialis). *Cryobiology,* **67,** 274-279.

Pauwels, K., Stoks, R., Verbiest, A. and De Meester, L. (2007) Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*. *Hydrobiologia,* **594,** 91-96.

Pegg, D. E. (1987) Ice Crystals in Tissues and Organs. In: D. E. Pegg and A. M. Karow, Jr. (eds) *The Biophysics of Organ Cryopreservation.* Vol. 147. Springer US, pp. 117-140.

Polge, C., Smith, A. and Parkes, A. (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature,* **164,** 666.

Putman, A., Martin-Creuzburg, D., De Meester, L. and Panis, B. (2015) A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality. *Journal of Plankton Research,* **DOI:10.1093/plankt/fbv043**.

Shirakashi, R., Köstner, C. M., Müller, K. J., Kürschner, M., Zimmermann, U. and Sukhorukov, V. L. (2002) Intracellular delivery of trehalose into mammalian cells by elektropermeabilization. *The journal of membrane biology,* **189,** 45-54.

Simon, J.-C., Pfrender, M. E., Tollrian, R., Tagu, D. and Colbourne, J. K. (2011) Genomics of Environmentally Induced Phenotypes in 2 Extremely Plastic Arthropods. *Journal of Heredity,* **102,** 512-525.

Steponkus, P. and Caldwell, S. (1993) Cryopreservation of Drosophila melanogaster embryos. *Cryo Letters,* **14,** 375-375.

Steponkus, P. L., Myers, S. P., Lynch, D. V., Gardner, L., Bronshteyn, V., Leibo, S. P., Rall, W. F., Pitt, R. E., Lin, T. T. and Maclntyre, R. J. (1990) Cryopreservation of Drosophila melanogaster embryos. *Nature,* **345,** 170-172.

Tautz, D. (2011) Not just another genome. *BMC Biology,* **9,** 8.

Toledo, J. D. and Kurokura, H. (1990) Cryopreservation of the euryhaline rotifer Brachionus plicatilis embryos. *Aquaculture,* **91,** 385-394.

Tollrian, R. and Leese, F. (2010) Ecological genomics: steps towards unraveling the genetic basis of inducible defenses in Daphnia. *BMC Biol,* **8,** 51.

Tsai, S., Rawson, D. M. and Zhang, T. (2009) Development of cryopreservation protocols for early stage zebrafish (Danio rerio) ovarian follicles using controlled slow cooling. *Theriogenology,* **71,** 1226-1233.

Walker, C. (2014) *Ecotoxicology: Effects of Pollutants on the Natural Environment.* CRC Press.

Zhang, T. (2004) Cryopreservation of gametes and embryos of aquatic species. In: B. J. Fuller, N. Lane and E. E. Benson (eds) *Life in the frozen state.* CRC Press LLC, pp. 415-435.

General discussion

The water flea *Daphnia magna* is an important model organism in many research areas, such as ecology, ecotoxicology, evolutionary biology and eco-genomics (Lampert, 2006; 2011; Altshuler et al., 2011; Seda and Petrusek, 2011; Tautz, 2011). Many of these applications use clonal lines produced via asexual reproduction. This gives researchers the opportunity to work with large numbers of genetically identical individuals (De Meester et al., 2004). For ecotoxicology, use of the same clonal lineages worldwide and for different tests over time increases comparability and reliability (Walker, 2014). Working with clonal lines for ecological and evolutionary biological research increases repeatability. Additionally, it allows researchers to distinguish between genetic responses and phenotypic plasticity, study genotype-environment interactions and genotype-genotype interactions in a straightforward way, and to perform their experiments with lines that have evolved special characteristics (Van Doorslaer, 2009; 2010; Tollrian and Leese, 2010; Simon, 2011; Jansen et al., 2015). Due to the publication of the *Daphnia* genome, clonal lines obtained from experiments can now also be characterized for their genome (http://daphnia.cgb.indiana.edu/; Colbourne et al., 2011). Yet, there is so far still one major hurdle: the clonal lines have to be kept continuously in culture, because there is no way to store them. While *Daphnia* produce dormant eggs that can be kept for long periods in a low-active state, these eggs are sexual so in effect, they destroy the original genotype. Apart from the maintenance costs, this is also very risky. Clones can be lost accidently or can be contaminated. In this PhD research, we tried to solve this problem by developing a cryopreservation protocol for the asexual eggs of *Daphnia magna*.

The dormant eggs (See Figure 4, Introduction) of *Daphnia* are drought and freeze resistant (see Box II, Introduction). While they are not suitable for the preservation of clonal lines, they can act as an excellent reference to determine what is needed for eggs to survive freezing, and thus cryopreservation. Therefore we first needed to determine what the main biochemical differences are between sexual and asexual eggs (Chapter 1-3; Discussed in section 1). Once these differences were identified, asexual eggs could be modified to try to bridge the gap between both egg types and to increase stress resistance of asexual eggs (Chapter 1-4; Discussed in section 2). In parallel, we started with the optimization of the protocol for cryopreservation. We first tested the toxicity of several cryoprotectants. In a second step, we incorporated the procedure to alter the biochemical properties of asexual eggs developed in chapters 1 and 2 in our cryopreservation protocols, and checked survival of modified and unmodified eggs after vitrification (Chapter 5; Discussed in section 3).

1 Biochemical composition of dormant and subitaneous *D. magna* eggs

It is already well-known for a long time that dormant and subitaneous eggs differ in their morphology and capacity to cope with stress (Makrushin, 1978; Seidman and Larsen, 1979; Zaffagnini, 1987; Radzikowski, 2013) and in the life history of the animals that hatch from them (Arbaciauskas, 1998).

Next to morphology, biochemical composition of the cytoplasm and membranes are the main factors influencing stress resistance of cells and tissues. Our results expanded the knowledge about the differences in biochemical composition of dormant and subitaneous eggs of *Daphnia magna* (see General introduction Table 2). To summarize (Table 1), we found increased levels of trehalose, putrescine, spermidine and EPA and ARA (when both eggs are produced on C20 PUFA-deficient diet) in dormant compared to subitaneous eggs while diaminopropane and total fatty acid concentrations were higher in parthenogenetic eggs. And it was already know that sexual eggs contain higher amounts of glycerol and Hsp 60 (Pauwels et al., 2007).

Table 1: Comparison of biochemical composition between sexual and asexual eggs

Biochemical components	Sexual eggs	Asexual eggs
Lipids[1]		
	More PUFA	More fatty acids in phospholipids
Sugars and sugar alochols		
	More Trehalose[2]	
	More Glycerol[3]	
Proteins[3]		
	More Hsp 60	
Polyamines[4]		
	More Spermidine	More Diaminopropane
	More Putrescine	

1 Abrusan et al., 2007; Putman et al., 2015 (Chapter 1)

2 Hengherr et al., 2011; Putman et al., subm. (Chapter 2)

3 Pauwels et al., 2007

4 Putman et al., subm. (Chapter 3)

Trehalose, neutral lipids, glycerol, heat shock proteins, putrescine and spermidine all have a stress protective role in the cytoplasm of the cells. Heat shock proteins, glycerol and trehalose act as molecular chaperones preserving the native state of proteins under stress conditions (Crowe, 1987; 2007; Parsell and Lindquist, 1993). The polycations spermidine and putrescine also protect negatively charged macromolecules such as proteins, DNA and RNA (Pegg and McCann, 1982; Tabor and Tabor, 1984). The cryoprotective substances trehalose and glucose additionally inhibit the formation of intracellular ice crystals by increasing the viscosity of the cytoplasm and by physically interrupting the formation of an ice crystal (Wolfe and Bryant, 1999; Fuller, 2004).

Phospholipids, glycerol, trehalose and polyamines also protect the cell membranes against damage caused by drought or cold temperatures by maintaining membrane fluidity and preserving the integrity of the membrane structure. The length and the saturation degree of the fatty acid tail of the

phospholipids determine membrane flexibility (Valentine and Valentine, 2004; Pruitt, 1990). Trehalose and glycerol stabilize membranes during cold or drought by the formation of hydrogen bounds (Crowe et al., 1987; Storey, 1997; Bryant et al., 2001; Crowe, 2007), while the polyamines spermidine and putrescine bind to negatively charged membrane components to stabilize them (Pegg and McCann, 1982; Tabor and Tabor, 1984). Another important asset of trehalose is that it lowers the phase transition temperature of the membrane (Hontoria et al., 1998).

We now have a more thorough understanding of the biochemical differences between dormant and subitaneous eggs and how these differences contribute to the striking contrast in stress resistance between the two egg types. There are still a number of candidate protective components for which we have no information, however. Besides phospholipids, also sterols are essential components of plasma membranes and in animal's cholesterol is the dominant sterol (Goad, 1981; Crockett, 1998).

Due to its ability to interact with phospholipids and membrane proteins, cholesterol has many functions in the plasma membrane. Cholesterol affects the stabilization of membrane functions, membrane fluidity and permeability by influencing the phase transition and ordering of membranes (Crockett, 1998; Ohvo-Rekilä et al., 2002). Arthropods lack the capacity to synthesize cholesterol and therefore have to rely on their diet for this compound (Goad, 1981). As the diet of *Daphnia* consists of phytoplankton, they have to convert phytosterols into cholesterol and not all of them are equally suitable (Martin-Creuzburg and Von Elert, 2004). Expect for the diet also the temperature influences the sterol content and the play a major role in thermal adaptation (Crockett 1998). Sperfeld and Wacker (2009) reported that female *Daphnia* incorporate more cholesterol in their body tissue and eggs at higher temperatures, Having so many functions, cholesterol deficit has been shown to have a large impact on the life history of *Daphnia* (Martin-Creuzburg and Von Elert 2004; 2009; Martin-Creuzburget al., 2005) however cholesterol will probably not contribute to the cold resistance of dormant eggs.

Another important biochemical component for which we still lack information in our comparison is protein composition. Stress responses in all organisms, including *Daphnia,* involve gene up- and down-regulation, consequently affecting the protein profile (Eads et al., 2007; Colbourne et al., 2011). Schwerin et al. (2009) showed distinct protein profiles for 10°C- and 20°C-acclimated *D. pulex* animals and it has been shown that synergistic effects between trehalose and stress proteins increase tolerance towards multiple stressors (Elliott et al., 1996; Crowe et al., 2001). Except for heat shock protein 60, which has been found in higher concentrations in sexual compared to asexual eggs by Pauwels et al. (2007), little is known about the protein profile of both egg types. Although we were able to develop a protocol to analyze the protein content of sexual and asexual eggs of *Daphnia magna*, we could not perform yet the analysis due to technical problems and time restrictions. The protocol uses 2D gel electrophoresis to separate the proteins and mass spectrometry to identify them.

2 Plasticity of subitaneous eggs

2.1 Alteration of biochemical composition via food conditions

From our comparison of the biochemical composition of the two egg types we can conclude that the largest differences between sexual and asexual eggs of *D. magna* are to be found in the much higher concentration of trehalose and the different fatty acid composition of the phospholipids in the sexual compared to the asexual eggs (Chapter 1-2). The total concentration of fatty acids and especially polyunsaturated fatty acids is higher in asexual eggs compared to sexual eggs when they are produced under the same conditions. There are, however, two exceptions; eicosapentaenoic acid (EPA, C20:5 n-3) as well as arachidonic acid (ARA, C20:4 n-6) were measured in higher concentrations in sexual eggs when these were produced on *S. obliquus,* a C20-PUFA deficient diet (Abrusan et al., 2007; Putman et al., 2015 (Chapter1)). EPA and ARA play important roles in the cell metabolism as precursors for prostaglandins and other eicosanoids, which are important for reproduction, the immune system and ion transport (Stanley, 2006; Heckmann et al., 2008). These long-chain polyunsaturated fatty acids are important for the maintenance of membrane fluidity and functioning under low temperatures (Hazel and Williams, 1990; Pruitt, 1990; Martin-Creuzburg et al., 2012). Our results indicate that dormant eggs may need a certain concentration of C20-PUFA to be able to survive harsh winter conditions. It has been shown that the fatty acid composition of subitaneous eggs is very plastic and strongly reflects the composition of the maternal diet (Wacker and Martin-Creuzburg, 2007). We observed that the plasticity of asexual eggs is higher than that of sexual eggs (Putman et al., 2015 (Chapter1)). To bridge the difference between the EPA and ARA content of sexual and asexual eggs produced on *S. obliquus,* we adjusted the diet and provided an alga containing high concentrations of C20 PUFA, *N. limnetica.* This resulted in a spectacular increase in EPA content in asexual eggs (Figure 1), so this manipulation was successful.

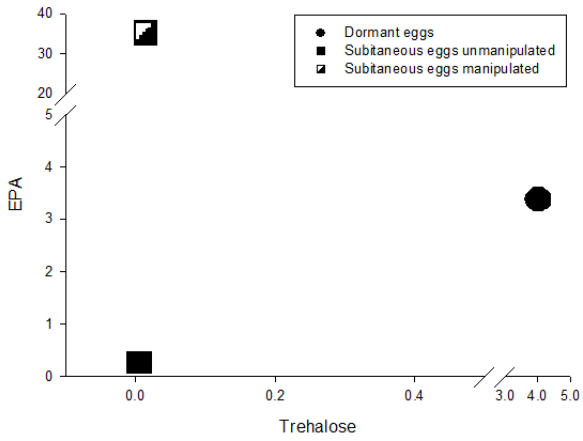

Figure 1: Comparison of the EPA and trehalose content (%) of dormant and subitaneous eggs. Dormant eggs and unmanipulated subitaneous eggs were produced under standard laboratory conditions with *Scenedesmus obliquus* as food. Manipulated subitaneous eggs were produced under the same standard conditions, but with *Nannochloropsis limnetica* and trehalose containing liposomes as food (Data from chapter 1-2).

The disaccharide trehalose has been linked to desiccation tolerance in dormant stages of many organisms, including the crustacean *Triops* (Hengherr et al., 2011), *Daphnia pulex* (Hengherr et al., 2011), *Daphnia magna* (Hengherr et al., 2011; Putman et al., subm. (Chapter 2)) and *Artemia franciscana* (Clegg, 1965). Additionally, trehalose is a crucial cryoprotectant in many cryopreservation protocols for embryos (Kuleshova et al, 1999; Eroglu et al., 2000; 2002; Lagares et al., 2009). Trehalose is a naturally occurring cryoprotectant in the eggs of *Daphnia magna*, so it likely to be less toxic than other cryoprotectants and it has many beneficial effects on the survival of cells during drought and freezing conditions. It would therefore be good if the concentrations of trehalose in the subitaneous eggs could be increased. In an attempt to do so, we raised female *Daphnia* on a diet supplemented with trehalose containing liposomes, a technique that has been applied successfully for the supplementation of *Daphnia* with fatty acids and sterols (Martin-Creuzburg et al., 2008; 2009; Martin-Creuzburg and Von Elert, 2009). Trehalose concentration in the eggs increased, but not significantly (Chapter 2) and the increase was marginal compared to the difference in trehalose concentration between subitaneous and dormant eggs (Figure 1). Increasing the liposome concentration supplemented to the food had no effect, probably because of a limitation in filtering rate of the water fleas. Raising the trehalose concentration in the liposomes even more was impossible as liposomes in this case did no longer form correctly. As trehalose could be a key component in the survival of subitaneous eggs undergoing cryopreservation, there is a need for additional mechanisms to increase trehalose levels in eggs. In mammalian cells electroporation and microinjection have been successfully used to transfer trehalose through the membranes (Eroglu et al., 2002; 2003; Shirakashi, 2002) and both techniques have already been optimized for *Daphnia* (Kato et al., 2010; 2011). It

would be thus be very interesting to test the effectiveness of these techniques for loading asexual eggs with trehalose.

2.2 Modification of biochemical composition via the manipulation of culture temperature

Temperature is an important environmental characteristic fluctuating in time and space, strongly affecting the physiology and life history of organisms, especially ectotherms as they rely on the external temperature for the regulation of their body temperature. Cell membranes are particularly sensitive to low temperatures as they tend to undergo a phase transition from liquid to gel, consequently losing flexibility, permeability and functionality (Wolfe and Bryant, 1999; 2001; Mazur, 2004). To avoid this, winter-active and cold-acclimated crustacea accumulate (long-chain) PUFA in their membranes (Farkas et al., 1984; Pruitt, 1990; Schlechtriem et al., 2006; Sperfeld and Wacker, 2012). We could thus reason that PUFA concentrations of eggs can be increased by culturing *Daphnia* at relatively cold temperatures. We confirmed these results for asexual eggs of *D. magna*: eggs generated at colder temperatures contained more phospholipids and incorporated more PUFA in their membranes. Many animals and plants induce cold-resistance strategies upon a short exposure to cold temperatures. Additionally to culturing at constant temperatures, we have therefore also tested the effect of a cold shock. Animals of the cold shock treatment were cultured at 18°C but placed at 4°C for 8h at day 3 of their development and at maturity. This experiment was carried out in parallel to the experiment described in chapter 4. In contrast to the continuous culturing at low temperatures, cold shocks did not have an influence on the fatty acid composition of neutral and phospholipids (Figure 2).

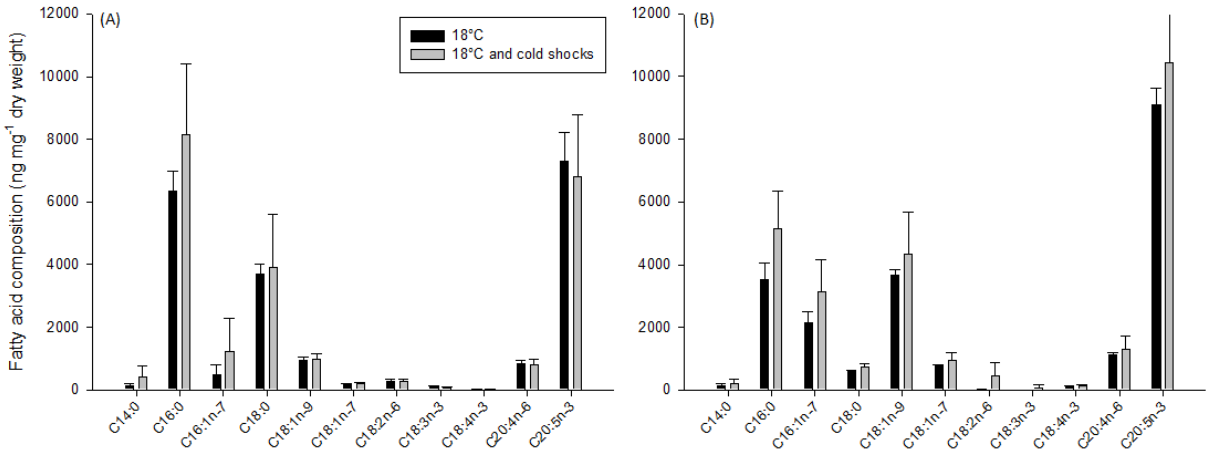

Figure 2: Fatty acid composition (µg mg^{-1} dry weight) of the neutral lipid (A) and phospholipid (B) fractions of asexual eggs of *D. magna* produced under different temperatures. Data are means of 4 replicates and error bars indicate one standard error.

It would also be interesting to check if low culturing or environmental temperatures influence the trehalose content of asexual eggs, because this would provide an easy way to increase the trehalose concentration. Earlier studies have shown that cold temperatures have a major influence on the concentration of trehalose in many organisms, such as bacteria, nematodes and insects. In all these organisms the trehalose content increased with decreasing temperature, with an increased cold resistance as result (Fields et al.; 1998; Kandror et al., 2002; Jagdale et al., 2003).

3 Cryopreservation

Optimization of every step in the vitrification protocol led to the survival of some subitaneous *Daphnia magna* eggs after vitrification (Chapter 5). Before exposure to liquid nitrogen these eggs must be dehydrated to increase their viscosity as much as possible. First, subitaneous eggs were loaded with trehalose through supplementation of the maternal diet with trehalose containing liposomes and the phospholipid composition was altered through feeding the mother C20 PUFA-rich food. Second, the eggs were loaded with the penetrating cryoprotectant glycerol through exposure to a 10% glycerol solution for 30 min. Third, they were osmotically dehydrated, and simultaneously loaded with methanol, through exposure to a vitrifying solution containing 10% glycerol, 30% methanol and 0.5M trehalose. In the last step, eggs were plunged into liquid nitrogen in a drop of the vitrification solution, applying the principles of ultra-fast freezing using droplet vitrification.

While we were successful in cryopreserving parthenogenetic eggs, survival rates so far are very low. To increase survival several steps in the protocol can still be improved. First, it should be tested whether the intracellular concentration of trehalose can be increased, either by changing the culture conditions for example in terms of temperature or by other techniques overcoming membrane impermeability for trehalose, like microinjection or electroporation. Second, the effect of the exact age of the eggs should be tested. In all experiments we aimed to use eggs of stage 1 following to Kast-Hutcheson et al. (2001), but the difference with stage 2 eggs is difficult to see. Stage 1 is the time between 0 and 15h after ovulation, with no evidence for cellular differentiation. In stage 2, 15-25h after deposition, embryos are already differentiated into a gastrula. The longer eggs stay in the brood pouch the more they differentiate, and the more differentiation the lower the changes to survive cryopreservation. The latter has been shown among others for rotifers (Toledo and Kurokora, 1990), blue mussel (Toledo et al., 1989) and sea urchins (Asahina and Takahashi, 1978) and is due to the fact that in differentiated structures not only the cell integrity but also the tissue integrity (with all intercellular connections) needs to be preserved. Additionally, it is more difficult for cryoprotectant substances to spread evenly over the tissue and it is more difficult to extract sufficient amounts of water from the centrally positioned cells. Therefore a comprehensive histological study of asexual and sexual eggs would provide valuable extra information about the structural differences between the egg types and it could allow checking the distribution of the cryoprotectants trehalose and glycerol in the

asexual eggs. Ideally, we should get a survival rate of >10% so that we can use the technique in a reliable way to preserve clonal lineages of *Daphnia*.

4 Conclusion

Our stepwise approach led to successful cryopreservation of subitaneous *Daphnia magna* eggs. Although survival rates are still low, this is a large step forward in the process towards safe storage of clonal lines. The comparison of the biochemical content of dormant, stress resistant eggs with subitaneous, sensitive eggs provided crucial information that was used to modify subitaneous eggs towards drought and freeze resistant eggs.

References

Altshuler, I., Demiri, B., Xu, S., Constantin, A., Yan, N. D. and Cristescu, M. E. (2011) An Integrated Multi-Disciplinary Approach for Studying Multiple Stressors in Freshwater Ecosystems: *Daphnia* as a Model Organism. *Integrative and Comparative Biology,* **51,** 623-633.

Arbaciauskas, K. (1998) Life-history traits of exephippial and parthenogenetically derived daphnids: indicators of different life-history strategies. *Archiv für Hydrobiologie Special Issues Advances in Limnology,* **52,** 339-358.

Asahina, E. and Takahashi, T. (1978) Freezing tolerance in embryos and spermatozoa of the sea urchin. *Cryobiology,* **15,** 122-127.

Bryant, G., Koster, K. L. and Wolfe, J. (2001) Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research,* **11,** 17-25.

Bryant, G., Koster, K. L. and Wolfe, J. (2007) Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research,* **11,** 17-25.

Clegg, J. S. (1965) The origin of threhalose and its significance during the formation of encysted dormant embryos of Artmia salina. *Comparative Biochemistry and Physiology,* **14,** 135-143.

Colbourne, J., Pfender, M., Gilbert, D., Thomas, W., Tucker, A., Oakley, T., Tokishita, S., Aerts, A., Arnold, G., Basu, M., Bauer, D., Caceres, C., Carmel, L., Casola, C., Choi, J., Detter, J., Dong, Q., Dusheyko, S., Eads, B., Frohlich, T., Geiler-Samerotte, K., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E., Kultz, D., Laforsch, C., Lindquist, E. and Lopez, J. (2011) The ecoresponsive genome of Daphnia pulex. *Science,* **331,** 555 - 561.

Crockett, E. L. (1998) Cholesterol Function in Plasma Membranes from Ectotherms: Membrane-Specific Roles in Adaptation to Temperature. *American Zoologist,* **38,** 291-304.

Crowe, J. (2007) Trehalose As a "Chemical Chaperone". In: P. Csermely and L. Vígh (eds) *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks.* Vol. 594. Springer New York, pp. 143-158.

Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Aurell Wistrom, C. (1987) Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochemical Journal,* **242,** 1-10.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Tsvetkova, N., Wolkers, W. and Tablin, F. (2001) The Trehalose Myth Revisited: Introduction to a Symposium on Stabilization of Cells in the Dry State. *Cryobiology,* **43,** 89-105.

De Meester, L., Gomez, A. and Simon, J. (2004) Evolutionary and ecological genetics of cyclical parthenogens. In: A. Moya and E. Font (eds) *Evolution from molecules to ecosystems.* Oxford university press, pp. 122-134.

Eads, B. D., Andrews, J. and Colbourne, J. K. (2007) Ecological genomics in Daphnia: stress responses and environmental sex determination. *Heredity,* **100,** 184-190.

Elliott, B., Haltiwanger, R. S. and Futcher, B. (1996) Synergy Between Trehalose and Hsp104 for Thermotolerance in Saccharomyces cerevisiae. *Genetics,* **144,** 923-933.

Eroglu, A., Lawitts, J. A., Toner, M. and Toth, T. L. (2003) Quantitative microinjection of trehalose into mouse oocytes and zygotes, and its effect on development. *Cryobiology,* **46,** 121-134.

Eroglu, A., Russo, M. J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H. and Toner, M. (2000) Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nature,* **18,** 163-167.

Eroglu, A., Toner, M. and Toth, T. L. (2002) Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. *Fertility and sterility,* **77,** 152-158.

Farkas, T., Nemecz, G. and Csengeri, I. (1984) Differential response of lipid metabolism and membrane physical state by an actively and passively overwintering planktonic crustacean. *Lipids,* **19,** 436-442.

Fields, P. G., Fleurat-Lessard, F., Lavenseau, L., Gérard, F., Peypelut, L. and Bonnot, G. (1998) The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in Sitophilus granarius and Cryptolestes ferrugineus (Coleoptera). *Journal of Insect Physiology,* **44,** 955-965.

Fuller, B. J. (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryoletters,* **25,** 375-388.

Goad, L. J. (1981) Sterol biosynthesis and metabolism in marine invertebrates. *Pure and Applied Chemistry, **53,** 837-852*

Hartwich, M., Martin Creuzburg, D., Rothhaupt, K.-O. and Wacker, A. (2012) Oligotrophication of a large, deep lake alters food quantity and quality constraints at the primary producer–consumer interface. *Oikos,* **121,** 1702-1712.

Hazel, J. R. and Williams, E. E. (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in lipid research,* **29,** 167-227.

Heckmann, L., Sibly, R., Timmermans, M. and Callaghan, A. (2008) Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. *Frontiers in Zoology,* **5,** 11.

Hengherr, S., Heyer, A. G., Brümmer, F. and Schill, R. O. (2011) Trehalose and Vitreous States: Desiccation Tolerance of Dormant Stages of the CrustaceansTriopsandDaphnia. *Physiological and Biochemical Zoology,* **84,** 147-153.

Hontoria, F., Crowe, J., Crowe, L. and Amat, F. (1998) Trehalose prevents imbitional damage in anhydrobiotic cysts of Artemia by depressing the phase transition temperature in lipids (with 7 figures). *Ergebnisse der limnologie,* **52,** 451-462.

Jagdale, G. B. and Grewal, P. S. (2003) Acclimation of entomopathogenic nematodes to novel temperatures: trehalose accumulation and the acquisition of thermotolerance. *International Journal for Parasitology,* **33,** 145-152.

Jansen, M., Coors, A., Vanoverbeke, J., Schepens, M., De Voogt, P., De Schamphelaere, K. a. C. and De Meester, L. (2015) Experimental evolution reveals high insecticide tolerance in Daphnia inhabiting farmland ponds. *Evolutionary Applications,* **8,** 442-453.

Jaromir, S. and Petrusek, A. (2011) Daphnia as a model organism in limnology and aquatic biology: introductory remarks. *Journal of limnology,* **70,** 337-344.

Kandror, O., Deleon, A. and Goldberg, A. L. (2002) Trehalose synthesis is induced upon exposure of Escherichia coli to cold and is essential for viability at low temperatures. *Proceedings of the National Academy of Sciences,* **99,** 9727-9732.

Kast-Hutcheson, K., Rider, C. V. and Leblanc, G. A. (2001) The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environmental Toxicology and Chemistry,* **20,** 502-509.

Kato, Y., Kobayashi, K., Watanabe, H. and Iguchi, T. (2010) Introduction of foreign DNA into the water flea, Daphnia magna, by electroporation. *Ecotoxicology,* **19,** 589-592.

Kato, Y., Shiga, Y., Kobayashi, K., Tokishita, S.-I., Yamagata, H., Iguchi, T. and Watanabe, H. (2011) Development of an RNA interference method in the cladoceran crustacean Daphnia magna. *Development Genes and Evolution,* **220,** 337-345.

Kuleshova, L. L., Macfarlane, D. R., Trounson, A. O. and Shaw, J. M. (1999) Sugars Exert a Major Influence on the Vitrification Properties of Ethylene Glycol-Based Solutions and Have Low Toxicity to Embryos and Oocytes. *Cryobiology,* **38,** 119-130.

Lagares, M. A., Castanheira, P. N., Amaral, D. C. G., Vasconcelos, A. B., Veado, J. C. C., Arantes, R. M. E. and Stahlberg, R. (2009) Addition of Ficoll and Disaccharides to Vitrification Solutions Improve in vitro Viability of Vitrified Equine Embryos. *Cryoletters,* **30,** 408-413.

Lampert, W. (2006) Daphnia: Model herbivore, predator and prey. *Polish journal of ecology,* **54,** 607-620.

Lampert, W. (2011) Daphnia: development of a model organism in ecology and evolution *Excellence in Ecology.* International Ecology Institute Oldendorf/Luhe.

Makrushin, A. V. (1978) Anhydrobiosis and yolk structure of eggs in cladocera. *Zoologichesky zhurnal,* **57,** 364-374.

Martin-Creuzburg, D., Wacker, A., Zeise, C. and Kainz, M. (2012) Dietary lipid quality affects temperature-mediated reaction norms of a freshwater key herbivore. *Oecologia,* **168,** 901-912.

Martin-Creuzburg, D. and Elert, E. V. (2009) Ecological significance of sterols in aquatic food webs. In: M. T. Arts, M. T. Brett and M. Kainz (eds) *Lipids in aqutic ecosystems.* pp. 43-64.

Martin-Creuzburg, D., Sperfeld, E. and Wacker, A. (2009) Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids. *Proceedings of the Royal Society B: Biological Sciences,* **276,** 1805-1814.

Martin-Creuzburg, D., Von Elert, E. and Hoffman, K. (2008) Nutritional constraints at the cyanobacteria–*Daphnia magna* interface: The role of sterols. *Limnology and oceanography,* **53,** 456-468.

Martin-Creuzburg, D., Wacker, A. and Von Elert, E. (2005) Life history consequences of sterol availability in the aquatic keystone species *Daphnia*. *Oecologia,* **144,** 362-372.

Martin-Creuzburg, D. and Von Elert, E. (2004) Impact of 10 Dietary Sterols on Growth and Reproduction of Daphnia galeata. *Journal of Chemical Ecology,* **30,** 483-500.

Mazur, P. (2004) Principles of Cryobiology. In: B. J. Fuller, N. Lane and E. E. Benson (eds) *Life in the frozen state.* CRC Press LLC, pp. 3-65.

Ohvo-Rekilä, H., Ramstedt, B., Leppimäki, P. and Peter Slotte, J. (2002) Cholesterol interactions with phospholipids in membranes. *Progress in lipid research,* **41,** 66-97.

Parsell, D. A. and Lindquist, S. (1993) The Function of Heat-Shock Proteins in Stress Tolerance: Degradation and Reactivation of Damaged Proteins. *Annual Review of Genetics,* **27,** 437-496.

Pauwels, K., Stoks, R., Verbiest, A. and De Meester, L. (2007) Biochemical adaptation for dormancy in subitaneous and dormant eggs of *Daphnia magna*. *Hydrobiologia,* **594,** 91-96.

Pegg, A. E. and Mccann, P. P. (1982) Polyamine metabolism and function. *American Journal of Physiology,* **243,** 212-221.

Pruitt, N. L. (1990) Adaptations to temperature in the cellular membranes of crustacea: membrane structure and metabolism. *Journal of Thermal Biology,* **15,** 1-8.

Putman, A., Martin-Creuzburg, D., De Meester, L. and Panis, B. (2015) A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality. *Journal of Plankton Research,* **DOI:10.1093/plankt/fbv043**.

Radzikowski, J. (2013) Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *Journal of Plankton Research,* **35,** 707-732.

Schlechtriem, C. (2006) Effect of temperature on the fatty acid composition and temporal trajectories of fatty acids in fasting Daphnia pulex (Crustacea, Cladocera). *Lipids,* **41,** 397.

Schwerin, S., Zeis, B., Lamkemeyer, T., Paul, R., Koch, M., Madlung, J., Fladerer, C. and Pirow, R. (2009) Acclimatory responses of the Daphnia pulex proteome to environmental changes. II. Chronic exposure to different temperatures (10 and 20degreesC) mainly affects protein metabolism. *BMC Physiology,* **9,** 8.

Seidman, L. A. and Larsen Jr, J. H. (1979) Ultrastructure of the envelopes of resistant and nonresistant Daphnia eggs. *Canadian journal of zoology,* **57,** 1773-1777.

Shirakashi, R., Köstner, C. M., Müller, K. J., Kürschner, M., Zimmermann, U. and Sukhorukov, V. L. (2002) Intracellular delivery of trehalose into mammalian cells by elektropermeabilization. *The journal of membrane biology,* **189,** 45-54.

Simon, J.-C., Pfrender, M. E., Tollrian, R., Tagu, D. and Colbourne, J. K. (2011) Genomics of Environmentally Induced Phenotypes in 2 Extremely Plastic Arthropods. *Journal of Heredity,* **102,** 512-525.

Sommer, U., Adrian, R., De Senerpont Domis, L., Elser, J. J., Gaedke, U., Ibelings, B., Jeppesen, E., Lürling, M., Molinero, J. C., Mooij, W. M., Van Donk, E. and Winder, M. (2012) Beyond the Plankton Ecology Group (PEG) Model: Mechanisms Driving Plankton Succession. *Annual Review of Ecology, Evolution, and Systematics,* **43,** 429-448.

Sperfeld, E. and Wacker, A. (2009) Effects of temperature and dietary sterol availability on growth and cholesterol allocation of the aquatic keystone species Daphnia. *Journal of Experimental Biology,* **212,** 3051-3059.

Sperfeld, E. and Wacker, A. (2012) Temperature affects the limitation of Daphnia magna by eicosapentaenoic acid, and the fatty acid composition of body tissue and eggs. *Freshwater biology,* **57,** 497-508.

Stanley, D. (2006) Prostaglandins and other eicosanoids in insects: biological significance. *Annual Review Entomology,* **51,** 25-44.

Storey, K. B. (1997) Organic Solutes in Freezing Tolerance. *Comparative Biochemistry and Physiology Part A: Physiology,* **117,** 319-326.

Tabor, C. W. and Tabor, H. (1984) Polyamines. *Annual Review of Biochemistry,* **53,** 749-790.

Tautz, D. (2011) Not just another genome. *BMC Biology,* **9,** 8.

Toledo, J., Kurokura, H. and Kasahara, S. (1989) Preliminary studies on the cryopreservation of the blue mussel embryos. *Japan Society Scientific Ficheries,* **55,** 1661-1661.

Toledo, J. D. and Kurokura, H. (1990) Cryopreservation of the euryhaline rotifer Brachionus plicatilis embryos. *Aquaculture,* **91,** 385-394.

Tollrian, R. and Leese, F. (2010) Ecological genomics: steps towards unraveling the genetic basis of inducible defenses in Daphnia. *BMC Biol,* **8,** 51.

Valentine, R. C. and Valentine, D. L. (2004) Omega-3 fatty acids in cellular membranes: a unified concept. *Progress in lipid research,* **43,** 383-402.

Van Doorslaer, W., Stoks, R., Swillen, I., Feuchtmayr, H., Atkinson, D., Moss, B. and De Meester, L. (2010) Experimental thermal microevolution in community-embedded Daphnia populations. *Climate research,* **43,** 81.

Van Doorslaer, W., Vanoverbeke, J., Duvivier, C., Rousseaux, S., Jansen, M., Jansen, B., Feuchtmayr, H., Atkinson, D., Moss, B., Stoks, R. and De Meester, L. (2009) Local adaptation to higher temperatures reduces immigration success of genotypes from a warmer region in the water flea Daphnia. *Global Change Biology,* **15,** 3046-3055.

Von Elert, E., Martin-Creuzburg, D. and Le Coz, J. R. (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London. Series B: Biological Sciences,* **270,** 1209-1214.

Wacker, A. and Martin-Creuzburg, D. (2007) Allocation of essential lipids in Daphnia magna during exposure to poor food quality. *Functional Ecology,* **21,** 738-747.

Walker, C. (2014) *Ecotoxicology: Effects of Pollutants on the Natural Environment.* CRC Press.

Wolfe, J. and Bryant, G. (1999) Freezing, drying and/or vitrification of membrane-solute-water systems. *Cryobiology,* **39,** 103-129.

Wolfe, J. and Bryant, G. (2001) Cellular cryobiology: thermodynamic and mechanical effects. *International journal of refrigeration,* **24,** 438-450.

Zaffagnini, F. (1987) Reproduction in *Daphnia*. In: R. H. Peters and R. De Bernardi (eds) *Daphnia.* Vol. 45. Memorie dell'Instituto Italiano di Idrobiologia, pp. 245-284.

Summary

The water flea *Daphnia magna* is an important model organism in many research areas, such as ecology, ecotoxicology, evolutionary biology and eco-genomics and most of these applications use clonal lines produced via asexual reproduction. To date, maintenance of the clonal lines can only be achieved through continuous culturing. This is a labor-intensive process and entails the risk of losing important lines because of contamination, disease or accidents. In this PhD research, we tried to solve this problem by developing a cryopreservation protocol for the asexual eggs of *Daphnia magna*.

The dormant eggs of *Daphnia*, produced by sexual reproduction, are drought and freeze resistant and are thereby an excellent reference to determine what is needed for eggs to survive cryopreservation. We determined the main biochemical differences between sexual and asexual eggs. We found that asexual eggs have higher concentrations of fatty acids than sexual eggs, however a certain concentration of long-chain PUFA, especially EPA and ARA, is maintained in sexual eggs even when they are not provided by the food and this in both neutral lipids and phospholipids. Sugar content of sexual and asexual eggs was very distinct, sexual eggs contained high amounts of trehalose (4.15% of their dry weight), while asexual only contain 0.006% trehalose. Also polyamine analysis revealed some difference between the two egg types. Asexual eggs always contained higher amounts of the metabolite diaminopropane and lower amounts of putrescine and spermidine in comparison with sexual eggs.

In a second step we tried to modify the composition of asexual eggs in order to increase their stress resistance. Fatty acid composition of asexual is strongly influenced by the maternal food, so to boost the PUFA composition of these eggs we simply switch to a diet containing more long-chain PUFA. PUFA composition of the asexual eggs of *D. magna* is also strongly influence by the culturing temperature of the females, they allocate more unsaturated fatty acids to their eggs at colder temperatures. In addition, we tried to increase trehalose concentration of the asexual eggs by supplementation of the maternal diet with trehalose containing liposomes, but changes were only minor.

In parallel, the protocol for cryopreservation was optimized. To obtain sufficient dehydration we choose for a two-step pretreatment for the eggs before applying droplet vitrification. The pretreatment consisted of a glycerol loading in a 10% glycerol solution for 30 min. This was followed by osmotic dehydration in a vitrification solution. The vitrification solution with the lest mortality after exposure for 10 or 20 min contains 10% glycerol, 30% methanol and 0.5M trehalose.

In the last step, we combined the procedure to alter the biochemical properties of asexual eggs and our most suitable cryopreservation protocol. This stepwise approach led to successful cryopreservation of subitaneous *Daphnia magna* eggs. Although survival rates are still low, this is a large step forward in the process towards safe storage of clonal lines.

Samenvatting

De watervlo *Daphnia magna* is een belangrijk modelorganisme in verscheidene onderzoeksdomeinen, zoals ondermeer ecologie, ecotoxicologie en evolutionaire biologie en de meeste van deze toepassingen maken gebruik van clonale lijnen geproduceerd via aseksuele reproductie. Tot op heden worden deze lijnen bijgehouden door ze continu in cultuur te houden. Dit is niet alleen arbeidsintensief, het is ook niet geheel zonder risico. Clones kunnen gecontamineerd worden of verloren gaan door ziekte of accidenteel verlies. Om dit probleem op te lossen hebben we tijdens dit doctoraatsonderzoek geprobeerd om een cryopreservatieprotocol te ontwikkelen voor de aseksuele eieren van *Daphnia magna*.

De dormante eieren van de watervlo, geproduceerd via seskuele reproductie, zijn wel droogte- en vriesresistent en kunnen daarom fungeren als referentiemateriaal. In een eerste deel hebben we dan ook de biochemische verschillen tussen beide eitypes bepaald. Onze resultaten toonden aan dat aseksuele eieren in het totaal meer vetzuren bevatten, maar seksuele eieren bevatten altijd een bepaalde concentratie aan lange ongesatureerde vetzuren, vooral EPA en ARA, ook als deze niet worden aangerijkt door het voedsel. De samenstelling van de suikers was zeer verschillend in seksuele en aseksuele eieren. Seksuele eieren bevatten zeer hoge concentraties aan trehalose (4.15% van hun drooggewicht), terwijl aseksuele eieren maar 0.006% bevatten. Ook de samenstelling van de polyamines vertoonde opvallende verschillen tussen beide types eieren. Aseksuele eieren bevatten meer van de metaboliet diaminopropaan en lagere hoeveelheden van spermidine en putrescine dan seksuele eieren.

In een tweede stap hebben we geprobeerd om de samenstelling van de aseksuele eieren te wijzigen opdat ze meer resistant zouden worden. Vetzuursamenstelling van de eieren wordt sterk beïnvloed door het maternale dieet, dus om de hoeveelheid meervoudig ongesatureerde vetzuren te verhogen hebben we de vrouwelijke *Daphnia* gevoederd met een dieet dat veel meervoudig ongesatureerde vetzuren bevat. Daarnaast wordt de vetzuursamenstelling van aseksuele eieren ook sterk beïnvloed door de temperatuur waarop de vrouwtjes worden gekweekt, deze alloceren meer meervoudig ongesatureerde vetzuren naar hun eieren wanneer ze gekweekt worden op koude temperaturen. Vervolgens hebben we geprobeerd om het gehalte aan trehalose in aseksuele eieren te verhogen door het maternale dieet te supplementeren met trehalose bevattende liposomen, maar dit reflecteerde slechts in een zeer kleine stijging.

In parallel met voorgaande stappen, hebben we ook het cryopreservatieprotocol geoptimaliseerd. Om voldoende uitdroging van de eieren te bekomen hebben we gekozen voor een voorbehandeling bestaande uit 2 stappen vooraleer de eieren werden ingevroren via ‘droplet vitrificatie’. Deze voorbehandeling bestaat uit het laden van gelycerol via de blootstelling aan een glyceroloplossing van 10% gedurende 30 min gevolgd door osmotische dehydratatie in een vitrificatieoplossing. De

vitrificatieoplossing resulterend in de hoogste overleving, na blootstelling eraan voor 10 of 20 min, bevat 10% glycerol, 30% methanol en 0.5M trehalose.

In de laatste stap, combineerden we de procedure voor het wijzigen van de samenstelling van de asksuele eieren met het meest geschikte cryopreservatieprotocol. Deze stapsgewijze aanpak resulteerde in succesvolle cryopreservatie van asksuele eieren van *Daphnia magna*. Ondanks het feit dat de ontluikingspercentages nog zeer laag zijn, is dit een grote stap voorwaarts in het proces naar het veilig bewaren van clonale lijnen.

List of abbreviations

ALA	Alpha-linolenic acid (C18:3n-3)
ANOVA	Analysis of variance
ARA	Arachidonic acid (C20:4n-6)
DAP	Diaminopropane
DHA	Docosahexaenoic acid (C22:6n-3)
DNA	Deoxyribonucleic acid
EG	Ethylene glycol
EPA	Eicosapentaenoic acid (C20:5n-3)
FA	Fatty acids
FAME	Fatty acids methyl esters
GC	Gas chromatography
GLMM	Generalized linear mixed model
HPLC	High performance liquid chromatography
HSD	Honestly Significant Difference
Hsp	Heat shock protein
LIN	Linoleic acid (C18:2n-6)
LRV	'Langerode vijver'
MANOVA	Multivariate analysis of variance
ME	Methyl ester
MUFA	Monounsaturated fatty acids
NSPD	Nor-spermidine
NSPM	Nor-spermine
POPC	1-palmitoyl-2-oleoyl-phosphatidylglycerol
POPG	1-palmitoyl-2-oleoyl-phosphatidylcholin
PUFA	Polyunsaturated fatty acids

PUT	Putrescine
RNA	Ribonucleic acid
SFA	Saturated fatty acids
SPD	Spermidine
SPM	Spermine
VS	Vitrification solution