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Cold storage of eggs of *Acartia tonsa* Dana: effects of light, salinity and short-term temperature elevation on 48-h egg hatching success

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Abstract

Copepods play a key role in the marine ecosystem where they form a vital trophodynamic link between primary and tertiary producers, and are the dominant prey of many marine fishes during their larval stage. Because of their naturally high nutritional quality, copepods are excellent alternatives to traditional live feed organisms for intensive marine fish production. For aquaculturists, it is important to have continuous access to live feed organisms when rearing altricial fish larvae. Cold stored subitaneous copepod eggs are highly relevant as inoculum for culturing live food.

In the present study, the effects of light, salinity and short-term elevations in temperature on the 48-h hatching success (HS, %) of cold stored (2 °C) *A. tonsa* eggs were evaluated. A light experiment exposed the eggs to three different environments with respect to light for a period of up to 7.5 months; one treatment exposed the eggs to an artificial light source, the second treatment had eggs stored in complete darkness, and the third treatment had eggs stored under the storage conditions applied at NTNU Sealab (SSC-treatment). A salinity experiment stored eggs in seawater of four different salinities (34, 50, 75 and 100 ‰) for a period of up to 7.5 months to evaluate if this was beneficial for long-term cold storage of *A. tonsa* eggs. A short-term temperature elevation experiment was conducted in order to simulate shipping conditions with insufficient cooling for consignments of *A. tonsa* eggs. Cold stored eggs were exposed to temperatures elevated to 9 and 17 °C for 12 and 24 hours, with and without oxygen present in the storage media, whereupon they were put back in cold storage for a period of one week before determining the HS.

The results for the light experiment showed that light had a profound negative effect on the HS of the cold stored *A. tonsa* eggs. No hatching was observed for eggs that were stored under constant light after 3 months of cold storage. Eggs stored in darkness showed a significantly higher HS at the final sampling of month 7.5 compared to the SSC treatment, and less dispersion in HS between the replicates throughout the experiment. The salinity experiment showed that the eggs could be stored in seawater with a salinity of 50 ‰ for up to 3 months with a high HS, whereas storage in seawater of 75 and 100 ‰ showed a low HS for most samplings and thus proved as a poor cold storage media. The short-term elevation in temperature experiment showed that the HS was significantly affected by the oxygen

concentration in the seawater upon a cold storage period of 3 weeks, and that short-term elevations in temperature, up to 17 °C for a duration of 24 hours, did not significantly affect the HS when hatched one week after the onset of incubation. The viability of the hatched nauplii post temperature exposures was assessed in a feeding incidence experiment. It showed that neither of the conducted temperature elevations utterly inhibited the nauplii from feeding.

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1. Introduction

1.1 Background

Today most marine fish species are either fully or over exploited by the worlds fisheries. From 2004 to 2009 the world`s total fisheries production has stabilized around 90 million tonnes (80 million tonnes from marine waters). The world`s total aquaculture production is in comparison steadily growing with an average annual growth rate of 8.3 %, from 41.9 million tonnes in 2004 to 55.1 million tonnes in 2009. Aquaculture accounted for 46 % of the world`s total fish supply in 2008. It is the fastest growing animal-food-producing sector worldwide, where per capita supply of animal protein increases with an annual rate of 6.6 % (FAO, 2010)

The source of marine raw material used in compound aquafeed is becoming scarcer as global fish stocks are declining. FAO have estimated that underexploited or moderately exploited fish stocks declined from 40 % in the 1970`s to 15 % in 2008. Fully exploited marine fish stocks were estimated to 53 % in 2008. The rest of the world`s fish stocks were either moderately exploited (12 %), underexploited (3 %), overexploited (28 %), depleted (2 %) or recovering from depletion (3 %) (FAO, 2010). Fish meal and fish oils derived from wild capture fisheries are very important aquafeed ingredients for nutritionally demanding carnivorous and omnivorous aquaculture species, because they are cost-effective providers of high quality animal protein and marine lipids (Tacon & Metian, 2008; Karalazos et al., 2011).

Fish develop into juveniles either directly (precocial) or indirectly (altricial) (Balon, 1999). Major aquacultural species like Atlantic salmon (*Salmo trutta trutta*) and rainbow trout (*Oncorhynchus mykiss*) are precocial species (Balon, 1986). They produce demersal eggs with a large endogenous food supply, where the free embryos (the period from hatching to first exogenous feeding) develop directly into juveniles with an adult phenotype. They can ingest and digest formulated diets as soon as exogenous feeding initiates (Bendiksen et al., 2011; Karalazos et al., 2011). Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglussus hippoglossus*) are examples of altricial species (Herbing, 2001). They produce small pelagic

eggs, where the free embryos have insufficient endogenous food supplies to support direct development, and therefore need to accumulate nutrients during a larval stage and undergo metamorphosis to become juveniles (Balon, 1999). The relatively undeveloped larvae have only a rudimentary digestive system and are incapable of processing formulated diets in a way that supports growth and survival (Govoni et al., 1986). They therefore appear to require live feed organisms in order to grow and survive (Conceição et al., 2010).

1.2 Live feed in aquaculture

Production of fish larvae is carried out under strictly monitored conditions with respect husbandry techniques, feeding strategies and microbial control (Lavens & Sorgeloos, 1996). The major bottleneck in larval rearing today is the first feeding period. The size of the larvae and the mouth opening should be considered when choosing live feed organisms, in addition to its nutritional content. Traditionally, brine shrimp (*Artemia* sp.) and rotifers (*Brachionus* sp.) have been used as first feed in larval rearing (Evjemo & Olsen, 1997). However, their nutritional qualities are suboptimal for supporting growth and survival in most fish species, as they contain low levels of essential *n*-3 highly unsaturated fatty acids (HUFA) like Docosahexaenoic acid (DHA, 22:6n-3) and Eicosapentaenoic acid (EPA, 20:5n-3) (Bell et al., 2003). Thus they need to be enriched with nutritionally adequate lipids before they are used as live food organisms for marine fish larvae (Rainuzzo et al., 1994; Baker et al., 1998).

In comparison, copepods are the natural prey organisms for several fish larvae in the marine food web and ideal as first-feed organisms for intensive production of marine fish larvae. Copepods have naturally high levels of essential *n*-3 HUFAs like DHA and EPA (Bell et al., 2003), they are rich in free amino acids important for metabolic fuel and body protein synthesis (Fyhn, 1989; Rønnestad et al., 1999; van der Meeren et al., 2008) , and they are size-flexible as different naupliar, copepodid or adult stages can be chosen according to the mouth size of the larvae. Traditionally, copepods have been harvested directly from nature, enclosed or semi-enclosed polls, or outdoor ponds and tanks (Støttrup & Norsker, 1997; van der Meeren & Naas, 1997; Støttrup, 2000). Since the early 1990s, however, intensive mass culturing of copepods have been attempted with variable results (Stottrup & McEvoi, 2003). It has proved difficult to produce copepods in sufficient amounts while making it

economically beneficial, but recently new technology (e.g. mass culture systems) and intensive rearing methods (e.g. monoalgal diets) have made production of copepods more cost-efficient (Sun & Fleeger, 1995; Schipp et al., 1999; Stottrup & McEvoi, 2003; Drillet et al., 2011).

1.2.1 Copepods

Copepods are small aquatic crustaceans claimed to be the most numerous multicellular organisms on earth (Mauchline, 1998). They occur in both fresh- and saltwater environments and have been estimated to constitute approximately 11,500 species divided between 10 orders, 200 families and 1650 genera (Humes, 1994). They are a vital trophodynamic link between primary and tertiary producers in the natural marine food web, and the dominant prey of many marine fishes during their larval stage (deYoung et al., 2004).

1.2.2 Copepods in aquaculture

The copepod species most commonly used in aquaculture are of the orders Harpacticoida, Cyclopoida and Calanoida (Støttrup, 2007). Copepods are superior to conventional live feed organisms such as brine shrimp (*Artemia* sp.) and rotifers (*Brachionus* sp.) because of their high nutritional quality with respect to proteins, vitamins, minerals, lipids and fatty acid compositions (Evjemo & Olsen, 1997; Evjemo et al., 2003; Drillet et al., 2006b). Several studies have shown that marine fish larvae fed exclusively, or as a supplement to *Artemia* sp. and rotifers, with copepods showed higher survival, improved growth, less malformations and higher rates of normal pigmentation (McEvoy et al., 1998; Evjemo et al., 2003; Imsland et al., 2006; Overrein, 2010). Copepods can also be used to rear more exotic species such as squid (Chen et al., 1996) and ornamental aquarium fishes, where traditional live feed organisms have proved to be insufficient (Harada, 1970; Holt, 2008; Olivotto et al., 2008).

1.2.3 *Acartia tonsa* Dana

A.tonsa is a pelagic marine calanoid copepod species from the family Acartiidae. It is a wide spread species, occurring mostly in estuaries and nearshore environments above 200 meters of depth (Cervetto et al., 1999). It is a euryhaline and eurythermal species, tolerating wide ranges of salinities and temperatures. The adults measure approximately 1.5 mm in length and the females are slightly larger than males (Mauchline, 1998).

A. tonsa is a heterotrophic suspension feeder, primarily feeding on phytoplankton, but it can also feed on ciliates, rotifers, bacteria and eggs and nauplii of their own species. They reproduce by gamogenesis, where males deposit spermatophores into seminal receptacle openings on the female. The females can produce between 18 and 26 eggs day $^{-1}$, depending on environmental factors, and they shed their eggs freely into the water masses, whereupon they descend to the sea bottom and deposit in the sediments or hatch. The eggs measure 70 – 80 µm in diameter, are spherically shaped and covered with short spines. The newly hatched nauplii measure approximately 70 µm in length, and needs to go through 6 naupliar and 5 copepodid stages to become sexually mature adults. Average generation time for *A. tonsa* is 12 – 14 days (Mauchline, 1998).

1.3 Dormancy in marine planktonic copepods

Dormancy is a temporary suspension of vital functions such as growth, development and activity in an organism to overcome unfavorable environmental conditions. It appears in aquatic invertebrates wherever their environments are influenced by seasonal or annual rhythmic fluctuations of factors important for life and survival (Alekseev et al., 2007). Planktonic copepods are able to undergo diapause in embryonal, larval, naupliar, copepodid and adult life stages, but usually only one type of diapause is peculiar to one order or suborder (Alekseev et al., 2007).

All types of resting stages are grouped under the category of dormancy, which in turn is separated into two major categories; quiescence and diapause (Laudien, 1973). Quiescence is induced directly by unfavorable environmental conditions such as salinity, oxygen concentration, temperature or light. It is a reversible process where normal development

resumes as soon as the environmental conditions become favorable again. Diapause is a more complicated process which is driven by neurohormonal mechanisms, and is not induced directly by unfavorable environmental conditions. Instead, initiation of diapause begins before the unfavorable conditions occur and normal development resumes first after a predetermined period of refractory or inactive state has been completed. It is most likely an evolutionary adaptation to periodic or seasonal fluctuations of photoperiod, temperature, food dynamics, predation pressure or oxygen concentrations (Alekseev et al., 2007).

1.3.1 Embryonal dormancy in *A. tonsa*

A. tonsa has been reported to produce two different types of eggs, subitaneous and diapause eggs (Uye, 1985). A study done by Castro-Longoria (2001) on *A. tonsa* Dana, 1848 eggs from the Southampton Water showed that the two types can be distinguished from each other by their morphological traits. In the study it is shown that, on average, the subitaneous eggs measured 76.5±2.9 µm in diameter and had short spines up to 4.8 µm in height on the chorion surface, compared to diapause eggs which were slightly larger (79.8±2 µm) and had apically branched spines measuring 6.8±1.9 µm in height (Castro-Longoria, 2001).

The subitaneous eggs are meant to hatch immediately *in situ* whereas the diapause eggs need to go through a refractory phase before they can hatch. However, quiescence can be induced in subitaneous eggs if they are exposed to suboptimal environmental conditions such as low oxygen levels, abrupt salinity changes or low temperatures (Uye & Fleminger, 1976; Højgaard et al., 2008). Quiescent subitaneous *A. tonsa* eggs are highly applicable to long-term storage for use in intensive production of nauplii as live feed organisms in marine aquaculture (Drillet et al., 2006a; Drillet et al., 2006b).

1.4 Cold storage of *A. tonsa* eggs

A study done by Drillet et al. (2006a) showed that subitaneous *A. tonsa* eggs remained viable with a high hatching success for up to a period of 12 months of cold storage (2 – 3 °C), and that cold storage does not affect the reproductive capacity of the following generations. The study pointed out that the total fatty acid content of the eggs decreased with the period of cold storage. This indicates that the embryo is still metabolically active during quiescence. The pool of free amino acids was proven to be relatively stable over a period of 12 months of cold storage, proposing that amino acids are not the primary energy source of the embryo.

The developmental time from nauplii to adult copepods could increase slightly with the duration of cold storage (10 and 14 days for fresh and 12 months cold stored eggs, respectively), most likely because the nauplii use up their energy reserves during cold storage and need to rebuild these after hatching before they can molt into the following naupliar stage (Drillet et al., 2006a).

Holmstrup et al. (2006) conducted a study on the effects of salinity, temperature and oxygen conditions on viability of cold stored *A. tonsa* eggs for a period up to 35 weeks. It showed that the eggs tolerated a wide range of salinities (from 10 to 50 ‰ up to 20 weeks, with the best hatching success observed at salinities between 10 and 20 ‰), that anoxic storage conditions were superior to oxygenated conditions and that temperatures should be kept at or below 5 °C.

1.5 Present study

1.5.1 Background for study

More experiments on how to optimize storage conditions of subitaneous *A. tonsa* eggs have been called for in several recent scientific studies (Drillet et al., 2006a; Holmstrup et al., 2006; Ohs et al., 2009; Conceição et al., 2010). They have suggested that the effect of factors as light, salinity, presence of organic compounds and disinfection methods on cold stored *A. tonsa* eggs needs further investigations. Additionally, a simulation of shipping conditions was planned, to assess the importance of a temperature stable environment for consignments of

A. tonsa eggs. This was thought to be highly relevant for commercialization of copepod egg production.

1.5.2 Aims of study

The main objective of this thesis was to evaluate the effects of light, salinity and short-term temperature elevation on cold stored *A. tonsa* eggs. The ultimate objective was to improve cold storage conditions for *A. tonsa* eggs produced for live feed purposes in intensive production of marine fish larvae in aquaculture. The effects of the experiments were evaluated based on the 48-h hatching success (%) of the *A. tonsa* eggs

The following sub-objectives were formulated:

I) Assess the effect of light on the 48-h hatching success (%) of cold stored *A. tonsa* eggs.
- Explored by exposing the eggs to three different light environments during cold storage.

II) Assess the effect of hypersaline seawater on the 48-h hatching success (%) of cold stored *A. tonsa* eggs.
- Explored by storing the eggs in four different seawater salinities.

III) Assess the effect of short-term elevation in temperature on the 48-h hatching success (%) of cold stored *A. tonsa* eggs in oxic (6.66 g/L) and anoxic seawater.
- Explored by exposing cold stored *A. tonsa* eggs to two different temperatures (9 and 17 °C) at two different durations (12 and 24-h). Eggs were stored in both anoxic and oxic (6.66 g/L) seawater to evaluate if the presence of oxygen in the storage media during the temperature elevations made a difference.

2. Materials and methods

The experiments conducted at NTNU Centre of Fisheries and Aquaculture (SeaLab), Brattørkaia 17B, Trondheim, Norway. The thesis forms part of the SINTEF project "Production of copepod eggs in a closed landbased facility", and was carried out between August 2009 and May 2011. Three different experiments were conducted where the effects of light, salinity and short-term elevation in temperature on the 48-h hatching success (HS, %) of cold stored *A. tonsa* eggs were explored. A schematic view of the main experiments is shown in Table 2.1

Table 2.1 Schematic view of the conducted main experiments for this thesis

Experiment	Treatment	Duration
Light	Standard storage condition (SSC)*	≤7.5 months
	Light	≤7.5 months
	Dark	≤7.5 months
Hypersaline seawater	Seawater at 34 ‰ (SSC)*	≤7.5 months
	Seawater at 50 ‰	≤7.5 months
	Seawater at 75 ‰	≤7.5 months
	Seawater at 100 ‰	≤7.5 months
Short-term temperature elevation	9 °C for 12-h, oxic	~3 weeks
	9 °C for 24-h, oxic	~3 weeks
	9 °C for 12-h, anoxic	~3 weeks
	9 °C for 24-h, anoxic	~3 weeks
	17 °C for 12-h, oxic	~3 weeks
	17 °C for 24-h, oxic	~3 weeks
	17 °C for 12-h, anoxic	~3 weeks
	17 °C for 24-h, anoxic	~3 weeks
	Control (2 °C, oxic)	~3 weeks
	Control (2 °C, anoxic)	~3 weeks

* The SSC treatment were the same for both experiments

2.1 Production of eggs for the experiment

The copepod eggs were produced at SINTEF/NTNU Sealab`s facilities and harvested from copepod cultures of the strain *Acartia tonsa* Dana (Clone DFH.AT1). Algae of the strain *Rhodomonas baltica* (Clone NIVA 5/91 Cryptophyceae: Pyrenomonadales) was used as copepod feed. The organisms will later be referred to as *R. baltica* and *A. tonsa* in the text.

The seawater used for production of algae and copepods was collected from the Trondheim Fjord at 70 meters of depth. It was sand filtered (20 µm), UV-treated and aerated before use. Seawater used for algae production was in addition chlorine treated according to Hoff and Snell (1987); chlorinated with sodium hypochlorite (25 mL, 10 – 15 % NaOCl 100 L^{-1}) under heavy aeration for 12 hours, then dechlorinated with sodium thiosulfate (3 g 25 mL^{-1} NaOCl) under heavy aeration for 6 hours. Conwy growth medium [1.5 mL L^{-1} chlorine treated seawater, (Walne, 1974)] was added before use.

2.1.1 Production of microalgae for *A. tonsa* feed

R. baltica was produced in three polycarbonate plastic cylinders (200 L) in seawater (34 ‰) added 1.5 mL Conwy growth medium (Walne, 1974) per liter, at temperatures between 20 - 22 °C and pH values ranging between 7.5 and 8.3 (measured with a pH/mV-meter, WTW pH 315i, Germany). The cylinders were continuously illuminated by six fluorescent tubes (58 W) from three sides, aerated and added 1 - 2 % CO_2. The culture was held at a density above 1 000 000 algal cells/mL (measured with Multisizer™ Coulter Counter (capillary diameter 100 µm), Beckman Coulter Inc., USA; measurements conducted by Marit Hansen), and the dilution rate of the algae culture was 45±5 % day^{-1} (adjusted according to algal density). The harvested volume was replaced with seawater added 1.5 mL Conwy growth medium (Walne, 1974) per liter.

2.1.2 Production of *A. tonsa* eggs

A. tonsa was cultivated in a cylindrical tank (1000 L) with aerated seawater (34 ‰) at temperatures between 19.5 - 20.6 °C. Fresh seawater was continuously supplied to the tank through a water inlet mounted below the water level, and exited through a screen-covered outlet placed at the centre of the tank bottom. Approximately 100 % of the tanks` total water volume was replaced each day (0.9 L/min), dissolved oxygen content was 59 % (measured with YSI ProODO Digital Professional Series), and pH values were stable around 7.5. Copepods were fed continuously with *R. baltica* through an automatic feeding pump (~250 L d^{-1}), and it was ensured that the algal density was approximately 30 000 cells/mL at all times to achieve a proper feed concentration (Marcus, 2007).

The eggs were gathered once a day by a rotating arm placed at the bottom of the tank and collected by siphoning into a plastic bucket. They were washed with seawater through a 120 µm screen to remove large particles prior to meticulously rinsing with seawater and concentrating them on a 64 µm screen. The acquired amount of eggs was transferred to a 200 mL EasyFlask (Nunclon™) which was filled up with seawater (34 ‰) and transferred to a SANYO Medi-Cool pharmaceutical refrigerator (model MPR-311D[H]) holding a temperature of 2 °C. The pharmaceutical refrigerator will for simplicity be referred to as a refrigerator later in the text.

2.2 Basic design and procedures

2.2.1 Basic design

Only fresh *A. tonsa* eggs (< 2 weeks in cold storage) were used in the experiments. Effects on viability of cold stored *A. tonsa* eggs are negligible during the first two weeks (Drillet et al., 2006a), thus the eggs can be regarded as fresh during this period. Eggs used in the light and hypersaline seawater experiments came from one day's production and harvest. This was also the case with the eggs used in the short-term temperature elevation experiment, although they were produced and harvested later on by Katrine Singsaas. The eggs in the replicates were treated as simple random samples of the entire batch of eggs from their respective production times. The experiments were carried out with low egg-densities in the replicates to reduce the possibility of overshadowing, ensuring equal exposures for all eggs.

Egg density in the EasyFlask was calculated by vigorously shaking it, extract a 50 µL sample, dilute it with 950 mL of seawater and extract 10 sub-samples of 50 µL. The sub-samples were photographed with a firewire microscope camera (Sony DFW-SX900 v.1.02D) and the number of eggs in the photos was counted with a Counter pen (Colony counter, Bel-Art products). Finally the density of eggs (eggs/mL) in the EasyFlask was calculated.

A fixed number of eggs was transferred to scintillation vials (8 mL Borosilicate glass vials) kept on ice to maintain low temperature during the transfer. The vials were then filled up with autoclaved anoxic seawater (34 ‰), with the exception of the experiment on "short-term temperature elevation" which had treatments with autoclaved sub-oxic seawater (6.66 mg O_2/L). Anoxic seawater was obtained by bubbling autoclaved seawater with nitrogen (N_2) through an airstone until anoxic conditions was reached. Anoxia was confirmed by surveillance of dissolved oxygen using an optical dissolved oxygen sensor (YSI ProODO Digital Professional Series). No water exchanges were maintained in the replicates during the experiments.

2.2.2 Procedure for calculating the 48-h hatching success of *A.tonsa* eggs

A 50 µL sample was extracted from the Scintillation vial with a pipette (40-200 µL, Thermo Labsystems Finnpipette Digital) and placed in a Petri dish under a stereomicroscope (Leica MZ 12_5) before it was photographed with a firewire microscope camera (Sony DFW-SX900 v.1.02D). Images were captured with Fire-I Application software (v.3.01.0.111) and stored as TIFF or JPEG files. Seawater (10 mL, 34 ‰) was added to the Petri-dish before sealing it with Parafilm (plastic paraffin film). Samples were left for 48 hours at 22 °C under continuous light (see Fig. 2.1 for light spectrum) to hatch. The parafilm was then removed and samples were fixed with Phytofix (Lugol`s solution) and placed under a stereomicroscope (Leica MZ 12_5). Hatched nauplii were extracted with a glass pipette connected to a peristaltic pump (ALITEA SX-Mini) and counted with a hand counter. Images were printed on paper sheets (A4) and the number of eggs in each sample was determined using a Counter-Pen (Colony counter, Bel-Art products). The 48-h egg hatching success (HS, %) was calculated using Equation 1.

$$\text{48-h egg hatching success (\%)} = (N_{\text{hatched nauplii}} / N_{\text{eggs}}) * 100 \quad (1)$$

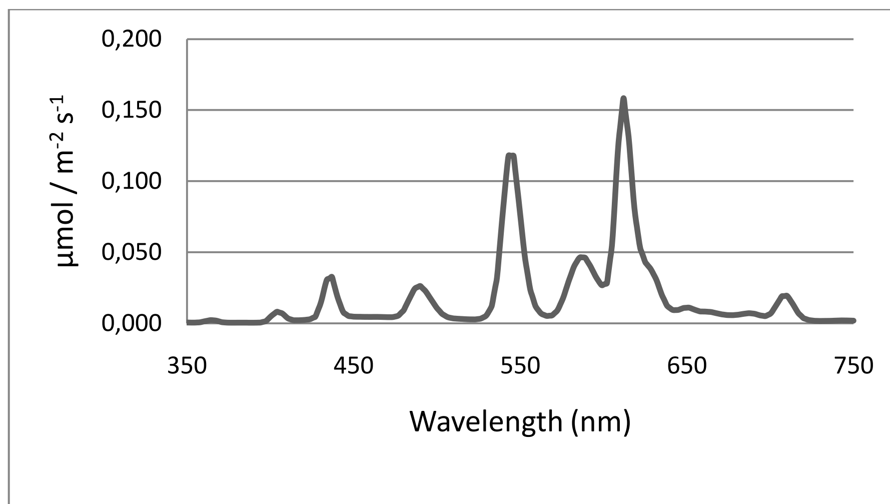

Figure 2.1 Quality of the light used during 48-h hatching of cold stored *A.tonsa* eggs (Figure modified from data by Zsolt Volent).

2.3 Effect of light

2.3.1 Aim of the experiment

The aim of the experiment was to investigate if exposure to light during cold storage had an effect on the 48-h hatching success (%) of *A. tonsa* eggs.

2.3.2 Experimental design

The *A. tonsa* eggs were stored in three different environments with respect to light, which for simplicity will be called the standard storage condition (SSC), light and dark treatment. Details of the treatments are elaborated in the following sections (2.3.3 – 2.3.5). Fresh eggs (1 mL, ~33 000 eggs/mL) were transferred from the EasyFlask to scintillation vials kept on ice. The vials were filled with autoclaved anoxic seawater (7.3 mL, 34 ‰) at a temperature of 2 °C, sealed with a screw cap and put in a refrigerator.

The duration of the experiment was set to 7.5 months with sampling after 1, 3, 4.5, 6 and 7.5 months. The experiment had a total of 45 replicates, 15 per treatment (3 for each sampling time), and the 48-h egg hatching success (%) was calculated from the means of the sub-samples (n=6). All replicates were disposed of after sampling. A schematic view of the samplings performed is shown in Table 2.2.

Table 2.2 Schematic view of samplings for the effect of light experiment

Treatment	**Time and replicates (n)**				
SSC	**1 month, n=3**	**3 months, n=3**	**4.5 months, n=3**	**6 months, n=3**	**7.5 months n=3**
	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6
Light	**1 month, n=3**	**3 months, n=3**	**4.5 months, n=3**	**6 months, n=3**	**7.5 months n=3**
	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6
Dark	**1 month, n=3**	**3 months, n=3**	**4.5 months, n=3**	**6 months, n=3**	**7.5 months n=3**
	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6

2.3.3 Light treatment

Eggs exposed to light were placed in a custom-made rack which allowed light to reach all vials (Figure 2.2). The rack was placed inside a cardboard box in a refrigerator. The light source was a custom-made panel consisting of four LED-light diode lists (IKEA 701.155.64) mounted directly onto the surface of the refrigerators` glass door with gaffer tape. A light quality measurement was conducted inside the box in the refrigerator to ensure that the desired light quality was obtained.

A.tonsa is mostly found in nearshore environments (Cervetto et al., 1999) and the eggs are usually concentrated in the upper sediments in shallow (< 20 m) estuarine waters. The aim for this experiment was to expose the eggs to light qualities assumed to be found in their natural environments. In short this means that red light is filtered out since it is absorbed quickly in the seawater column. Figure 2.3 shows the light spectrum used in this experiment.

2.3.4 Dark treatment

Eggs in the dark treatment were placed in an opaque cardboard box which allowed no light to reach the storage vials. The ceiling light was switched off when the replicates were removed from the box for sampling, to fully ensure a dark environment during the entire experiment.

2.3.5 Standard storage conditions (SSC) treatment

Eggs in the SSC treatment were placed in a rack inside the refrigerator which to some extent allowed ceiling light and daylight (shone through windows situated in the room) to reach the vials (see Figure 2.1 for light spectrum of ceiling light). This represent standard storage conditions for *A. tonsa* eggs at SINTEF/NTNU SeaLab`s production facilities.

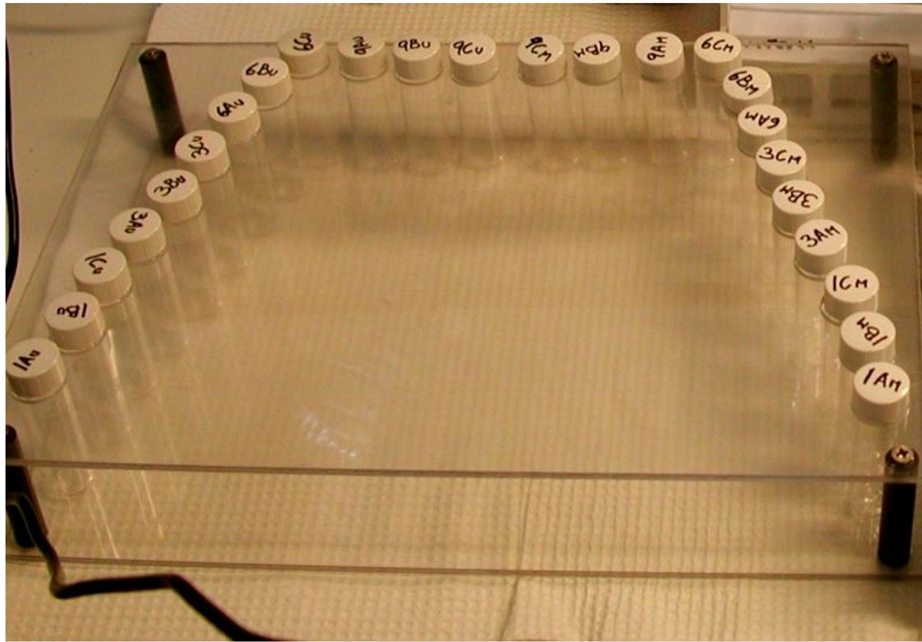

Figure 2.2 The custom made rack used in the light exposure experiment (Photo by Andreas Hagemann).

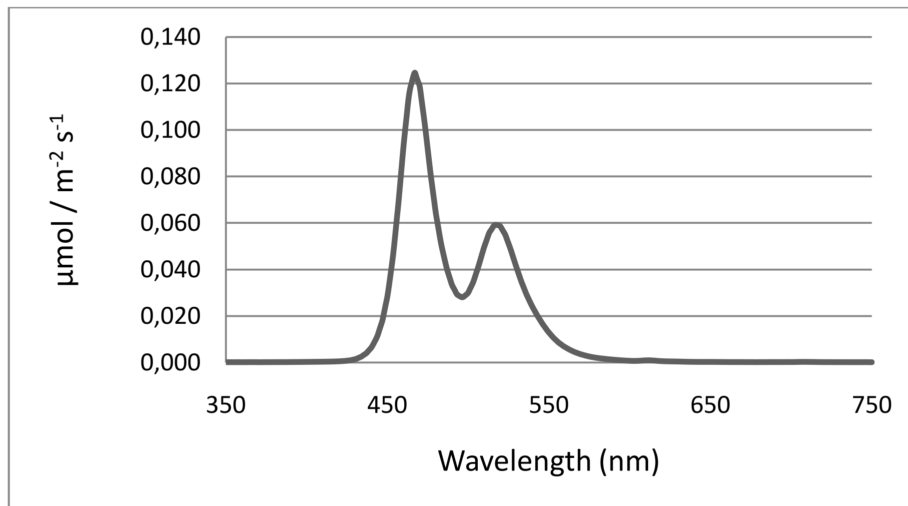

Figure 2.3 Light spectrum for the LED-light panel used in the light treatment. Wavelength ranged between ~450 - ~540 nanometers and is of the quality assumed to be found in the ocean < 20 meters of depth (Figure modified from data by Zsolt Volent).

2.4 Effect of salinity

2.4.1 Aim of the experiment

The aim of the experiment was to look at the effects of three different seawater salinities on the 48-h hatching success (%) of cold stored *A. tonsa* eggs. Salinities of 50, 75 and 100 ‰ were chosen based on previous studies performed by Ohs et al. (2009) where it was found that hypersaline seawater up to a concentration of 100 g/L have potential for short and long-term storage of subitaneous *A.tonsa* eggs (Ohs et al., 2009).

2.4.2 Experimental design

Three separate glass beakers containing 100 mL autoclaved anoxic seawater (34 ‰) at a temperature of 2 °C had 1.6, 4.1 and 6.6 grams of Ocean Reef Salt (www.prodac.no) added. The solutions were mixed until the salt was completely dissolved, yielding seawater of 50, 75 and 100 ‰. The solutions were controlled using a hand-held refractometer (ATAGO S/Mill-E) to ensure that the desired salinities were obtained. Fresh eggs (1 mL, ~33 000 eggs/mL) were pipetted from the EasyFlask to scintillation vials kept on ice. Vials were filled with autoclaved anoxic seawater (7.3 mL) at a temperature of 2 °C holding salinities of 50, 75 and 100 ‰, sealed with a screw cap and put in the refrigerator. The control treatment for this experiment was the SSC treatment from the *effect of light* experiment.

The duration of the experiment was set to 7.5 months with sampling after 1, 3, 6 and 7.5 months. The experiment had a total of 36 replicates, 12 per treatment (3 for each sampling time), and the 48-h egg hatching success (%) was calculated from the means of the subsamples (n=6). All replicates were disposed of after sampling. A schematic view of the samplings performed is shown in Table 2.3.

Table 2.3 Schematic view of samplings for the effect of hypersaline seawater experiment

Treatment	**Time and replicates (n)**			
Seawater of 34 ‰ (SSC) *	**1 month, n=3**	**3 months, n=3**	**6 months, n=3**	**7,5 months, n=3**
	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6
Seawater of 50 ‰	**1 month, n=3**	**3 months, n=3**	**6 months, n=3**	**7,5 months, n=3**
	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6
Seawater of 75 ‰	**1 month, n=3**	**3 months, n=3**	**6 months, n=3**	**7,5 months, n=3**
	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6
Seawater of 100 ‰	**1 month, n=3**	**3 months, n=3**	**6 months, n=3**	**7,5 months, n=3**
	HS (%), n=6	HS (%), n=6	HS (%), n=6	HS (%), n=6

*SSC treatment is the same as for the e*ffect of light* experiment

2.5 Effect of short-term elevation in temperature during cold storage

2.5.1 Aim of the experiment

The aim of the experiment was to investigate the effects of short-term elevation in temperature during cold storage on the 48-h hatching success (%) of *A.tonsa* eggs. This experiment simulate conditions that could occur during shipping of eggs in commercial contexts where the sender fail to cool eggs properly during transport, or a technical failure in the production facilities` refrigerator. The experiment also assessed the importance of oxygen conditions during cold storage upon temperature elevations.

2.5.2 Experimental design

Two different temperatures (9 and 17 °C), two different exposure periods (12 and 24 hours) and two different seawater qualities (sub-oxic (6.66 g/L) and anoxic seawater (34 ‰) at a temperature of 2 °C) were chosen for this experiment. Oxygen content of the seawater used in the oxic treatment was measured with an optical dissolved oxygen sensor (YSI ProODO Digital Professional Series).

The temperature elevations were conducted in two Sanyo Incubator cabinets (MIR-253) holding temperatures of 9 °C (± 0.3 °C) and 17 °C (± 0.3 °C). The time needed to reach the desired temperatures was measured in scintillation vials filled with pure seawater (2 °C). The vials were placed inside the preheated incubator cabinets (9 and 17 °C) and two Thermocouple Thermometers (DIGI-SENSE COLE PARMER model 91199-40) were submerged into the vials through a hole in the screw cap. The temperature of the seawater was continuously noted until the desired temperatures were reached. Time taken to acclimatize is shown in Figure 2.4. This was taken into account when exposure times were determined, where the incubation times were extended by 25 and 35 minutes for the 9 °C and 17 °C treatments, respectively. There were no light present in the incubator cabinets during heating.

One week old eggs (1 mL, ~31000 eggs/mL) were pipetted from the EasyFlask to scintillation vials kept on ice. The vials were filled up with seawater (7.3 mL, 34 ‰, 2 °C) of the desired oxic quality (6.66 mg O_2/L and anoxic), and put back in the refrigerator. After one week the

vials were transferred from the refrigerator to the incubator cabinets to be heated. Before the onset of incubation, a water exchange was conducted in all the replicates of the oxic treatment to ensure that oxygen was present in the vials during the incubation, in case the oxygen had been depleted during the week in storage. When the predetermined exposure times were reached, the vials were put back into the refrigerator for a period of one week. The control treatment replicates were kept in the refrigerator (2 °C) throughout the entire experiment. At the end of the experiment, the 48-h hatching success (%) of the *A. tonsa* eggs was determined.

The experiment had a total of 50 replicates, 5 for each treatment, and the 48-h egg hatching success (%) was calculated from the means of the subsamples (n= 16-20) of each treatment. All replicates were disposed of after sampling. A schematic view of the conducted samplings is shown in Table 2.4.

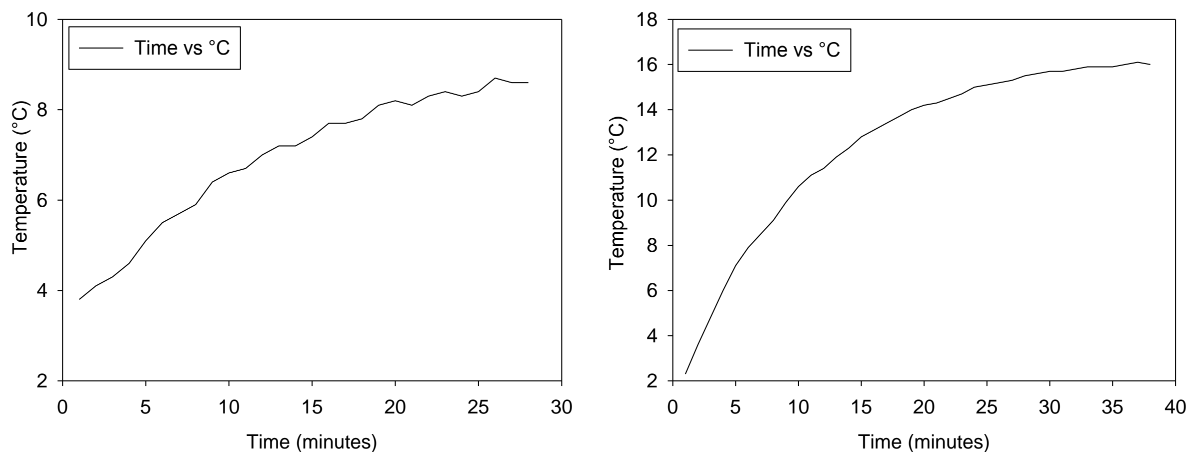

Figure 2.4 Time needed for the pure seawater (2 °C) in the Scintillation vials to reach 9 °C (left) and 17 °C (right) in the preheated incubator cabinets.

Table 2.4 Schematic view of samplings for the effect of short-term temperature elevation on cold stored *A. tonsa* eggs experiment

Duration	Temperature	Seawater quality	Replicates, n=5	Seawater quality	Replicates, n=5
12 h	9°C	Oxic	HS (%), n=4	Anoxic	HS (%), n=4
24 h	9°C	Oxic	HS (%), n=4	Anoxic	HS (%), n=4
12 h	17°C	Oxic	HS (%), n=4	Anoxic	HS (%), n=4
24 h	17°C	Oxic	HS (%), n=4	Anoxic	HS (%), n=4
Control	2°C	Oxic	HS (%), n=4	Anoxic	HS (%), n=4

2.5.3 Viability of hatched nauplii after exposure to short-term elevated temperatures during cold storage

To assess if the nauplii from all *short-term temperature elevation* treatments were viable after hatching, a feeding incidence (frequency of nauplii that had ingested food) experiment was conducted. Eggs from all treatments were transferred to 10 separate plastic beakers with seawater (200 mL, 34 ‰) at a temperature of 20 °C and aeration, and fed *R.baltica* twice a day for three consecutive days. The beakers were then poured into a 64 µm sieve and washed gently with seawater (34 ‰) before the nauplii were transferred to Petri dishes and anaesthetized with tricaine methane sulphonate (MS-222, Ethyl 3-aminobenzoate methanesulfonate, prod.nr. EI0521, Sigma-Aldrich Norway AS). Finally the samples were placed under a stereomicroscope, and the first 50 nauplii encountered were visually examined for traces of algae in the gut before the feeding incidence was determined. The visual confirmation of feed ingestion was for this experiment considered as proof of viability.

2.6 Statistics

All statistical analyses were performed in SigmaPlot® for Windows v.11.0 with a significance level of $\alpha = 0.05$. Percentage data (HS, %) were arcsine transformed in Microsoft Office Excel for Windows (Microsoft Inc.) prior to the analyses. The effects of the treatments were analyzed with one-way ANOVAs, Kruskal-Wallis one-way ANOVAs on ranks or Mann-Whitney rank sum tests. Holm-Sidak multiple comparisons or *t*-tests were used for pairwise comparisons of treatments when significant differences were found (post-hoc). All tables were made in Microsoft Office Excel for Windows (Microsoft Inc.). All graphs were made in SigmaPlot for Windows Version 11.0 (Systat Software, Inc., 2008).

3. Results

In order to describe the pattern of the 48-h hatching success (HS, %) of the *A. tonsa* eggs over time for each treatment, all graphs for the *light* and *salinity* experiments are followed by a trend analysis based on the means of the HS tests (n=18) at each time of sampling (1 - 7.5 months). The coefficient of variation (CV= (SD/ $\overline{X}$)*100) was calculated to illustrate the dispersion between the replicates (n=3); thus the trend data are given as mean ± CV. The reason for the replicates being plotted individually was to provide information about replicate dispersion that a mean of the replicates would not have shown.

3.1 Effect of light

The initial 48-h egg hatching success (HS, %) was 91±7 % (mean ± 95 % CL) for all treatments. Each of the three replicates (A, B and C) have for all samplings been plotted individually to illustrate occasional large variations. The treatments were separated by different light environments; a light treatment where eggs were exposed directly to an artificial light source, a dark treatment which had no light exposure on the eggs, and a standard storage conditions (SSC) treatment where ceiling light and/or daylight to some extent reached the eggs. The eggs were stored at a temperature of 2 °C

3.1.1 Standard storage conditions (SSC) treatment

Figure 3.1 shows the 48-h hatching success (HS, %) of the cold stored *A. tonsa* eggs for the SSC treatment. The results are plotted as means with 95 % confidence limits for replicate A, B and C after 1, 3, 4.5, 6 and 7.5 months of cold storage. The number of eggs in the sub-samples ranged between 49 and 120 (mean = 81).

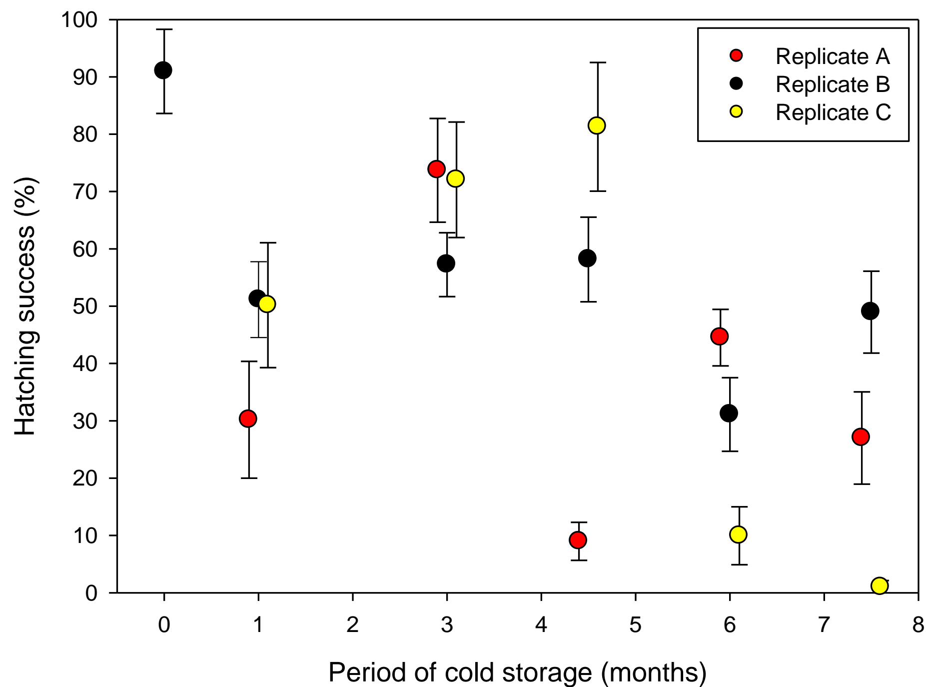

Figure 3.1 Percentage of *A. tonsa* eggs hatched after being stored under standard storage conditions for different periods of time (mean ± 95 % CL).

The mean value of HS for the indicated replicates decreased from 91±7.7 % for the initial sampling to 43.8±29.7 % at sampling of month 1, increased to 67.7±15.9 % from month 1 to month 3, and decreased subsequently to 49.5±64.4 %, 28.5±54.2 % and 25.6±81.6 % at month 4.5, 6 and 7.5, respectively (mean ± CV).

Statistical analyses of the trend showed that the initial HS was significantly higher than the HS of all subsequent samplings ($p < 0.001$; Holm-Sidak). The sampling of month 1 showed a significantly lower HS than the HS of both the initial and month 3 samplings ($p < 0.001$; *t*-test). There was no significant difference in HS between the samplings of month 3 and 4.5 ($p = 0.141$; Mann-Whitney rank sum). The HS decreased significantly from month 4.5 to month

6 ($p = 0.028$; Mann-Whitney rank sum), and remained at the same level from month 6 to month 7.5 ($p = 0.624$; Mann-Whitney rank sum). Because of the large dispersion between the replicates, some additional comparisons between the replicates are elaborated in the following section.

The initial 48-h HS was significantly higher ($p < 0.001$; Holm-Sidak) than the HS of all other replicates. The HS for month 1 samplings were significantly lower than both initial ($p < 0.001$; Holm-Sidak) and 3 month samplings ($p < 0.005$; Holm-Sidak) with two exceptions (3B vs. 1B and 1C; $p = 0.228$ and 0.283; Holm-Sidak). Sampling after 4.5 months showed no correlation for HS between the replicates ($p > 0.05$; Pearson product moment correlation), where replicate 4A was significantly lower than all 3 and 4.5 month replicates ($p < 0.001$; Holm-Sidak) and replicate 4B and 4C lay in both the upper and lower range of the 3 month samples. The HS decreased significantly from 4.5 to 6 months ($p < 0.05$; Holm-Sidak), with the exception of replicate 4.5A which was significantly lower than nearly all 4.5 and 6 months samples (equal to 6C; $p = 0.05$; Holm-Sidak).

3.1.2 Light treatment

Figure 3.2 shows the 48-h hatching success (%) of *A. tonsa* eggs for the light treatment. The results are plotted as means with 95 % confidence limits for replicate A, B and C after 1 and 3 months of cold storage. The number of eggs in the sub-samples ranged between 40 and 92 (mean = 65).

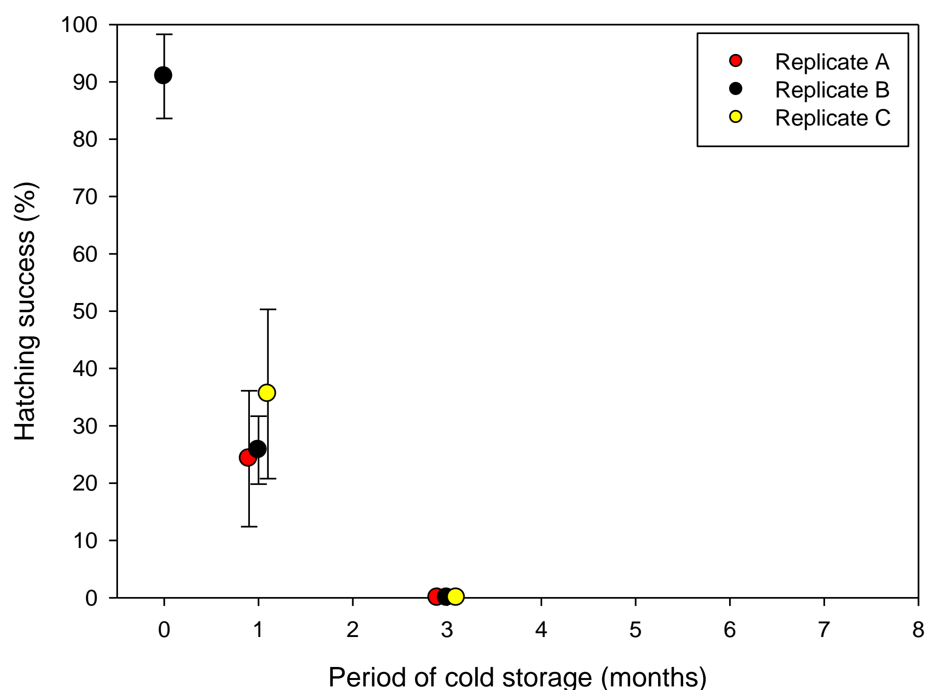

Figure 3.2 Percentage of *A. tonsa* eggs hatched after being stored under constant light at 2 °C for different periods of time (mean ± 95 % CL).

The mean value of HS for the indicated replicates decreased from 91±7.7 % at month 0 to 28.5±40.2 % at month 1 (mean ± CV). No hatching was observed after 3 months and the experiment was terminated.

Statistical analyses showed that the initial HS was significantly higher ($p < 0.001$; *t*-test) than the HS for all subsequent samplings. Replicate A, B and C in samplings of month 1 were equal ($p = 0.179$; one-way ANOVA), significantly lower ($p < 0.001$; Holm-Sidak) than the initial HS and significantly higher samplings of month 3 ($p < 0.001$; Holm-Sidak).

The vials with eggs exposed to light contained a lot of green epigrowth and the eggs showed tendencies of clustering after a few weeks of storage. This was visible to the naked eye on

inspection. Sub-samples examined under a light microscope revealed a great number of ciliates in the storage media, together with the green epigrowth. The contaminants were not subjected to species identification. Figure 3.3 illustrate the tendencies of clustering and epigrowth found when the eggs were stored under constant light, whereas the eggs from the SSC treatment showed a more even distribution without clustering or epigrowth. The conditions illustrated in picture A characterized the situation for all the replicates stored under constant light.

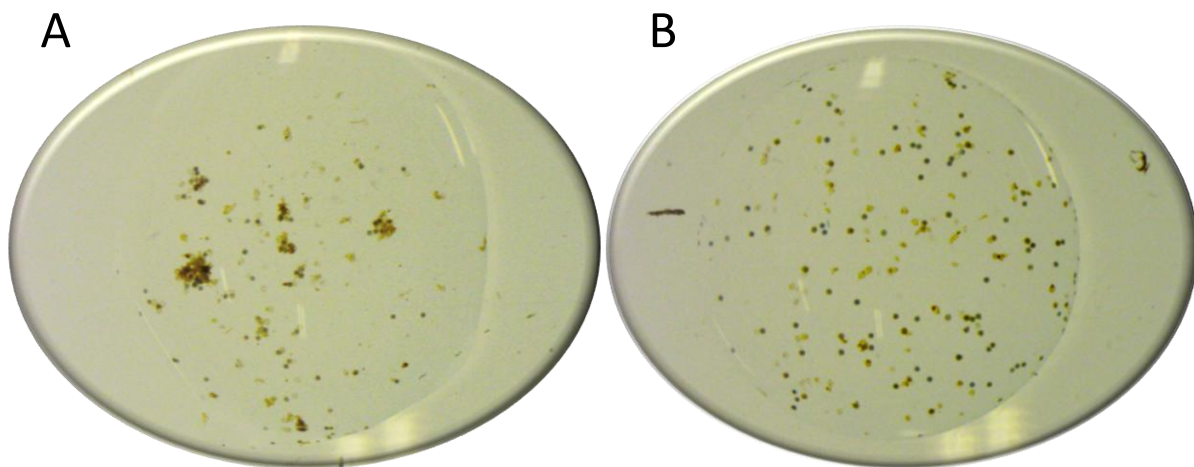

Figure 3.3 Pictures of two samples (50 µL) of *A. tonsa* eggs from the light (A) and SSC (B) treatment after 1 month of cold storage (Photos by Andreas Hagemann).

3.1.3 Dark treatment

Figure 3.4 shows the 48-h HS (%) of the *A. tonsa* eggs in the dark treatment. The results are plotted as means with 95 % confidence limits for replicate A, B and C after 1, 3, 4.5, 6 and 7.5 months of cold storage. The number of eggs in the sub-samples ranged between 42 and 121 (mean = 85).

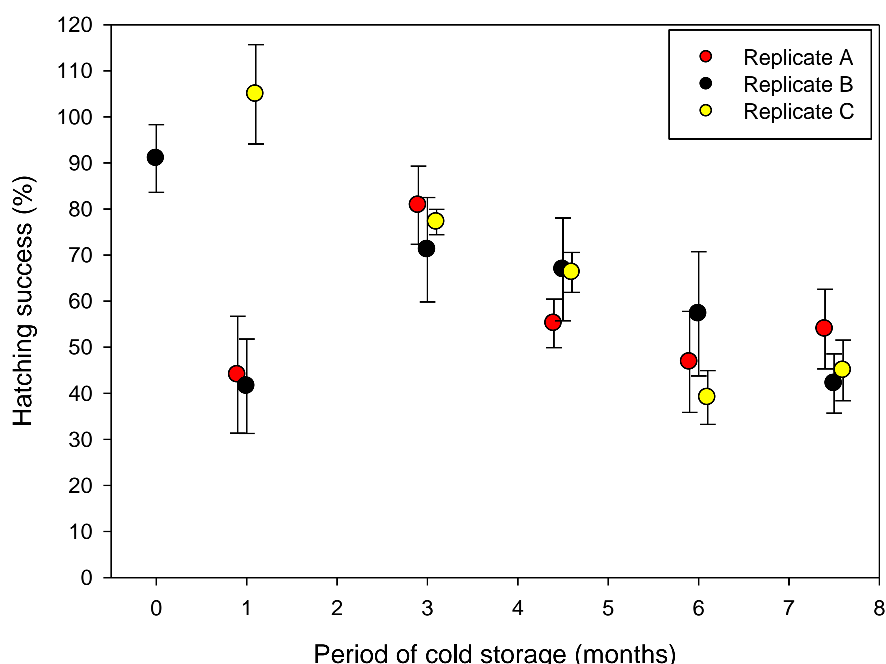

Figure 3.4 Percentage of *A. tonsa* eggs hatched after being stored in darkness at 2 °C for different periods of time (mean ± 95 % CL).

The mean value of HS for the indicated replicates decreased from 91±7.7 % at month 0 to 63.5±50.1 % after 1 month, increased to 76.4±11.1 % after 3 months, and decreased subsequently to 62.8±13.9 % and 47.7±25.5 % after 4.5 and 6 months of storage, respectively. Final sampling showed a HS of 47±17.7 % (mean ± CV).

Statistical analyses of the trend showed that the initial HS was significantly higher than the HS of all subsequent samplings ($p < 0.05$; Holm-Sidak). The HS for samplings of month 1 and 3 were equal ($p = 0.073$; Mann-Whitney rank sum test). From 3 to 4.5 months, the HS dropped significantly ($p < 0.001$; *t*-test), although there were no significant differences in the

HS between the samplings of month 1 and 4.5 ($p > 0.05$; Mann-Whitney rank sum test). The HS dropped significantly from 4.5 to 6 months ($p < 0.001$; *t*-test), and remained at the same level from 6 to 7.5 months of storage ($p = 0.789$; *t*-test). Because of the large dispersion between the 1 month replicates, some additional analyses of comparisons between the replicates are elaborated in the following section.

The initial HS was significantly different ($p < 0.001$; Holm-Sidak) from most other replicates. Although the highest HS was found in replicate 1C, the initial HS was significantly higher than the rest of the replicates and equal to replicate 1C ($p = 0.051$; *t*-test). The replicates for the samplings of month 3 showed a significant increase in HS compared to replicate 1A and 1B ($p < 0.001$; Holm-Sidak). Replicate 1C should be treated as an outlier, as a HS above 100 % is impossible and an indication that a human error occurred during sampling.

3.2 Effect of salinity

The initial 48-h hatching success (HS, %) was 91±7 % (mean ± SD) for all treatments. Each of the three replicates (A, B and C) have for all samplings been plotted individually to illustrate occasional large variations. The control treatment for this experiment was the SSC treatment for the *effect of light* experiment (cf. 3.1). The treatments were different with respect to the salinity of the seawater in the vials. The four treatments had salinities of 34, 50, 75 and 100 ‰, and were stored for a period of up to 7.5 months at a temperature of 2 °C.

3.2.1 Storage of *A. tonsa* eggs in seawater with a salinity of 50 ‰

Figure 3.5 shows the 48-h HS (%) of *A. tonsa* eggs in the hypersaline seawater at 50 ‰ treatment. The results are plotted as means with 95 % confidence limits for replicate A, B and C after 1, 3, 6 and 7.5 months of cold storage. The number of eggs in the sub-samples ranged between 39 and 141 (mean = 83).

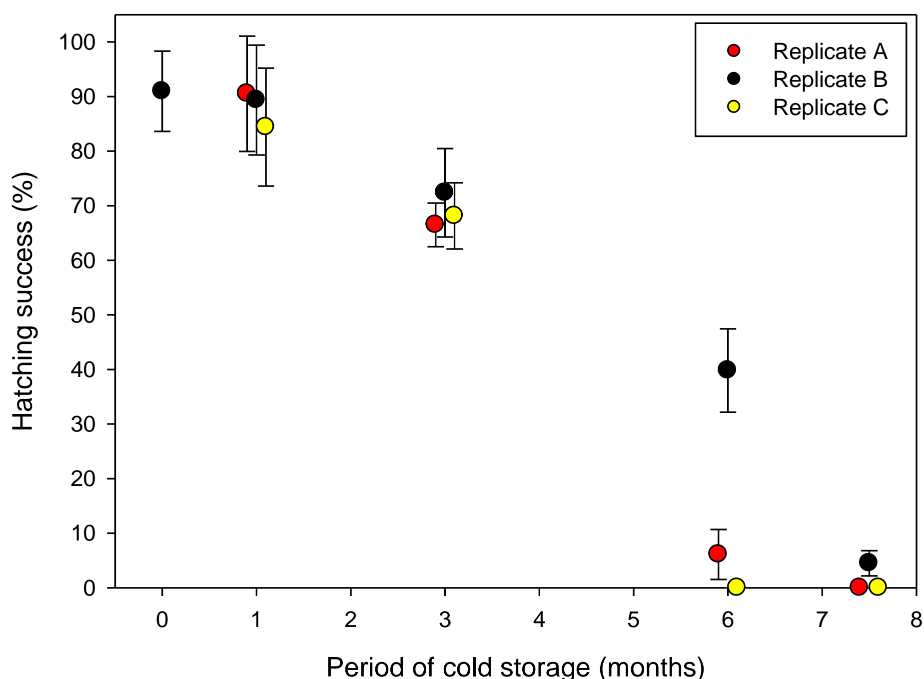

Figure 3.5 Percentage of *A. tonsa* eggs hatched after being stored in seawater with a salinity of 50 ‰ for different periods of time (means ± 95 % CL).

Subsequently, the mean value of HS for the indicated replicates decreased slightly from 91±7.7 % at the initial sampling to 88.1±11.1 % and 69±9 % at samplings of month 1 and 3, respectively. At sampling of month 6, the HS had dropped drastically to 15.3±121.5 %, and decreased further to 1.5±166.1 % after 7.5 months of storage (mean ± CV).

Statistical analyses of the trend showed that the initial HS was equal to the HS of month 1 samplings ($p = 0.504$; Mann-Whitney). There was a significant subsequent decrease in HS from samplings of month 1 to month 3 ($p < 0.001$; Mann-Whitney), month 3 to month 6 ($p < 0.001$; Mann-Whitney) and month 6 to month 7.5 ($p = 0.014$; Mann-Whitney).

Statistical analyses showed no differences in HS between the initial and month 1 replicates ($p = 0.726$; one-way ANOVA). The month 1 replicates were equal to each other ($p = 0.626$ an; one-way ANOVA), as were the 3 month replicates ($p = 0.213$; one-way ANOVA). Samplings of month 7.5 were for the most part equal to the 6 month samplings, except for replicate 6B which proved significantly different from the rest of the 6 and 7.5 month replicates ($p < 0.001$; Holm-Sidak).

3.2.2 Storage of *A. tonsa* eggs in seawater with a salinity of 75 ‰

Figure 3.6 shows the 48-h HS (%) of cold stored *A. tonsa* eggs in the hypersaline seawater (75 ‰) treatment. The results are plotted as means with 95 % confidence limits for replicate A, B and C after 1, 3, 6 and 7.5 months. The number of eggs in the sub-samples ranged between 57 and 110 (mean = 85).

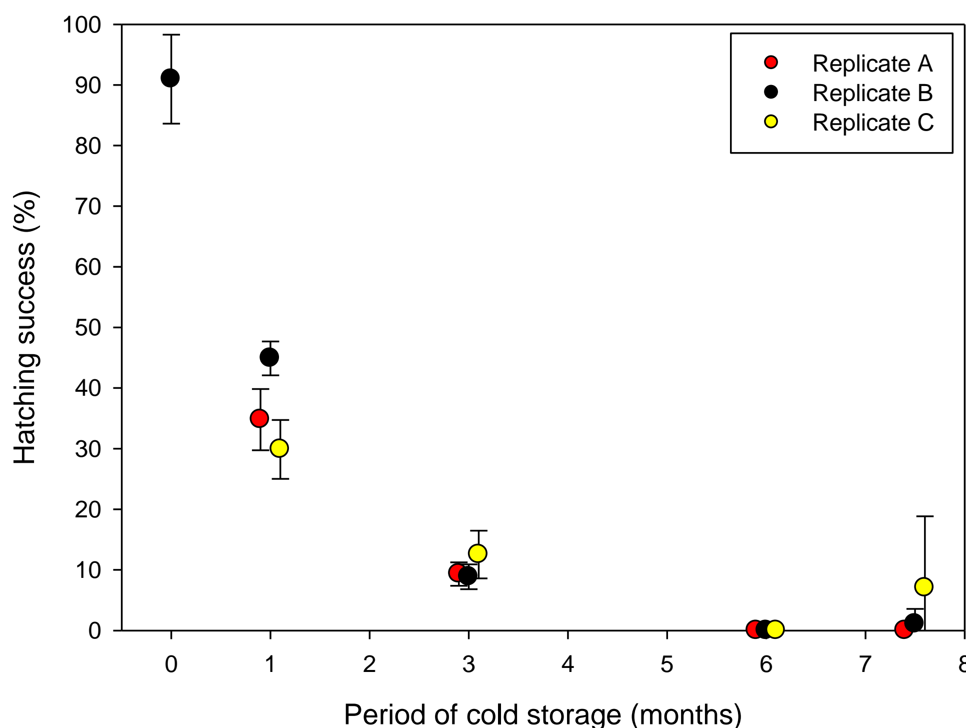

Figure 3.6 Percentage of *A. tonsa* eggs hatched after being stored in seawater with a salinity of 75 ‰ for different periods of time (means ± 95 % CL).

The mean value of HS for the indicated replicates decreased subsequently to 36.5±22 % at samplings of month 1, 10.2±34.5 % at month 3 and 0±0 % at month 6. Samplings of month 7.5 (n=6) showed a HS of 2.7±151.6 % (mean ± CV).

Statistical analyses of the trend showed that the initial HS was significantly higher than the HS of all subsequent samplings ($p < 0.001$; Holm-Sidak). The HS decreased significantly for all samplings from month 1 to month 6 compared to the preceding samplings ($p < 0.001$; Holm-Sidak). The HS of eggs stored for a period of 6 and 7.5 months were equal ($p = 0.416$; Holm-Sidak).

3.2.3 Storage of *A. tonsa* eggs in seawater with a salinity of 100 ‰

Figure 3.7 shows the 48-h HS (%) of *A. tonsa* eggs in the hypersaline seawater (100 ‰) treatment. The results are plotted as means with 95 % confidence limits for replicate A, B and C after 1 and 3 months of cold storage. The number of eggs in the sub-samples ranged between 57 and 99 (mean = 76).

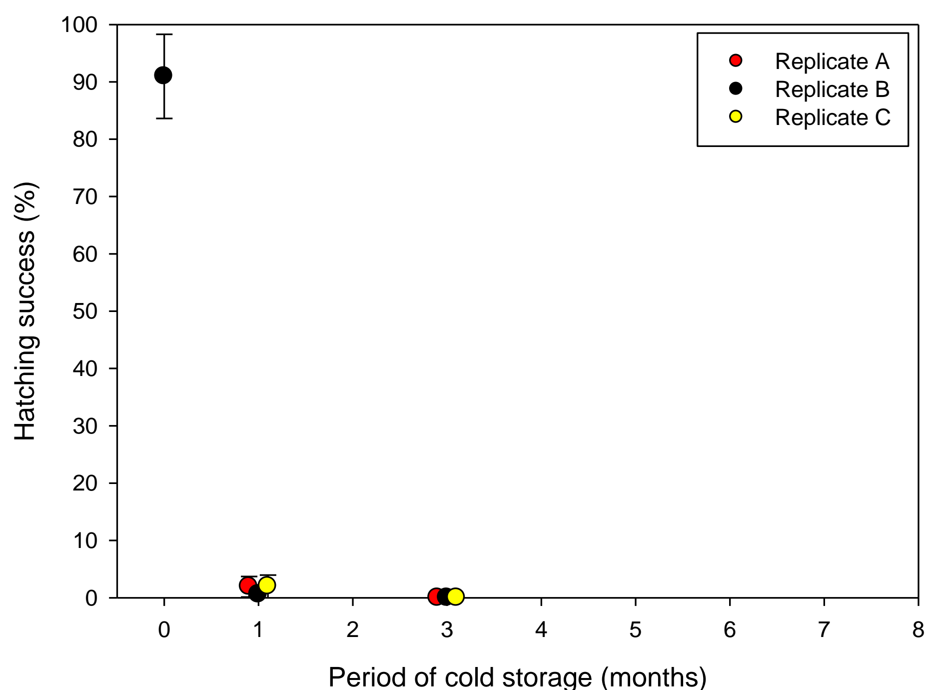

Figure 3.7 Percentage of *A. tonsa* eggs hatched after being stored in seawater with a salinity of 100 ‰ for different periods of time (means ± 95 % CL).

The mean value of HS for the indicated replicates decreased from the initial 91±7.7 % to 1.5±108 % after 1 month of storage (mean ± CV). No hatched nauplii were observed after 3 months, and the experiment was terminated.

Statistical analyses showed that the initial HS was significantly higher than all subsequent samplings ($p < 0.001$; Holm-Sidak). Samplings of month 3 showed a significantly lower HS than samplings of month 1 ($p < 0.001$; Mann-Whitney rank sum).

3.3 Effect of short-term elevation in temperature during cold storage

3.3.1 Effect of short-term temperature elevation on *A. tonsa* eggs stored in oxic seawater

Figure 3.8 shows the 48-h hatching success (HS, %) of the *A. tonsa* eggs in the oxic seawater treatments after exposure to elevated temperatures. The results are plotted as means with 95 % confidence limits. All groups passed normality (p = 0.204) and equal variance (p = 0.869) tests prior to testing for significant differences between the treatments. The number of eggs in the sub-samples (n = 16-20) for all treatments ranged between 111 and 229 (mean = 171).

The HS for the five treatments ranged between 84.4 and 88.1 %. The highest HS was found for the control treatment. Statistical analyses showed that there were no significant differences in HS between the treatments (p = 0.269; one-way ANOVA).

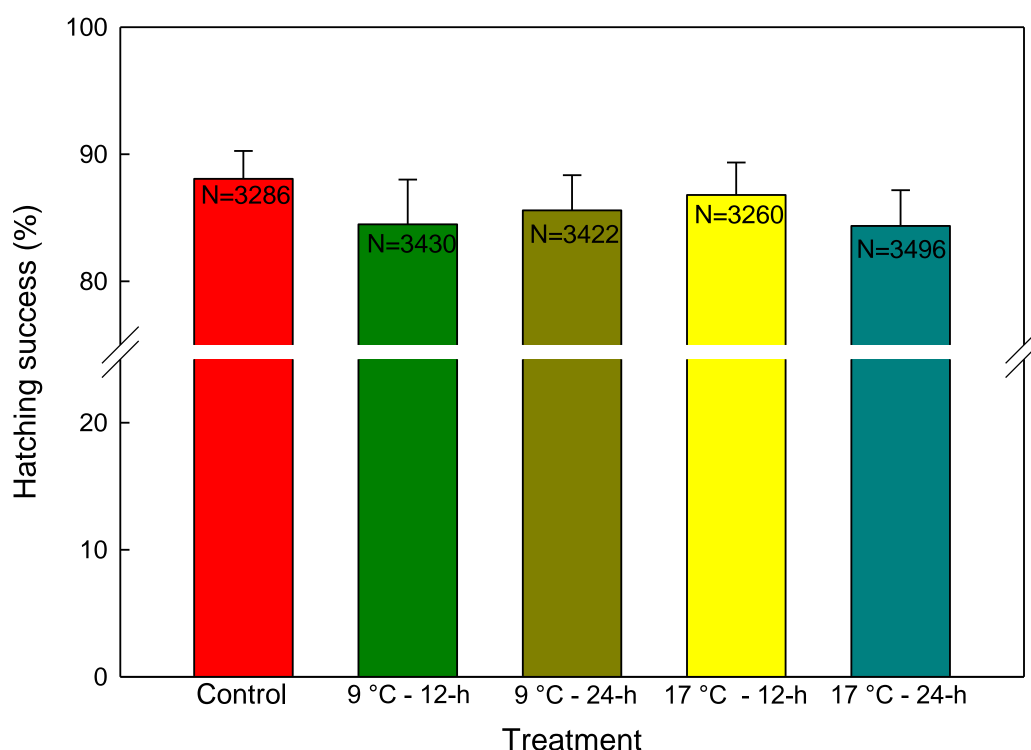

Figure 3.8 Percentage of *A. tonsa* eggs stored in oxic seawater that hatched after the eggs had been exposed to different periods of elevated temperatures (mean ± 95 % CL). N gives the total number of eggs in each treatment.

3.3.2 Effect of short-term temperature elevation on *A. tonsa* eggs stored in anoxic seawater

Figure 3.9 shows the HS (%) of the *A. tonsa* eggs in the anoxic seawater treatments after exposure to elevated temperatures. The results are plotted as means with 95 % confidence limits. All groups passed normality (p = 0.773; Shapiro-Wilk) and equal variance (p = 0.232) tests prior to testing for significant differences between the treatments. The number of eggs in the sub-samples (n = 19-20) for all treatment ranged between 128 and 232 (mean = 170).

The HS for the treatments ranged between 91 and 92.2%. The highest HS was found for the *17 °C – 24-h* treatment. Statistical analyses showed that there were no significant differences in HS between the treatments (p = 0.710; one-way ANOVA).

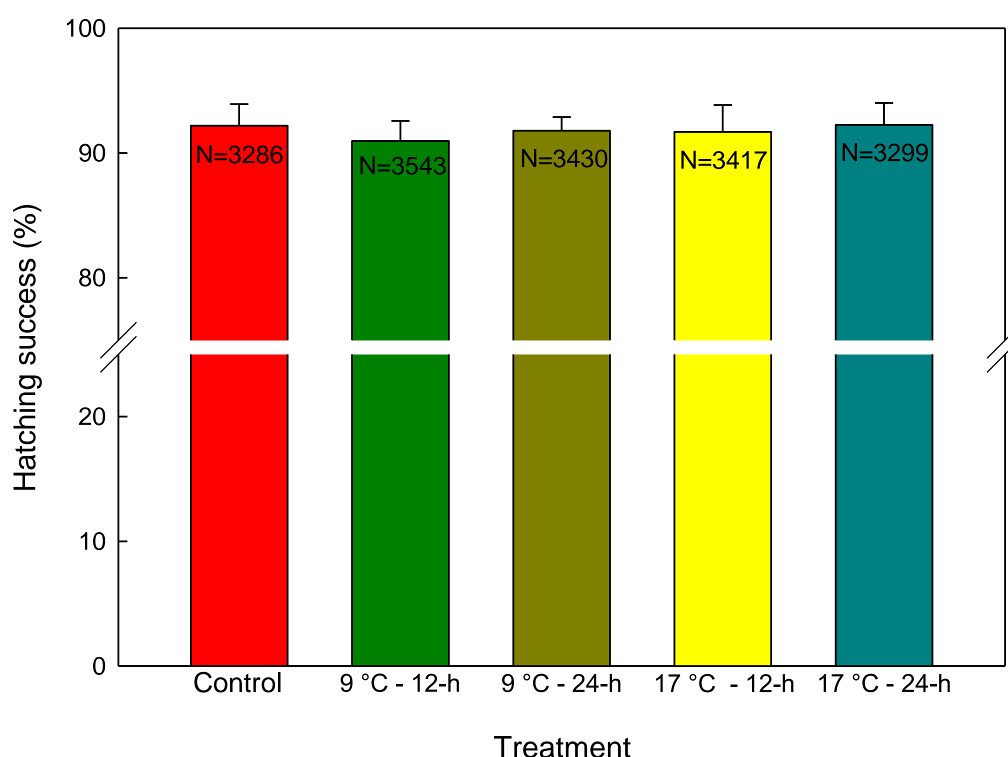

Figure 3.9 Percentage of *A. tonsa* eggs stored in anoxic seawater that hatched after the eggs had been exposed to different periods of elevated temperatures (mean ± 95 % CL). N gives the total number of eggs in each treatment.

3.3.3 Comparison between oxic and anoxic seawater conditions upon exposure to elevated temperatures

Statistical analyses of the HS between the oxic and anoxic seawater treatments were performed. Table 3.1 shows the *p*-values and the method of analysis for all comparisons done between the identical treatments with different seawater qualities.

Table 3.1 P-values and method of analysis for comparisons between identical groups in anoxic and oxic seawater treatments.

Oxic		Anoxic	Method of analysis	P-value	Significance
Control	vs.	Control	*t*-test	0.004	Yes
9 °C – 12-h	vs.	9 °C – 12-h	*t*-test	0.001	Yes
9 °C – 24-h	vs.	9 °C – 24-h	Mann-Whitney Rank Sum test	< 0.001	Yes
17 °C – 12-h	vs.	17 °C – 12-h	*t*-test	0.003	Yes
17 °C – 24-h	vs.	17 °C – 24-h	*t*-test	< 0.001	Yes

Statistical analyses showed that there were significant differences ($p \leq 0.004$) in HS of the *A. tonsa* eggs stored in oxic and anoxic seawater for all treatments. To evaluate the effect of the presence of oxygen during cold storage of *A. tonsa* eggs, a global mean of all treatments with 95 % confidence limits was calculated. The results are shown in Figure 3.10.

The global mean HS and the coefficient of variation for the *A. tonsa* eggs in the oxic (N=16894) and anoxic (N=16975) seawater treatments were 85.9±6.5 % and 91.8±3.9 %, respectively (mean ± CV). Statistical analyses showed that the HS of the *A. tonsa* eggs was significantly higher for the anoxic treatment ($p < 0.001$; *t*-test).

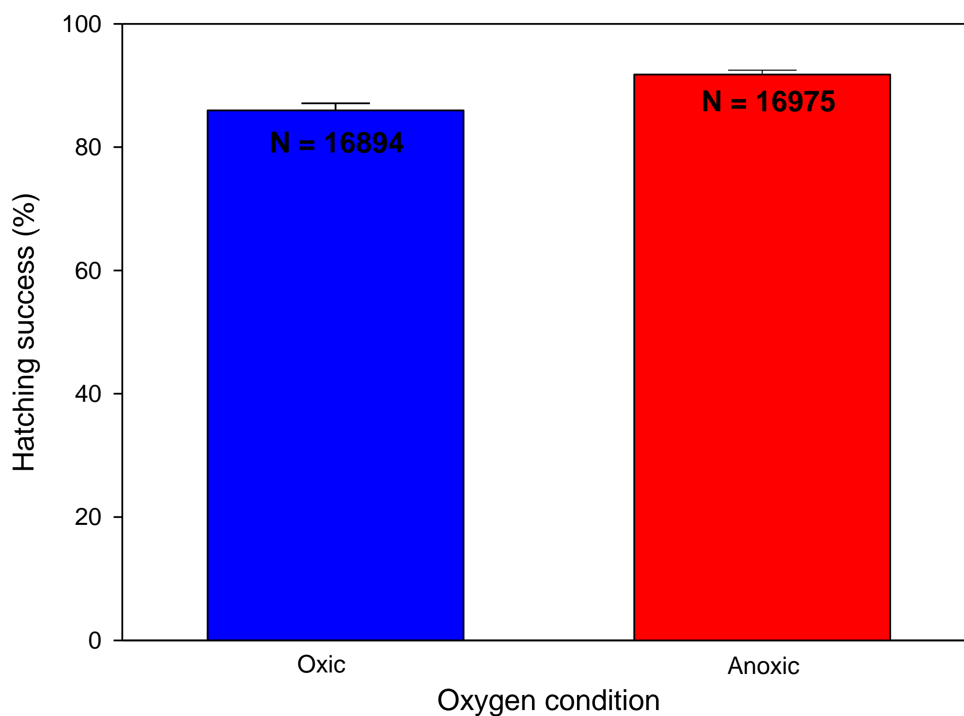

Figure 3.10 Global mean HS for *A. tonsa* eggs stored in anoxic and oxic seawater for a period of approximately 3 weeks (mean ± 95 % CL). N gives the total number of eggs in each treatment.

3.3.4 Impact of short-term elevated temperatures on viability of hatched *A. tonsa* nauplii

The high HS of the *A. tonsa* eggs from both oxic and anoxic seawater treatments showed that they coped well with the short-term elevated temperatures in terms of hatchability. The viability in terms of feeding incidence of the hatched nauplii was investigated, and the results are shown in Figure 3.10.

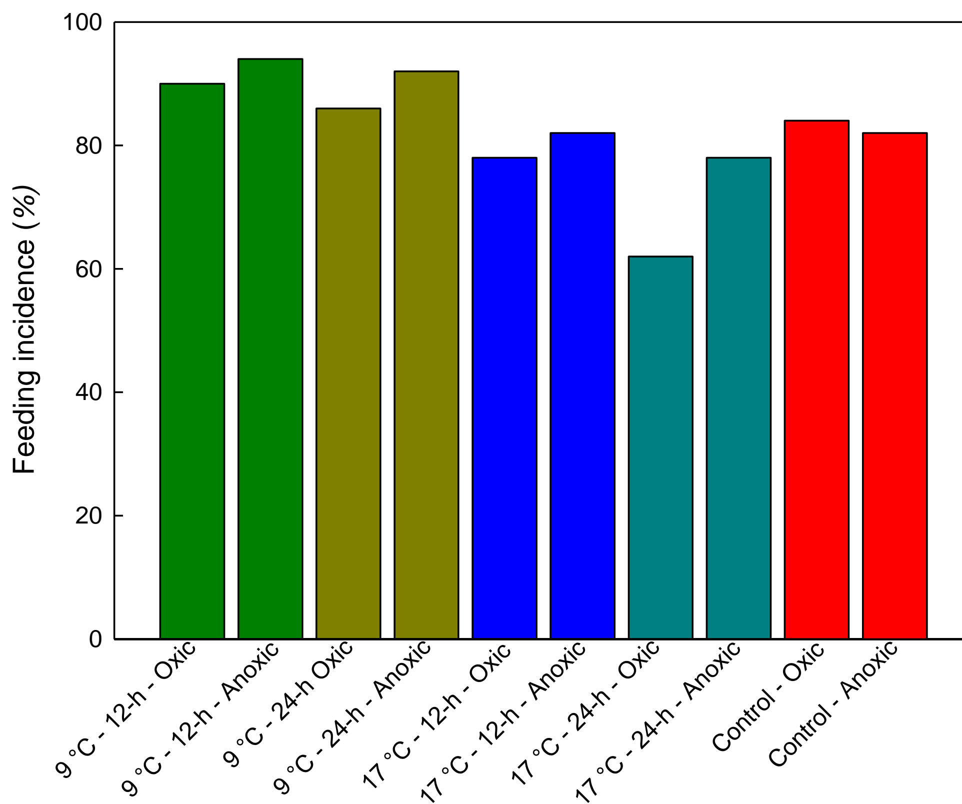

Figure 3.10 Feeding incidence of nauplii (n= 50) from all treatments that had ingested *R. baltica* 3 days post hatching

The lowest feeding incidence was found for the nauplii exposed to the *17 °C - 24-h – Oxic* treatment, where 62 % of the nauplii examined had traces of *R. baltica* in the gut. The numbers might have been higher since investigation through a stereo microscope was insufficient, and it might be difficult to detect minor amounts of algae in the gut or digestive tracts. None of the treatments seemed to utterly inhibit the nauplii from feeding post hatching.

4. Discussion

This thesis describes three different experiments where the effects of light, hypersaline seawater and short-term elevations in temperature on the 48-h hatching success of cold stored *A. tonsa* eggs were assessed. The experiments showed that the eggs were affected by the different light environments, that storing *A. tonsa* eggs in seawater with a salinity of 50 ‰ was beneficial for eggs utilized one month after the onset of cold storage, and that the eggs were not affected by the conducted short-term temperature elevations.

4.1 General remarks

Several studies on optimizing storage of *A. tonsa* eggs for use in aquaculture have been conducted. Drillet et al. (2006a) showed that the eggs could be stored for up to 12 months at 2 – 3 °C in seawater (30 ‰) without affecting the viability of the following generations. Their study showed a hatching rate > 70 % after 11 months of storage under these conditions. However, their procedure for calculating egg hatching success (HS) differed from the method employed in this study, as they counted the exact number of dark pigmented (viable) eggs in each replicate and incubated the eggs for a period of 4 days, before finally counting the unhatched eggs left in the replicate and determine the HS. The HS results presented in this thesis are based on the 48-h hatching success of the *A. tonsa* eggs incubated at room-temperature (22 °C), where the exact number of hatched nauplii was counted and divided by the initial number of eggs in the sample. Thus, there could have been unviable eggs present in the sample prior to the egg incubation. Hansen et al. (2010) showed that most eggs hatch within 48 hours when incubated at temperatures > 17 °C, implying that eggs not hatching within this time window are either unviable, delayed-hatching or diapause eggs.

Delayed-hatching eggs have been described in a few studies (Chen & Marcus, 1997; Onoue et al., 2004), but this type of egg cannot be physiologically distinguished from subitaneous and diapause eggs (Drillet, 2010). Alekseev et al. (2007) ignored this egg type in their paper where they grouped diapause into superpause, mesopause and oligopause according to the

duration of the refractory period. However, an unpublished study presented in a thesis of Drillet (2010) showed that production of delayed hatching eggs was triggered by low food availability and that this type of egg can be stored for many months with a high hatching success, although they hatched slowly under favorable conditions (≤ 1 month). Unhatched eggs in this study were not subjected to a prolonged incubation period to see if hatching occurred at a later time, nor did this study discriminate between viable and unviable eggs prior to testing for HS. The eggs that did not hatch within 48 hours were considered as non-hatching (unviable).

Peck and Holste (2006) showed that the HS is strongly influenced by salinity and photoperiod, both during rearing and egg incubation. Their study suggested salinities of 14 – 20 ‰ and photoperiods between 16 – 20 hours as optimal conditions for both maximum egg production and 48-h HS. Based on a logistic equation, they predicted the HS to decrease linearly by 4 % for every 20 days of storage at 4 °C. As a reference to later comparisons, they predicted a HS of 47 % after six months of cold storage for eggs with an initial HS of 82 % produced using a photoperiod of 12-h and 20 ‰ seawater. A paper by Holmstrup et al. (2006) reported that salinities between 10 and 20 ‰ in combination with temperatures below 5 °C and anoxia were the best storage conditions for *A. tonsa* eggs. The storage conditions used for the experiments in this thesis might be sub-optimal with respect to the salinity (34 ‰) of the storage media according to the two studies mentioned above, but this was considered as unimportant as the effects of the treatments were evaluated entirely by comparing the HS of the treatments up against each other.

Some of the treatments showed a large dispersion between the replicates. This was mainly observed for samplings where the HS was low. Although this could be due to an uneven distribution of different types of eggs, human errors, different microbial environments or different abiotic factors, large dispersions between replicates are commonly observed in studies of fish egg mortality (see 4.3). When samples of organisms are in the process of dying, it is not bound to happen synchronously throughout the replicate for all organisms. Thus, a sampling a few days earlier or later could yield different results and a more central tendency.

The *effect of light* and the *effect of salinity* experiments were conducted with 3 replicates from which six sub-samplings for HS were conducted. These experiments showed a large variation in HS between the replicates, whereas the sub-samples showed less variation in HS. Because of this finding, the *effect of short-term temperature elevation* experiment was adjusted accordingly, as the number of replicates was increased to five, and the number of sub-samplings was reduced to four. Due to minute dispersion between the replicates, the results were presented as a mean of all sub-samplings for the replicates. The number of sub-samples vary slightly (n= 16–20) for the treatments because some of the sub-samplings were handled poorly (e.g. Petri-dish was overturned, spillage) and was therefore dismissed.

The amount of eggs in the sub-samples for the *effect of light* and *effect of salinity* experiments were lower than expected. Preferably there should have been at least 100 eggs in each sampling for HS (personal opinion). This was considered when deciding the density of eggs in the replicates, as all replicates had ~33000 eggs added to a total volume of 8.3 mL, yielding ~4000 eggs/mL and ~4 eggs/µL. Thus, a sample of 50 µL should contain approximately 200 eggs. However, this was not the case as the average number of eggs in the samples from the two experiments ranged between 75 and 85. In comparison, the *short-term temperature elevation* experiment had a slightly lower number of eggs in the vials (~31000 eggs), but a much higher mean number of eggs in the sub-samples (~170 eggs). The low number of eggs could be explained by difficulties with suspending the eggs evenly in the vials upon shaking them, due to clustering of the eggs and the biofilm that forms around the eggs after long periods of storage.

4. 2 Effect of light

No previous studies on the effect of light during cold storage of *A. tonsa* eggs have been found. This study used light of a light quality assumed to be present at depths where *A. tonsa* eggs commonly are found (< 20 meter, see Fig. 2.1). As a consequence, the results of this experiment should therefore be interpreted with respect to this particular light quality.

The *A. tonsa* eggs showed a negative response to light during cold storage. The HS decreased rapidly during the 3 months of storage for the light treatment, and already after one month

the HS was significantly lower than the HS of month 1 samplings for the SSC and dark treatment ($p < 0.026$; Holm-Sidak). A low HS was observed in samplings of month 1 for both the SSC and the dark treatment as well, but there it was followed by an increased HS at samplings of month 3, which was not the case for the light treatment where the HS decreased to zero at sampling of month 3. Zero hatching could very well have occurred after less than 3 months of cold storage, but no samplings between 1 and 3 months were conducted. The eggs from the light treatment replicates were clustered and had a lot of contaminants both on and around them (see Fig 3.3), but aside from that the eggs were dark pigmented and thus looked viable. Empty egg-shells or hatched nauplii were not found in the samples, so the low HS cannot be explained by the eggs already being hatched before sampling for HS. Instead, it is possible that the contaminants in the replicates created a biofilm around the eggs preventing them from hatching, they were unable to hatch simply because of the dense mass enclosing the eggs thus blocking the exit path of the emerging nauplii, or that the eggs died and deteriorated because of predation from ciliates and/or bacteria. These theories are simply speculations, and cannot be validated from the data in this study. Nevertheless, cold storage under this light condition proved to be a bad storage strategy.

The temporary decrease in HS for samplings of month 1 in the SSC and dark treatment (43.8 % vs. 63.5 %, respectively) was peculiar. If we consider replicate 1C as an outlier, as it showed a HS above 100 % which indicates that a sampling error occurred, removing it from the sample yields a mean HS of 46.3 and 42.3 % at month 1 samplings for the dark and SSC treatment, respectively. Thus the mean HS for sampling of month 1 was less than half of the initial HS, and much lower than what should be expected from Peck and Holste`s equation (Peck & Holste, 2006). The increased HS observed for samplings of month 3 (76.4 and 67.7 % for dark and SSC treatment, respectively) was significantly higher than the HS for month 1 samplings for both treatments when replicate 1C (outlier) from the dark treatment was removed from the sample ($p < 0.001$; *t*-test). This pattern of a temporary decreased HS between month 0 and 3 during cold storage has not been described in any other studies. This drop could be due to several reasons that can`t be experimentally proven in this study, although some speculations are elaborated below.

A. tonsa have been reported to produce both subitaneous and diapause eggs (Chen & Marcus, 1997; Castro-Longoria, 2001) in different ratios depending on the environmental conditions. Diapause eggs need to go through a refractory phase for a predetermined period of time before they can resume normal development and hatch (Marcus, 1996), so if diapause eggs constitute a large share of the egg sample and only the quiescent subitaneous eggs are hatchable after one month of cold storage, then the HS will reflect this. If that is the case however, the amount of diapause eggs should be equal for all replicates assuming simple random sampling. Thus, if the increased HS for sampling of month 3 is explained by the contribution of hatched diapause eggs, then this should also be apparent in the initial HS test. In this experiment, the initial HS was 91 %, so if the remaining 9 percent are diapause eggs it is still hard to argue for the HS of 43 and 63 % for samplings of month 1 in the SSC and dark treatment. Also, because the mechanism behind the termination of the refractory phase of diapause eggs is poorly documented, there is no scientific ground for claiming that diapause was broken at samplings of month 3. From an ecological perspective however, as the duration of diapause is genetically controlled and reflects the natural environment of the species, an obligate refractory period of 3 months could be long enough to overcome the harshest winter months in the Danish waters; thus diapause eggs could have contributed to an increased HS after 3 months of storage.

Delayed hatching eggs can constitute a large share of the total egg sample, especially when food availability is suboptimal during the copepod rearing (Drillet, 2010). Drillet (2010) showed that delayed-hatching eggs hatched up to a month later than the subitaneous eggs. If this type of egg can hatch within 48-h after spending time in cold storage, the increased HS at samplings of month 3 could be due to the contribution of the delayed-hatching eggs readily hatching. Although the delayed-hatching eggs might not enter quiescence, they will never the less be affected by the low temperatures in terms of a lowered metabolic rate which could further extend the delayed hatching duration. Thus, if eggs meant to hatch after a month *in situ* are stored at 2 °C, the hatching of the eggs could be set back a month or two due to the lowered metabolism, then delayed-hatching eggs could have contributed to an increased HS after 3 months.

Nevertheless, such an apparent fluctuation of HS between month 0 and 3 of cold storage have not been reported in other studies, where a steady decline in HS over time have been

the foreseen trend. The eggs from the *short-term temperature elevation* experiment (see below) did not show a low HS after a cold storage period of approximately 3 weeks. These two experiments however, used eggs produced at different times. Therefore they could have differed with respect to different shares of subitaneous, delayed-hatching and diapause eggs in the main sample. Also, an extended cold storage period (of ≤ 2 weeks) to 1 month might have shown the same trend for HS as the light and dark treatments.

Comparing the mean HS for the samplings of month 7.5 from the SSC and dark treatment (25.6 % vs. 47 %, respectively), the best cold storage condition seems to be for the dark treatment. In addition to a higher HS by the end of the experiment, the dark treatment showed less dispersion between the replicates and a higher mean HS throughout most samplings (the HS for samplings of month 4.5 were equal for the two treatments, $p = 0.438$; Mann-Whitney rank sum). This indicates that light should be excluded from the storage environment. However, the HS of the SSC treatment could have been influenced by the light source from the light treatment. Although precautions were taken when the light source was installed to ensure that this light was not spread throughout the refrigerator, there were some occurring instances of the light source, or the box assembled to stop the light from spreading, being moved during the experimental period. If the setup was not reset properly after the distortion, the light could spread beyond the targeted eggs and affect the SSC treatment replicates. At the last sampling for the SSC treatment, a HS close to zero was found for all replicates. These replicates were the ones placed closest to the light source. Due to suspicions that the replicates had been affected by the light source, a new sampling for HS was conducted, only with another set of replicates (the experiment had some extra replicates in case of instances like this occurring) placed further away from the light source. This sampling showed a mean HS of 25.6 %, and is what was as results. Thus, the light source could have affected all SSC treatment replicates throughout the experiment, but most likely only after the light source had been moved somewhere between month 4 and 5. If that was the case, then the large dispersion between the replicates from month 4.5 to month 7.5 could be explained by different exposures to the light. This is plausible as the light treatment clearly showed that the *A.tonsa* eggs were negatively affected by this light exposure.

4.3 Effect of hypersaline seawater

Seawater with a salinity of 50 ‰ proved as a good cold storage media for periods up to 3 months. In a study by Holmstrup et al. (2006) it was found that storage of *A. tonsa* eggs in anoxic seawater (2 °C) with a salinity of 50 ‰ gave a good HS for up to 9 weeks of storage (HS of ~40 %.), but a poor HS after 9 weeks. Their study differed from the experiments performed for this thesis which used fresh eggs for the experiments, whereas their study used eggs that had been stored under fully oxygenated conditions for 9 weeks before the onset of the experiment. This study showed a much higher HS for samplings up to month 3 (88 and 69 % for month 1 and 3 samplings, respectively) than their study, but this was probably due to fresher eggs and anoxic storage conditions throughout the experiment.

The 50 ‰ treatment showed an unchanged HS for sampling of month 1 compared to the initial HS, which is remarkable compared to the dark and SSC treatments which showed a significant decrease in HS at the same time of sampling. The reason for this is not known, but one could speculate if this has something to do with a microbial factor as the high salinity might prevent nonhalotolerant, halotolerant and mild- and moderate halophile microorganisms, in addition to ciliates and algae, from growing thus yielding a better storage environment. Bacterial cells have been shown to colonize egg surfaces in excessive amounts, and are thought to reduce the exchange of gas and metabolic waste across the chorion (Salvesen & Vadstein, 1995). A publication by Salvesen et al. (1997) showed that surface disinfection of Atlantic halibut (*Hippoglossus hippoglossus* L.) and turbot (*Scophthalmus maximus* L.) eggs had a positive effect on viability of the yolk-sac larvae and the egg hatching success. Eggs treated with optimal doses of disinfectants lead to a higher amount of eggs hatching, a reduced hatching time and a more synchronous hatching compared to untreated eggs (Salvesen et al., 1997). Their study showed that bacterial factors could highly affect egg viability, and should therefore be of concern when storing eggs of *A. tonsa* as well. It could also be that the refractory period of diapause and delayed-hatching eggs is shortened as a consequence of a harsher environment, yielding eggs that are ready to hatch after one month of storage in 50 ‰ seawater. Nevertheless, as no microorganisms were identified and the termination of embryonic diapause (competent phase) for *A. tonsa* is poorly documented, conclusion on this matter cannot be made. However, the results clearly

showed that storing of eggs in seawater of 50 ‰ is a good strategy if the intention is to use the eggs shortly after production, compared to all other treatments for the *light* and *salinity* experiments.

Both the 75 ‰ and 100 ‰ seawater treatments proved as insufficient storage media for longer periods of time, where the HS over time was poor for most samplings. The exception to this was the sampling of month 1 for the 75 ‰ treatment, which was equal to samplings of month 1 for both the dark and SSC treatment (p = 0.098; one-way ANOVA). The osmotic effect is high at these salinities, which in turn might cause the eggs to dehydrate and die. High salinity seawater can draw water out of the eggs; the lower the water activity (a_w) the bigger the water loss. The only way for organisms to overcome a low water activity environment is to increase the internal solute concentrations, which the eggs are probably incapable of, although compatible solutes preventing water loss might be present. The fact that some eggs were able to hatch after 7.5 months of cold storage in 75 ‰ seawater demonstrates the resilience these organisms exert, although the few hatched nauplii might have emerged from diapause eggs which because of its thicker egg-shell (chorion) probably have a broader salinity tolerance than subitaneous eggs.

4.4 Effect of short-term elevations in temperature

This experiment was conducted in order to simulate transport conditions for consignments of eggs during shipping. The aim was to assess the impact of insufficient cooling of the eggs during transportation, ascertaining the importance of proper shipping conditions with respect to cooling.

The 48-h hatching success of the *A. tonsa* eggs was not significantly affected by any of the conducted temperature elevations. It was assumed, at least for the oxic treatments, that the eggs would hatch during the 24-h incubation, but since light is a key element for the eggs to hatch, the absence of light during the incubation probably hindered the eggs from doing so (Peck et al., 2008). The assessment of the feeding incidence of the hatched nauplii post incubation showed that neither of the treatments hindered the nauplii from molting into the first feeding naupliar stage or from feeding. Thus, as long as light is excluded from the

storage environment, short periods of elevated temperatures of up to 17 °C for 24 hours, should not be a reason of concern with respect to the viability of either the eggs (hatching) or the hatched nauplii (feeding). However, the temporary increase in temperature level might increase the embryo`s metabolic rate leading to a more rapid consumption of their energy source. Thus, since the embryo is still metabolically active during quiescence, the period of storage with a maintained viability could be shortened after the temperature elevation.

The seawater used in the oxic treatment was not aerated before use, thus yielding sub-oxic 2 °C seawater with 6.66 mg O_2 L^{-1}. Aerating the seawater prior to the experiment was considered unnecessary, as it within aquacultural contexts only would be higher oxygen concentrations in the storage media for freshly harvested eggs stored in oxic seawater. Even then, the media will turn anoxic within a short period of time, thus situations with oxygen concentrations higher than what was used in this experiment will seldom, and should not, occur. Eggs should be stored in anoxic seawater immediately after harvest.

For the survival experiment (see 3.3.4), some additional information about the experimental circumstances should be elaborated. The nauplii were fed only twice a day for three days straight, and the quality of the algae was sub-optimal as they were acquired from the bottom of the feeding tank for the copepod culture. The algae sedimented rapidly and thus became unavailable to the nauplii. It is therefore thought that more stable food availability and higher quality algae could have resulted in a higher percentage of feeding nauplii. Nevertheless, the experiment proved that the nauplii were able to molt into the first feeding developmental stage in spite of the temperature impact, and most of the examined nauplii were able to feed, further confirming that the cold stored eggs were not negatively affected by the conducted temperature elevations.

During the experimental period of 3 weeks, it was shown that the HS was significantly affected by the oxygen concentration of the seawater during cold storage. *A. tonsa* eggs stored in anoxic seawater showed a significantly higher HS than the eggs stored in oxic seawater, both for the isolated treatments and the global mean HS for the two oxygen conditions.

5. Conclusions and future perspectives

Based on the results of this study the following conclusions can be made;

- Light should be excluded from the cold storage environment.
- Cold stored *A. tonsa* eggs are not affected by short-term elevations in temperature up to 17 °C for 24 hours when light is absent, not even if oxygen is present in concentrations up to 6.66 mg O_2 L^{-1}.
- Cold storage of *A. tonsa* eggs in seawater with a salinity of 50 ‰ can be beneficial if the intention is to use the eggs ~ 1 - 3 months after the onset of cold storage.
- Cold storage of *A. tonsa* eggs in anoxic seawater yields a significantly higher 48-h HS than storage in oxic seawater after a period of 3 weeks.
- *A. tonsa* eggs stored in seawater of 34 ‰ should not be used for live feed purposes after exactly 1 month of cold storage, as this could result in a poor 48-h HS.
- *A. tonsa* eggs do not hatch under favorable temperature and oxygen conditions when light is absent.

Future perspectives;

- Cold storage experiments with surface disinfected eggs of *A. tonsa* should be conducted to evaluate the impact of microorganisms on egg viability.
- The implication and mechanisms of delayed-hatching eggs in *A. tonsa* cultures should be further investigated.
- A simple methodological procedure for determining the share of different types of eggs produced in a copepod culture should be developed, both to predict the HS more accurately, and as a means of evaluating the state of the culture.
- More detailed studies of HS between 0 and 3 months under similar storage conditions should be investigated to validate the HS pattern found in this study. The sampling for HS should be adjusted to an extended period of hatching to assess the amount of delayed-hatching eggs in the sample.

6. References

Alekseev, V., Ravera, O., Stasio, B., 2007. Introduction to Diapause. in: Alekseev, V.,Stasio, B.,Gilbert, J. (Eds.), Diapause in Aquatic Invertebrates Theory and Human Use. Springer Netherlands, pp. 3-10.

Baker, E.P., Alves, D., Bengtson, D.A., 1998. Effects of Rotifer and Artemia Fatty-Acid Enrichment on Survival, Growth and Pigmentation of Summer Flounder Paralichthys dentatus Larvae. Journal of the World Aquaculture Society. 29, 494-498.

Balon, E., 1986. Types of feeding in the ontogeny of fishes and the life-history model. Environmental Biology of Fishes. 16, 11-24.

Balon, E., 1999. Alternative Ways to Become a Juvenile or a Definitive Phenotype (and on Some Persisting Linguistic Offenses). Environmental Biology of Fishes. 56, 17-38.

Bell, J.G., McEvoy, L.A., Estevez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture. 227, 211-220.

Bendiksen, E.Å., Johnsen, C.A., Olsen, H.J., Jobling, M., 2011. Sustainable aquafeeds: Progress towards reduced reliance upon marine ingredients in diets for farmed Atlantic salmon (Salmo salar L.). Aquaculture. 314, 132-139.

Castro-Longoria, E., 2001. Comparative observations on the external morphology of subitaneous and diapause eggs of Acartia species from Southampton Water. Crustaceana. 74, 225-236.

Cervetto, G., Gaudy, R., Pagano, M., 1999. Influence of salinity on the distribution of Acartia tonsa (Copepoda, Calanoida). Journal of Experimental Marine Biology and Ecology. 239, 33-45.

Chen, D.S., Dykhuizen, G.V., Hodge, J., Gilly, W.F., 1996. Ontogeny of Copepod Predation in Juvenile Squid (Loligo opalescens). The Biological Bulletin, Vol 190, Issue 1 69-81, Copyright © 1996 by Marine Biological Laboratory

Chen, F., Marcus, N.H., 1997. Subitaneous, diapause, and delayed-hatching eggs of planktonic copepods from the northern Gulf of Mexico: morphology and hatching success. Marine Biology. 127, 587-597.

Conceição, L.E.C., Yúfera, M., Makridis, P., Morais, S., Dinis, M.T., 2010. Live feeds for early stages of fish rearing. Aquaculture Research. 41, 613-640.

deYoung, B., Heath, M., Werner, F., Chai, F., Megrey, B., Monfray, P., 2004. Challenges of Modeling Ocean Basin Ecosystems. Science. 304, 1463-1466.

Drillet, G., Iversen, M.H., Sørensen, T.F., Ramløv, H., Lund, T., Hansen, B.W., 2006a. Effect of cold storage upon eggs of a calanoid copepod, Acartia tonsa (Dana) and their offspring. Aquaculture. 254, 714-729.

Drillet, G., Jørgensen, N.O.G., Sørensen, T.F., Ramløv, H., Hansen, B.W., 2006b. Biochemical and technical observations supporting the use of copepods as live feed organisms in marine larviculture. Aquaculture Research. 37, 756-772.

Drillet, G., 2010. Copepods and their resting eggs, a potential source of nauplii for aquaculture. Ph.D. Thesis. Department of Environmental, Social and Spatial Changes,. Roskilde University, Denmark, 169.

Drillet, G., Frouël, S., Sichlau, M.H., Jepsen, P.M., Højgaard, J.K., Joarder, A.K., Hansen, B.W., 2011. Status and recommendations on marine copepod cultivation for use as live feed. Aquaculture. 315, 155-166.

Evjemo, J.O., Olsen, Y., 1997. Lipid and fatty acid content in cultivated live feed organisms compared to marine copepods. Hydrobiologia. 358, 159-162.

Evjemo, J.O., Reitan, K.I., Olsen, Y., 2003. Copepods as live food organisms in the larval rearing of halibut larvae (Hippoglossus hippoglossus L.) with special emphasis on the nutritional value. Aquaculture. 227, 191-210.

FAO, 2010. The State of World Fisheries and Aquaculture. Rome 2010. FAO Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations. 197.

Fyhn, H.J., 1989. First feeding of marine fish larvae: Are free amino acids the source of energy? Aquaculture. 80, 111-120.

Govoni, J., Boehlert, G., Watanabe, Y., 1986. The physiology of digestion in fish larvae. Environmental Biology of Fishes. 16, 59-77.

Hansen, B.W., Drillet, G., kozmér, A., Madsen, K.V., Pedersen, M.F., Sørensen, T.F., 2010. Temperature effects on copepod egg hatching: does acclimatization matter? Journal of Plankton Research. 32, 305-315.

Harada, T., 1970. The present status of marine fish cultivation research in Japan. Helgoland Marine Research. 20, 594-601.

Herbing, I.H.v., 2001. Development of feeding structures in larval fish with different life histories: winter flounder and Atlantic cod. Journal of Fish Biology. 59, 767-782.

Hoff, F.H., Snell, T.W., 1987. Plankton culture manual. Florida Aqua Farms, Inc, 126 pp.

Holmstrup, M., Overgaard, J., Sørensen, T.F., Drillet, G., Hansen, B.W., Ramløv, H., Engell-Sørensen, K., 2006. Influence of storage conditions on viability of quiescent copepod eggs (Acartia tonsa Dana): effects of temperature, salinity and anoxia. Aquaculture Research. 37, 625-631.

Holt, G.J., 2008. Research on Culturing the Early Life Stages of Marine Ornamental Fish. Blackwell Publishing Company.

Humes, A.G., 1994. How many copepods? Hydrobiologia. 292-293, 1-7.

Højgaard, J.K., Jepsen, P.M., Hansen, B.W., 2008. Salinity-induced quiescence in eggs of the calanoid copepod Acartia tonsa (Dana): a simple method for egg storage. Aquaculture Research. 39, 828-836.

Imsland, A.K., Foss, A., Koedijk, R., Folkvord, A., Stefansson, S.O., Jonassen, T.M., 2006. Short- and long-term differences in growth, feed conversion efficiency and deformities in juvenile Atlantic cod (Gadus morhua) startfed on rotifers or zooplankton. Aquaculture Research. 37, 1015-1027.

Karalazos, V., Bendiksen, E.Å., Bell, J.G., 2011. Interactive effects of dietary protein/lipid level and oil source on growth, feed utilisation and nutrient and fatty acid digestibility of Atlantic salmon. Aquaculture. 311, 193-200.

Laudien, H., 1973. “Resting Stages in Development and Their Induction or Termination by the Effect of Temperature,” in Temperature and Life (Springer Verlag, 1973), pp. 390–399.

Lavens, P., Sorgeloos, P., 1996. Manual on the Production and Use of Live Food forAquaculture. FAO Technical Paper, No.361, Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, . 295.

Marcus, N.H., 1996. Ecological and evolutionary significance of resting eggs in marine copepods: past, present, and future studies. Hydrobiologia. 320, 141-152.

Marcus, N.H., Wilcox, Jeffrey A., 2007. Guide to the meso-scale production of the copepod Acartia tonsa. Technical publication - Florida Sea Grant College Program. 156, 29.

Mauchline, J., 1998. The Biology of Calanoid Copepods: The Biology of Calanoid Copepods (Advances in Marine Biology, Vol. 33) Elsevier Academic Press, NewYork. pp 710.

McEvoy, L.A., Naess, T., Bell, J.G., Lie, Ø., 1998. Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (Hippoglossus hippoglossus) fed enriched Artemia: a comparison with fry fed wild copepods. Aquaculture. 163, 237-250.

Ohs, C.L., Rhyne, A.L., Stenn, E., 2009. Viability of subitaneous eggs of the copepod, Acartia tonsa (Dana), following exposure to various cryoprotectants and hypersaline water. Aquaculture. 287, 114-119.

Olivotto, I., Buttino, I., Borroni, M., Piccinetti, C.C., Malzone, M.G., Carnevali, O., 2008. The use of the Mediterranean calanoid copepod Centropages typicus in Yellowtail clownfish (Amphiprion clarkii) larviculture. Aquaculture. 284, 211-216.

Onoue, Y., Toda, T., Ban, S., 2004. Morphological Features and Hatching Patterns of Eggs in Acartia Steueri (Crustacea, Copepoda) from Sagami Bay, Japan. Hydrobiologia. 511, 17-25.

Overrein, I., 2010. Copepod lipids in aquaculture, Doctorial Thesis. Department of Biotechnology, NTNU.

Peck, M.A., Holste, L., 2006. Effects of salinity, photoperiod and adult stocking density on egg production and egg hatching success in Acartia tonsa (Calanoida: Copepoda): Optimizing intensive cultures. Aquaculture. 255, 341-350.

Peck, M.A., Ewest, B., Holste, L., Kanstinger, P., Martin, M., 2008. Impacts of light regime on egg harvests and 48-h egg hatching success of Acartia tonsa (Copepoda: Calanoida) within intensive culture. Aquaculture. 275, 102-107.

Rainuzzo, J.R., Reitan, K.I., Olsen, Y., 1994. Effect of short- and long-term lipid enrichment on total lipids, lipid class and fatty acid composition in rotifers. Aquaculture International. 2, 19-32.

Rønnestad, I., Thorsen, A., Finn, R.N., 1999. Fish larval nutrition: a review of recent advances in the roles of amino acids. Aquaculture. 177, 201-216.

Salvesen, I., Vadstein, O., 1995. Surface disinfection of eggs from marine fish: evaluation of four chemicals. Aquaculture International. 3, 155-171.

Salvesen, I., Øie, G., Vadstein, O., 1997. Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: evaluation of concentrations and contact times. Aquaculture International. 5, 249-258.

Schipp, G.R., Bosmans, J.M.P., Marshall, A.J., 1999. A method for hatchery culture of tropical calanoid copepods, Acartia spp. Aquaculture. 174, 81-88.

Stottrup, J.G., McEvoi, L.A.E., 2003. Live Feeds in Marine Aquaculture. Blackwell Science,Oxford.

Støttrup, J.G., Norsker, N.H., 1997. Production and use of copepods in marine fish larviculture. Aquaculture. 155, 231-247.

Støttrup, J.G., 2000. The elusive copepods: their production and suitability in marine aquaculture. Aquaculture Research. 31, 703-711.

Støttrup, J.G., 2007. Production and Nutritional Value of Copepods, Live Feeds in Marine Aquaculture. Blackwell Science Ltd, pp. 145-205.

Sun, B., Fleeger, J.W., 1995. Sustained mass culture of Amphiascoides atopus a marine harpacticoid copepod in a recirculating system. Aquaculture. 136, 313-321.

Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture. 285, 146-158.

Uye, S., Fleminger, A., 1976. Effects of various environmental factors on egg development of several species of Acartia in Southern California. Marine Biology. 38, 253-262.

Uye, S., 1985. Resting egg production as a life history strategy of marine planktonic copepods. Bulletin of Marine Science. 37, 440-449.

van der Meeren, T., Naas, K.E., 1997. Development of rearing techniques using large enclosed ecosystems in the mass production of marine fish fry. Reviews in Fisheries Science. 5, 367 - 390.

van der Meeren, T., Olsen, R.E., Hamre, K., Fyhn, H.J., 2008. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture. 274, 375-397.

Walne, P.R., 1974. Culture of Bivalve Mollusks: 50 Years Experience at Conwy. Fishing News Books, 189 pp.