Copepods and their resting eggs, a potential source of nauplii for aquaculture

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There are no such things as applied sciences, only applications of science.

Louis Pasteur (1822 - 1895)

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Abstract

English

Copepods are the most numerous metazoans on earth and can represent up to 80% of the zooplankton in the ocean, becoming the natural food sources for many fish larvae. Copepods are adapted to fluctuating environment, with some producing resting eggs that can survive decades in the sediment before hatching and returning to the water column. Understanding the processes behind this resting strategy does not only give insights about the ecological implications of such stages, but it also raises the possibility to store the best live prey for feeding by the early stages of fish larvae, cephalopods and other aquatic animals. Using copepods as a live prey could have an important effect in the aquaculture industry where new species could be reared through their entire life cycle and it could also be used as a tool to decrease the pressure in sensitive environments such as coral reefs, where fish are increasingly being removed to feed the aquarium trade with live individuals. During my PhD I mainly studied the resting eggs potentials from different populations of the copepod *Acartia tonsa* and I evaluated its cultivation potential from a zoo-technical to a biochemical perspective.

French

Les copépodes sont les métazoaires les plus nombreux et peuvent représenter jusqu'à 80% du zooplancton dans les systèmes marins où ils sont la nourriture principale des larves de poissons. Les copépodes sont adaptés aux fluctuations environnementales et certains utilisent la dormance comme stratégie pour survivre pendant les périodes critiques. Les œufs de dormance peuvent subsister plusieurs décennies dans le sédiment avant d'éclore et de retourner dans la colonne d'eau. Comprendre le phénomène de la dormance chez les copépodes ne permet pas uniquement de mieux appréhender les questions écologiques, cela permet aussi à la société humaine de développer des méthodes efficaces pour stocker les œufs de copépodes afin de les utiliser ultérieurement pour nourrir les larves de poisson/céphalopodes dans les écloseries. Cette utilisation ne touche pas *in fine*, uniquement le monde aquacole, c'est aussi une façon de remédier aux pêches intensives/destructives sur les récifs coralliens où des poissons vivants sont prélevés pour alimenter le marché de l'aquariophilie. Durant ma thèse, j'ai étudié les capacités de stockage des œufs *d'Acartia tonsa* provenant de différentes populations et j'ai

analysé différents paramètres de cultures aussi bien zootechniques que biochimiques pour ces mêmes populations.

Danish

Vandlopper (copepoder) anses for at være verdens talrigeste flercellede dyr og kan ræpresentere 80% af havets dyreplankton. Copepoder er et af de vigtigste fødeemner for mange fiskelarver. Copepoder er tilpasset fluktuerende miljøer og nogle producerer hvileæg, der kan overleve i dekader i sedimentet før de klækker og larverne svømmer op i vandsøjlen. At forstå processerne bag denne hvilestrategi giver ikke blot indsigt i de økologiske betydninger af hvilestadier, men giver akvakulturindustrien mulighed for at oplagre det bedste fødeemne til senere at kunne fodre tidlige stadier af fiskelarver, blæksprutter og andre akvatiske dyr. Ved at anvende copepoder som levende foder kan nye arter indenfor akvakulturen blive kultiveret gennem hele deres livscyklus. Copepoder som levende foder kan tillige mindske presset på følsomme økosystemer f.eks. koralrev hvor der idag opfiskes fisk til akvarieindustrien. I mit PhD projekt har jeg studeret hvileæg fra forskellige populationer af den kystnære calanoide copepod *Acartia tonsa* (Dana). Jeg har vurderet copepodens kultiveringspotentiale i et bredt spektrum der spænder fra et zooteknisk- til et biokemisk perspektiv.

1. Introduction

"Aquaculture is the farming of freshwater and saltwater organisms including fish, molluscs, crustaceans and aquatic plants. Unlike fishing, aquaculture, also known as aquafarming, implies the cultivation of aquatic populations under controlled conditions. Mariculture refers to aquaculture practiced in marine environments. Particular kinds of aquaculture include algaculture (the production of kelp/seaweed and other algae), fish farming, shrimp farming, oyster farming, and the growing of cultured pearls". (Wikipedia 2009).

1.1. Historical aspect of Aquaculture

The Common Carp (*Cyprinus carpio*) from the Cyprinid family is a freshwater fish whose production is recognized to be the oldest of all aquaculture farmed species. "Carp is the most ancient species to be cultivated in Europe, being introduced from China, where the first instruction book, written by Fan Lee, was published in year 500 BC" (Aquamedia, 2009). Central Europe Monasteries from today's Czech and Slovak republics are considered as the introducers of carp aquaculture in Europe for continuous supply of proteins. The growing understanding and knowledge of the species biology, and the positive results obtained, was spread throughout Europe. Even the Emperor Charles the Great (768-814), asked for fish ponds to be built, wherever possible, to overcome the problems of supply and demand for animal protein of the growing European population (Aquamedia, 2009). The history of the common carp freshwater invasions because of aquaculture development is often linked to political decisions. For example, referred as the German Carp in the US, the Common Carp, which appeared in American history starting in the late 1870's, can teach us more about environmental history. It was for instance imported by the Federal government in 1877 as supply for protein production and became the most abundant commercial fish within two decades. By 1900, however, the Illinois Fish Commission was one of the few agencies still promoting carp and most other states had abandoned carp stocking and even implemented bounties for it (Sandiford, 2009). Learning about aquaculture history may also help to tackle future decisions for management of the aquaculture industry.

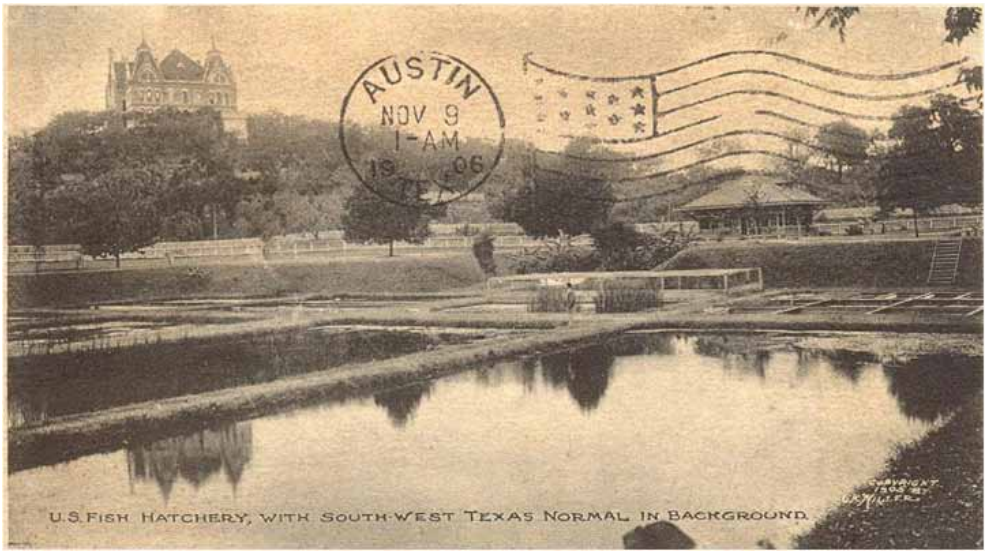

Figure 1: *The original San Marcos National Fish Hatchery, the first warmwater hatchery west of the Mississippi River, was established in 1897 and was located near the headwaters of the San Marcos River. For over 60 years, the hatchery was involved with production and development of efficient cultural techniques of warmwater sportfishes (Photo: The Edwards aquifer website).*

The culture of Brown Trout (*Salmo trutta*) is a little younger compared to Common Carp. By the 17th century, river stock depletion of Brown Trout was a problem and, in 1741, Stephen Ludwig Jacobi established the first trout hatchery in Germany (Aquamedia, 2009). From this point, technological improvement has helped to increase the production of aquaculture. Technical improvements have increased productivity of existing fish farms and also assisted the aquacultural development of new species. Today fish aquaculture is based on hundreds of fishes of which the entire life cycle can be totally controlled. First feeding and fish meal production of many marine fish species are still a bottleneck in the commercial development of some species which could be economically interesting. However, the fast development of the aquaculture industry is likely to bring enough new knowledge and technology to undertake these problems and new challenges for fish farmers will come up.

1.2. Contemporary Aquaculture

Capture fisheries and aquaculture supplied the world with about 110 million tons of food fish in 2006 providing a per capita supply of 16.7 kg (live weight equivalent). Of this total, aquaculture accounted for 47%. Aquaculture is considered as the fastest growing food sector in the world increasing with an average growth rate of 6.9% (SOFIA-Report, 2009).

The value of aquaculture production varies from one region to another and is not necessarily linked to the quantity of product produced. For instance, China is by far the highest aquaculture producer in the world in quantity (66.7% of the world tonnage). However, the value of its production is lower (48.8% of the world production). On the other extreme, Europe which produces only 4.2% of the world production in tonnage has an evaluated production of 9.1% of the world production value. (SOFIA-Report, 2009, see Figure 2)

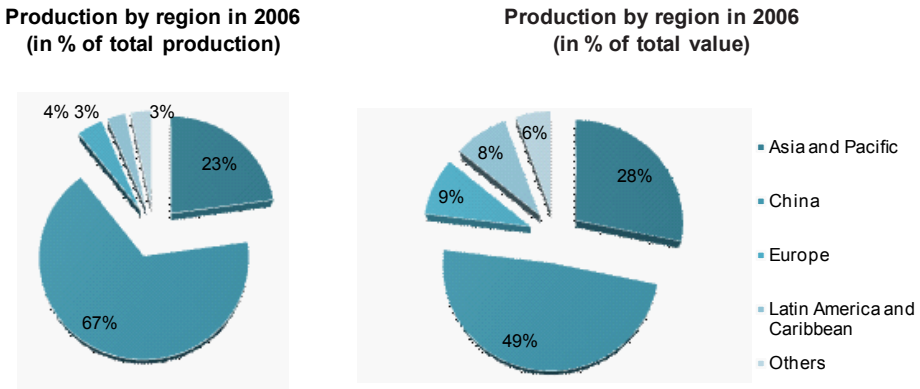

Figure 2: *Aquaculture production and its value in the world depending of the production region. SOFIA-Report, 2009.*

Marine fish aquaculture represents only 3% of the volume produced globally but 8% of its value, making marine finfish a very valuable sector (see figure 3). This is likely due to the increased demand of proteins for human consumption and the decline of natural fish stocks. Capture of fish products has decreased while aquacultural production of marine species has continuously increased (Table 1).

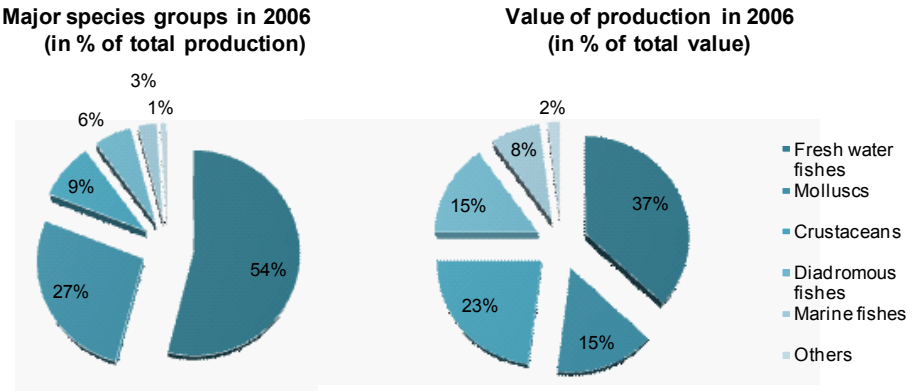

Figure 3: *Major groups reared in aquaculture in term of production and value. SOFIA-Report, 2009*

Table 1: *Production and capture in million tons, marine environment; SOFIA-Report, 2009*

Inland	**2002**	**2003**	**2004**	**2005**	**2006**
• Capture	8.7	9.0	8.9	9.7	10.1
• Aquaculture	24	25.5	27.8	29.6	31.6
Marine					
• Capture	84.5	81.5	85.7	84.5	81.9
• Aquaculture	16.4	17.2	18.1	18.9	20.1
Total					
• Capture	93.2	90.5	94.6	94.2	92.0
• Aquaculture	40.4	42.7	45.9	48.5	51.7

2. Live preys in aquaculture

2.1. The need for live prey

Many commercial marine finfish species that are raised through their entire life cycle are unable to grow if fed exclusively on formulated diets during their first developmental stages even though some research works show encouraging results with fish meals only (Fernández-Díaz and Yúfera, 1997; Cahu and Infante, 2001). Development of fish meals suitable for all stages would definitely assist the growth of the aquaculture industry. However, because the knowledge about first feeding on formulated diets is still limiting, many finfish larvae rely on live prey for a period of a few weeks until weaning is possible.

Live prey are necessary for first feeding for several reasons. Some fish species produce fish eggs containing a large amount of vittelin reserves which allow the larvae to develop until they reach a large size and therefore have a fully developed gut and a large mouth. Fish larvae developing from their yolk reserves until they look like mini-adults are called precocial larvae. This is the case for Salmonids which can be grown from the first feeding on fish meals. Some other fish like the Sea Bream, the Sea Bass and the Cod produces many small eggs with a low amount of vittelin reserves which do not allow the larvae to develop until they are capable of feeding on fish meals. These larvae are called altricial larvae. Alitrical larvae need to feed on small prey due to the small size of their mouth (see first feeding size of fish larvae in figure 4 and table 2). Also, because the gut of the altricial larvae is not fully developed at the time of first feeding, it is an advantage to get live prey which brings some exo-enzymes to the fish, helping the digestion of the prey.

Another reason for using live prey for first feeding is that moving prey are more attractive to fish than inert particles. The inert food particles from fish meals also tend to agglomerate on the water surface making food unavailable to fish larvae that do not feed there (Bengtson, 2003). This is of crucial importance when some altricial fish larvae need to feed within a relatively short time due to depletion of their energy reserves, which is only a few hours for some species.

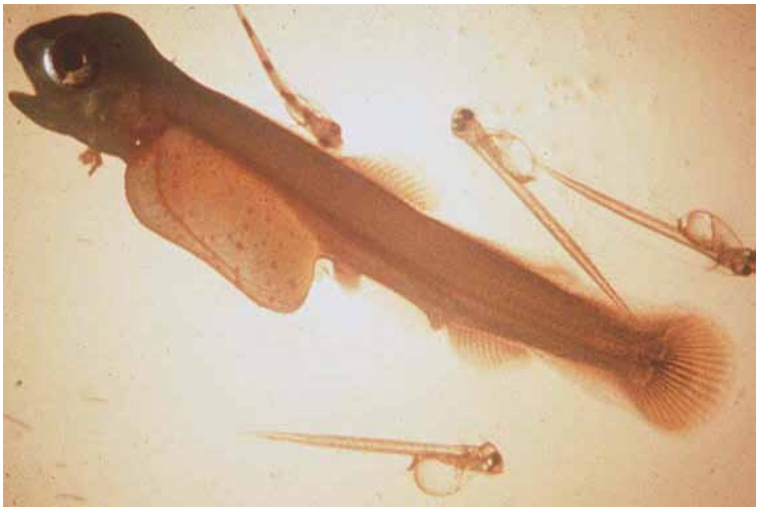

Figure 4: *Atlantic salmon and gilthead seabream larvae at first feeding. (Photo from Lavens and Sorgeloos, 1996)*

Table 2: *Size of eggs and larval length at hatching in different fish species (copied from Lavens and Sorgeloos (1996)*

Species	***Egg diameter (mm)***	***Length of larvae (mm)***
Atlantic salmon (*Salmo salar*)	5.0 - 6.0	15.0 - 25.0
Rainbow trout (*Oncorhynchus mykiss*)	4.0	12.0 - 20.0
Common carp (*Cyprinus carpio*)	0.9 - 1.6	4.8 - 6.2
European sea bass (*Dicentrarchus labrax*)	1.2 - 1.4	7.0 - 8.0
Gilthead seabream (*Sparus aurata*)	0.9 - 1.1	3.5 - 4.0
Turbot (*Scophthalmus maximus*)	0.9 - 1.2	2.7 - 3.0
Sole (*Solea solea*)	1.0 - 1.4	3.2 - 3.7
Milkfish (*Chanos chanos*)	1.1 - 1.25	3.2 - 3.4
Grey mullet (*Mugil cephalus*)	0.9 - 1.0	1.4 - 2.4
Greasy grouper (*Epinephelus tauvina*)	0.77 - 0.90	1.4 - 2.4
Bream (*Acanthopagrus cuvieri*)	0.78 - 0.84	1.8 - 2.0

2.2 Artemia

Until now, most of the hatcheries have been using the brine shrimp (*Artemia* spp.) and rotifers as live food because of their ease to use, but many industries suffer

from high mortality during the first developmental stages of their fish larvae despite the use of these live foods. Annually, over 2000 metric tons of dry *Artemia* cysts are marketed worldwide for on-site hatching and *Artemia* have the advantage of being present in many areas of the world (Lavens and Sorgeloos, 1996). However, because at the present time most of the world *Artemia* cyst harvest originate from the Great Salt Lakes (Utah, USA) where climatic changes may generate unforeseen lack of live food, the availability and prices of *Artemia* cyst may endure economic inflation (Lavens and Sorgeloos, 2000). Because *Artemia* spp. show a poor natural biochemical composition, enrichment is necessary before use in first feeding (Støttrup, 1986; Barclay and Zeller, 1996; Rodríquez et al., 1996; Rainuzzo et al., 1997; Sargent et al., 1997). Furthermore, the size of *Artemia* is too large for the first days of feeding for many fish larvae with small mouths, however in such cases smaller live prey such as rotifers may be substituted. More about *Artemia* and its use in aquaculture can be found in (Sorgeloos et al., 2001; Støttrup and McEvoy, 2003).

2.3. Rotifers

Though rotifers have long been considered as pests in the aquaculture industry, their use as live prey has increased in common hatchery facilities. The two main species used are *Brachionus plicatilis* and *Brachionus rotundiformis*. Rotifers are parthenogenetic, reproduce at high rates and their densities achieved in culture are extremely high. Dhert et al. (2001) present the situation and advancement of rotifer culture and manipulation in Europe. The availability of large quantities of rotifers as a live food source has contributed to the successful hatchery production of more than 60 marine finfish species and 18 species of crustaceans. However, as for Artemia, the biochemical composition of rotifers is poor and the need for enrichment is required (See figure 5).

2.4. Other potential live preys

There are more potential live preys that could be developed for the aquaculture and aquarium industries, however none of the alternative prey have been intensively used by the industries due to the difficulty to grow, store, or transport them (see table 3). Among these potential alternatives, copepods are the best prospective

candidate and the development of many studies is increasing their potential as live prey.

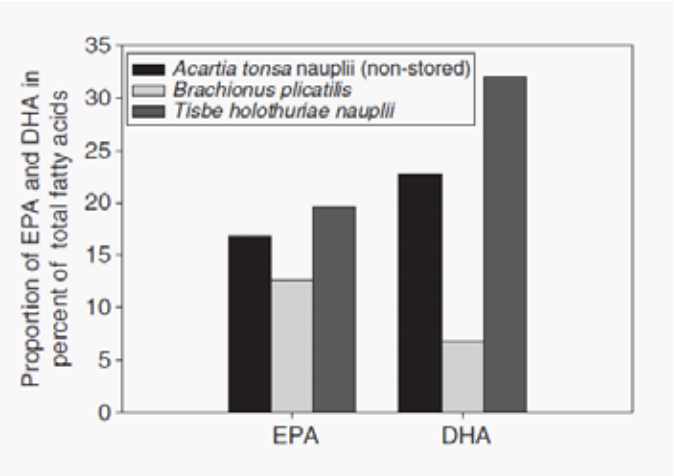

Figure 5: *Eicosapentaenoic acid and docosahexaenoic acid content as a percentage of the total fatty acid content of three live prey organisms. These two fatty acids are known to be essential to the proper development of many fish larvae. Brachinus plicatilis (rotifer) and the two copepods species were reared under the same conditions and fed similar algae (Rhodomonas salina). Figure from Drillet et al. (2006b)*

Table 3: *Potential live prey for aquaculture (Goshorn and Epifanio, 1991; Lavens and Sorgeloos, 1996; Purcell et al., 2005).*

Marine and brackish systems	*Fresh water systems*
• Nematodes	• Daphnia
• Trochophora larvae	• Moina
• Polychaete larvea	
• Copepods	
• Appendicularians	

3. Copepods as a live prey

Copepods are the most numerous metazoan on earth and are natural food for fish larvae (Möllmann et al., 2004). They can represent up to 80% of the zooplankton biomass in the water column (Mauchline et al., 1998).

It is well accepted that many copepods are a valuable source of food for fish larval rearing although they are not often used in aquaculture industry. Many results considering biochemical composition and the size of the copepods suggest that the use of copepods will be established in the close future. Støttrup (2000) and Payne et al. (2001) strongly suggest that the inclusion of copepods in the aquaculture industry will increase the number of successfully reared fish species.

Raised copepods as well as harvested zooplankton contain biochemical characteristics which makes them a good alternative or supplemental live prey for larval rearing (see table 4 and Næss et al., 1995; Shansudin et al., 1997; Støttrup and Norsker, 1997; McEvoy et al., 1998; Rønnestad et al., 1998; Payne and Rippingale, 2000; Støttrup, 2000; Payne et al., 2001; Evjemo et al., 2003; see review by Støttrup, 2003; Rajkumar and Vasagam, 2006; Wilcox et al., 2006). Also, copepods have a slower passage through the gut of fish larvae than *Artemia* spp., which leads to a more complete digestion and more efficient nutrient uptake (Pedersen, 1984). This may be due to the fact that copepods have higher digestive enzyme contents than *Artemia* which can be used by the fish larvae as exo-enzymes (Munilla-Moran et al., 1990).

Copepods are already used at a semi-extensive scale (Toledo et al., 1999; Engell-Sørensen et al., 2004). They are part of the natural fish preys present in aquaculture ponds (Sipaùba-Tavares et al., 2001), and promising results from some sustainable intensive cultures have been reported (Sun and Fleeger, 1995; Schipp et al., 1999; Payne and Rippingale, 2001b). Recently, methods to trap copepods in extensive production ponds were developed (Lindley and Phelps, 2009) and production of copepod nauplii all year around in a fish farm was shown possible in Denmark at 57˚N (Sørensen et al., 2007).

Table 4: *Fatty acid composition (% of total lipids ± SD) of wild zooplankton (mainly copepods), un-enriched, and enriched Artemia (Super Selco) during a feeding experiment of the Atlantic halibut (Hippoglossus hippoglossus). The data are from Næss et al. (1995) and reveal very clearly the higher quality of copepods in comparison to Artemia.*

Fatty acid	Wild zooplankton D0	Wild zooplankton D10	Wild zooplankton D19	Unenriched *Artemia*	Enriched *Artemia*
14:0	3.4 ± 0.2	4.3 ± 0.1	4.2 ± 0.2	0.8 ± 0.0	0.9 ± 0.0
16:0	16.9 ± 1.0	16.4 ± 0.2	15.4 ± 0.2	12.6 ± 0.8	11.0 ± 0.3
16:1*n*-9	0.7 ± 0.0	0.6 ± 0.0	0.4 ± 0.1	0.9 ± 0.0	1.0 ± 0.1
16:1*n*-7	1.7 ± 0.1	4.8 ± 0.1	4.6 ± 0.2	4.0 ± 0.1	3.9 ± 0.4
16:2*n*-4	0.3 ± 0.0	1.0 ± 0.1	0.5 ± 0.1	0.2 ± 0.3	0.1 ± 0.0
18:0	3.7 ± 0.4	2.6 ± 0.0	1.8 ± 0.2	7.4 ± 0.6	4.9 ± 0.3
18:1*n*-9	2.9 ± 0.5	6.6 ± 0.3	11.7 ± 0.4	22.5 ± 0.4	19.7 ± 0.7
18:1*n*-7	3.3 ± 0.2	1.7 ± 0.1	1.1 ± 0.0	10.6 ± 0.1	7.5 ± 0.3
18:2*n*-6	2.0 ± 0.1	2.4 ± 0.1	2.8 ± 0.0	6.8 ± 0.0	6.8 ± 0.3
18:3*n*-3	1.5 ± 0.4	2.6 ± 0.3	3.1 ± 0.0	20.3 ± 1.5	18.4 ± 2.7
18:4*n*-3	1.5 ± 0.1	4.6 ± 0.1	7.8 ± 0.2	2.3 ± 0.3	2.7 ± 0.4
20:1*n*-9	0.2 ± 0.1	0.7 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	1.0 ± 0.2
20:1*n*-7	0.6 ± 1.4	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:4*n*-6	0.8 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	2.3 ± 0.1	1.7 ± 0.0
20:4*n*-3	0.5 ± 0.0	1.5 ± 0.0	1.8 ± 0.0	0.6 ± 0.1	1.0 ± 0.0
20:5*n*-3	21.1 ± 1.3	18.1 ± 0.2	14.5 ± 0.2	3.6 ± 0.3	9.8 ± 2.5
22:0	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	1.1 ± 0.1	0.5 ± 0.0
22:1*n*-1	0.0 ± 0.4	0.6 ± 0.0	0.3 ± 0.0	tr	0.2 ± 0.1
22:5*n*-3	0.8 ± 0.2	0.8 ± 0.0	0.7 ± 0.0	0.1 ± 0.0	0.8 ± 0.2
22:6*n*-3	32.9 ± 2.3	24.8 ± 0.5	24.0 ± 0.6	0.2 ± 0.2	5.0 ± 1.8
Sum *n*-3 PUFA	58.3	52.4	51.9	27.1	37.8
Sum *n*-6 PUFA	2.8	3.0	3.4	9.1	8.5
n-6/*n*-3 PUFA	0.0	0.1	0.1	0.3	0.2
22:6*n*-3/20:5*n*-3	1.6	1.4	1.7	0.1	0.5
Total lipid (μg/mg WW)	13.0	19.0	30.0	13.0	29.0

Another possibility for the aquaculture industry is to develop intensive culture of copepods to sustain their need and offer copepods with a known history and a controlled quality to the fish larvae. A list of more than 60 copepod species have been laboratory raised (Mauchline et al., 1998) and data on many continuous cultures can be found on the World Copepod Culture Database (http://copepod.ruc.dk/main.htm) which we launched at Roskilde University in order to facilitate the communication between groups working with copepod cultures. However, the copepod industry is still not fully developed primarily due to the lack of knowledge about large scale cultivation. Nevertheless, some have achieved simple and effective methods to produce a large number of copepods (*Amphiascoides atopus*, *Acartia* spp. and Gladioferens imparites) that could probably be adapted to the need of

a potential copepod industry (Sun and Fleeger, 1995; Schipp et al., 1999; Payne and Rippingale, 2001b). Støttrup and Norsker (1997) presented a continuous culture of the harpacticoid copepod *Tisbe holothuriae* for raising turbot larvae and took into consideration the daily effort (man hours) which is very relevant in an aquaculture context. McKinnon et al. (2003) suggest the smallest size of paracalanid copepods as a particular advantage whereas Shansudin et al. (1997) emphasize the potential value of in situ harvested copepods covering a broad range of prey sizes. To summarize, there is a lot of room for the improvement of copepod culture techniques and the multiple groups working on copepods as live feed produce regularly scientific reports and manuscript that are available to others helping to hasten the development of new techniques. As part of my PhD, I summarized these developments and the obvious bottlenecks with copepod culturing and proposed different potential solutions or ideas that could help scholars and the industries interested in this subject (Drillet et al., Submitted).

4. Copepod eggs, a storage stage for aquaculturists

As aquacultural development makes culturing more reliable, another bottleneck in the expansion of copepod use in aquaculture appears: the lack of storage capacity. This is particularly true for Harpacticoid and Cyclopoid copepods which are egg carriers and therefore do not release their eggs in the water column but instead release nauplii from eggs sacs. (Payne and Rippingale, 2001a) stored fresh nauplii of *Gladioferens imparites* for up to 12 days at 8°C with 99% survival, but mortality was almost 100% after 36 days. Storing eggs from free spawning copepods seems, however, more promising because eggs from many calanoids can survive rougher treatments than their nauplii. Copepod eggs have been shown to survive decades in the sediment of natural environment (332 years in freshwater systems and 40-69 years in marine systems; Marcus et al., 1994; Hairston et al., 1995; and Mie Schislau pers. comm.) and this gives researchers a lot of room for the development of storage techniques for copepod eggs. The idea of storing copepod eggs for aquaculture has been long going and may be possible for all species belonging to the superfamily of Centropagoidea. This technique, for example, has been used at the Danish Technical University (DTU-Aqua) for decades in order to keep a culture starter during vacation periods and is used regularly at Roskilde University (see Figure 6).

Figure 6: *Copepod eggs stored in scintillation vials (Roskilde University, 2004 – Guillaume Drillet)*

During my PhD I focused on different aspects of egg storage and their consequences for aquaculture. I first studied the effect of cold storage on the survival and biochemical contents of *Acartia* tonsa eggs and showed that cold storage

does not affect the reproductive capacities of the following generation of adults (Drillet et al., 2006a).

In a second study, I focused on a comparison of the biochemical composition of two copepods species and one rotifer. I could confirm that copepods are of higher nutritional quality than rotifers and that the hatching from egg to nauplii is a process that partially transforms the biochemical composition of these two stages (Drillet et al., 2006b; Figure 7).

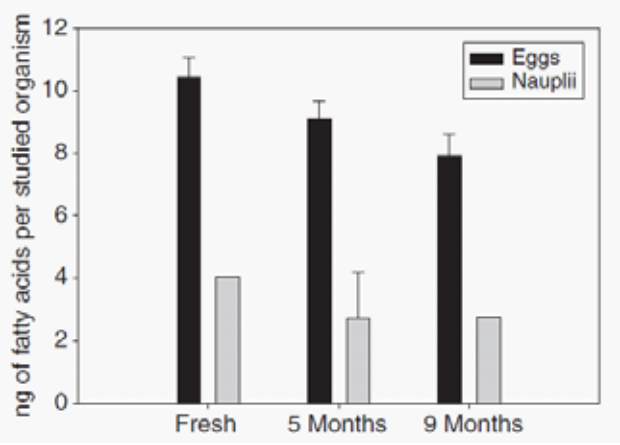

Figure 7: *Acartia tonsa total amount of fatty acids (average (ng) ± SD) in eggs and nauplii for each different period of egg storage at 1-3 °C. The fatty acids contents of the eggs and their hatched nauplii are shown. The data show the decrease of fatty acid content of eggs during cold storage and the loss of fatty acid during the hatching process. Data from Drillet et al. (2006b)*

In a third study which I carried out at Florida State University (USA), I looked at the effect of glucose as a potential source of energy that could diffuse through egg shell and increase the survival of eggs. In this study I used three different antibiotic treatments (Streptomycin, Penicillin and Kanamycin). I could not achieve an increase in egg survival with the glucose treatment, however, the antibiotics treatment showed a positive short-term effect and a toxic long-term effect (Drillet et al., 2007). During this study, I also observed that under almost similar culturing conditions, the eggs from a Florida population of *A. tonsa* could not survive more than a month at cold temperature (a few ºC) while the *A. tonsa* population from Denmark could survive many months of cold storage. Finally, one parallel study allowed me to show the effects of egg development on egg storage capacity (Figure 8).

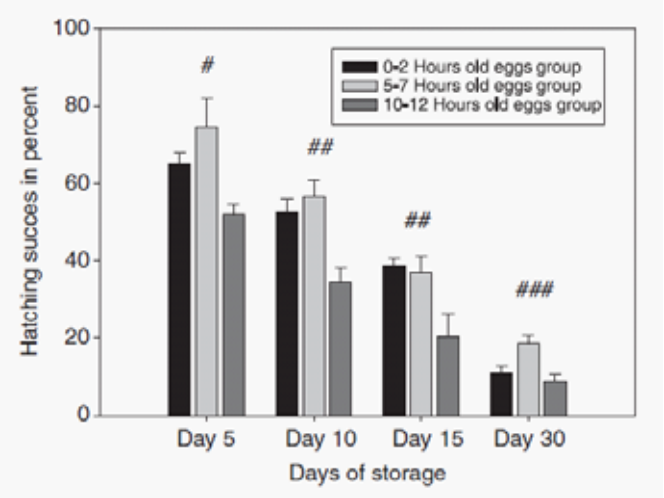

Figure 8: *Hatching success of three different egg development groups after 4 increasing periods of cold storage (mean ± standard deviation). Eggs were 0-2, 5-7 and 10-12 h old when they were first cold stored at 1 °C. #All the hatching success (HS) were statistically different from each other; ##the HS from the 0-2 h and 5-7 h groups were statistically different from the HS of the 10-12 h group; ###HS from the 5-7 h group was statistically different from the HS of the10-12 h group (P<0.05). Data from Drillet et al. (2007).*

I used a common garden experiment approach to clarify if the difference between populations was inherent to the population or was due to slight differences in the handling of the copepod cultures and eggs. I showed that out of four populations from the Baltic Sea (Denmark and Germany) and the Gulf of Mexico (Florida and Alabama) three were distinct populations that could be considered cryptic species. All populations showed differences in life history traits and egg storage capacities. For these reasons, I consider that a selection of diverse populations with distinct particularities for the aquaculture industry is possible and should be performed (Drillet et al., 2008a b; Figure 9 and 10).

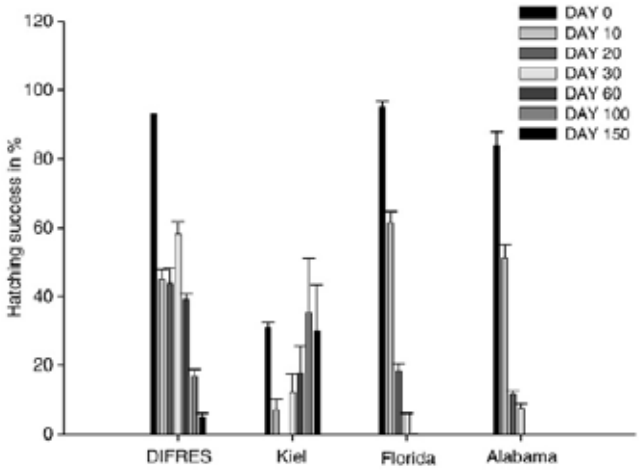

Figure 9: *Hatching success at 17 °C of eggs from the four A. tonsa strains produced at 17 °C and stored subsequently at 5.4±0.7 °C for a range of storage durations. For each strain, bar color indicates increasing periods of storage (inset legend). Figures represent the mean±S.E (From Drillet et al. (2008a).*

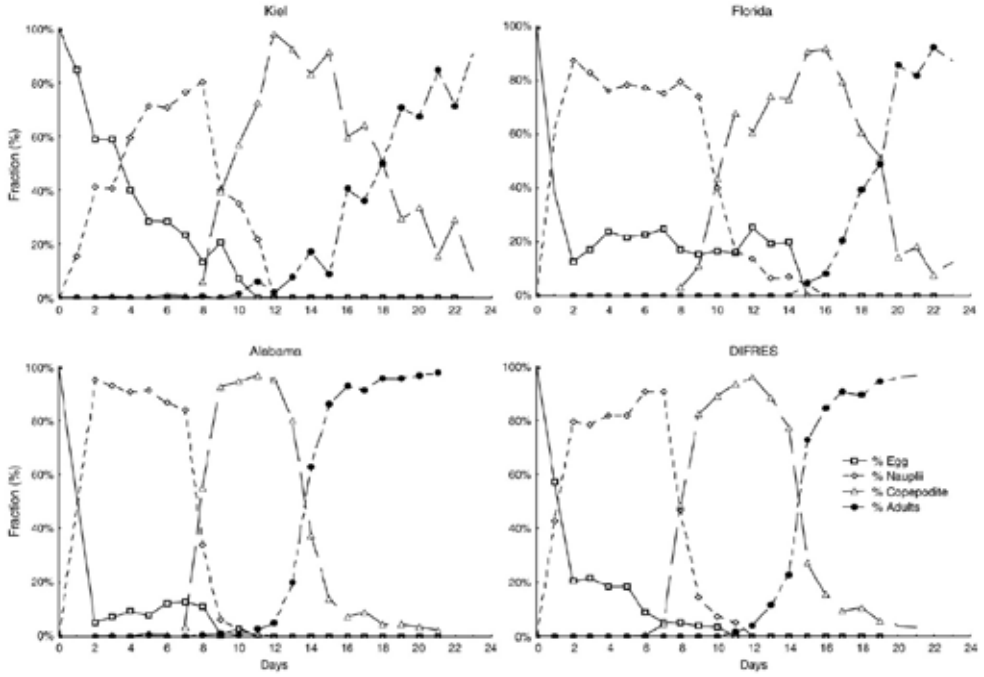

Figure 10: *Fractional developmental stages of four A. tonsa populations (DIFRES, Kiel, Florida and Alabama) from egg to adult. Data points represent the means of four replicates (incubation bottles). The copepods were fed ad libitum with Rhodomonas salina and were grown in closed bottles at 17°C. (data from Drillet et al. (2008b).*

5. Why use Acartia tonsa as a lab rat?

During my PhD I focused on the Calanoid copepod *A. tonsa* (Figure 11). It is a cosmopolitan planktonic species that is abundant in neritic and coastal waters of the global ocean (Razouls et al., 2005-2009). As dominant members of coastal planktonic communities, *Acartia* species serve as important food sources for fish populations in the wild (Fox et al., 1999; Möllmann et al., 2004; Maes et al., 2005). The calanoids of the *Acartia* genus have been identified as particularly interesting because of their small size (0.1 – 1.5 mm), short generation time and ease to grow (O'Bryen and Lee, 2005). Also, *Acartia* tonsa is known to produce dormant eggs in either true diapause or quiescent stages in order to survive periods of poor environmental conditions (Zillioux and Gonzalez, 1972; Castro-Longoria, 2001). For these reasons *A. tonsa* may be particularly valuable for use in marine aquaculture. However, the taxonomy of the genus *Acartia* is known to be incomplete (Bradford-Grieve, 1999), and genetically distinct clades have been reported within the nominal species *A. tonsa* (Caudill and Bucklin, 2004; Chen and Hare, 2008). These clades have been shown to be ecologically differentiated in some cases (Chen and Hare, 2008). Given these observations, it appears likely that the nominal species *A. tonsa* includes a number of genetically divergent lineages or cryptic species that may have specific habitat or culturing requirements.

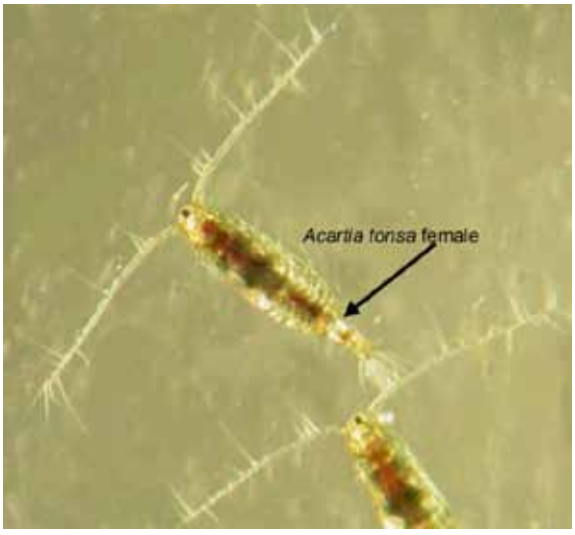

Figure 11: *Photo of a female Acartia tonsa (Dana). (photo from www.flseagrant.org)*

6. Terminology of copepod eggs

One observation that striked me while working on resting eggs with an applied perspective is that there are some slight differences between the terminology used in different research groups and biology domains…

As reported by Belmonte and Rossi (1998), "many organisms can interrupt or slow down their life functions and this ability has been labeled in many ways: dormancy, quiescence, rest, encystment, diapause, crytobiosis, lethargy, tanatosis, catalepsy… inter alia ". Dormancy is an important strategy used by marine planktonic copepods to survive poor environmental conditions. Copepods have been described to enter dormancy at various stages of development, including over-wintering copepodites and resting eggs that settle into sediment layers (Mauchline et al., 1998). Close to fifty copepods (calanoid) have been described to produce diapause eggs (Engel and Hirche, 2004 and ref therein). Resting eggs can be important in the population dynamics of coastal species due to their role as benthic seed banks for pelagic populations (Katajisto, 1998). Recovery of resting eggs from the sediment is thought to vary depending on physical and biological conditions in the sediment, but hatching success can be approximated to values around 21%, with seed banks containing on the order of 106 - 107 viable eggs m-2 (Grice and Marcus, 1981; Marcus, 1984; Marcus et al., 1994; Dahms, 1995; Marcus, 1996; Katajisto, 1998; Alekseev et al., 2007).

Based on the work of Mansingh (1971), resting eggs have been classified into two distinct categories by Grice and Marcus (1981):

1. Subitaneous eggs are produced to hatch immediately after spawning but are able to rest for some time if the environmental conditions are not suitable for them. If they do rest, they switch to a so called "quiescent" stage and they will hatch as soon as the environmental conditions become favorable again
2. Diapause eggs go through a refractory period during which hatching is not possible even if environmental conditions are suitable. These eggs, after the refractory period, are still able to stay quiescent if the environmental conditions are not suitable

A third type of dormant egg called ”delayed-hatching” was later observed in Labidocera scotti and Pontella meadi by Chen and Marcus (1997) as another resting strategy, although no physiological data has distinguished them from the two other egg types. This third type was also described for a population of Acartia in the Baltic Sea (Katajisto, 2006). Therefore we could add a third category to the list described above (Figure 12).

3. Delayed hatching eggs...

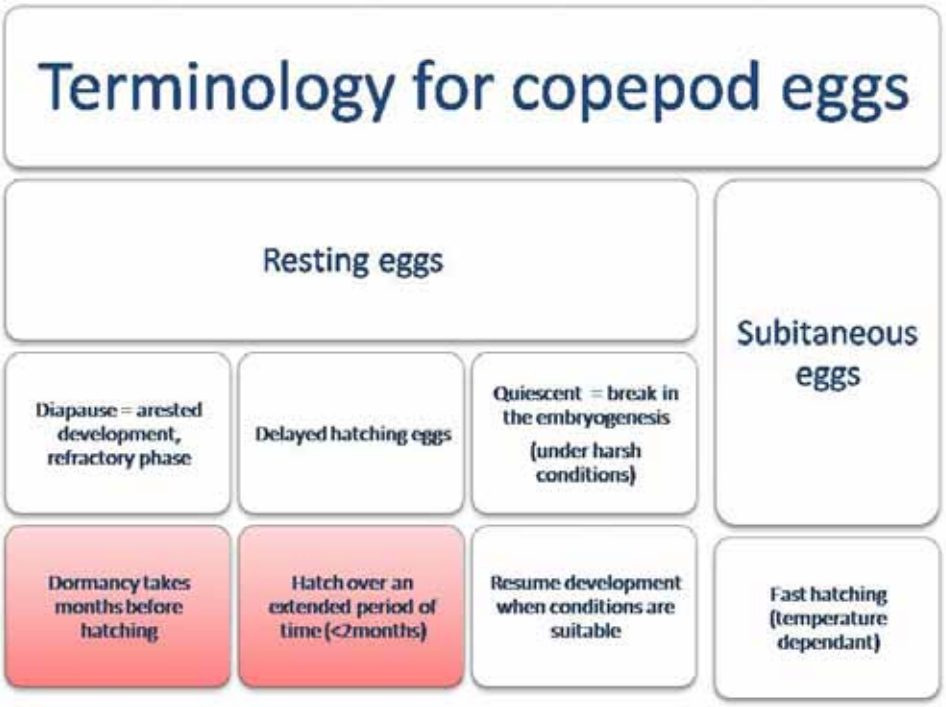

Figure 12: *Terminology of copepod eggs. The figure is a combination of descriptions from Grice and Marcus 1981 and Chen and Marcus (1994). Delayed hatching eggs would, however, be considered as true diapause eggs following Prof. Victor Alexseev's terminology of resting stages in crustaceans (2007, Diapause=Dash box). The boxes colored in red represent groups of eggs for which hatching is maternally controlled.*

One of the key aspects about studies on resting stages of zooplankton is that the terminology used to separate the different physiological states (as diapause, delayed hatching and subitaneous) varies from one research group to another. This is likely due to different thinking schools or approaches within the research field. However, it may bring some misunderstanding among the researchers

sharing their data. For example, the delayed hatching eggs group was ignored in a recent review from (Alekseev et al., 2007) who instead separated the diapause groups into levels (superpause, mesopause and oligopause). None of these two thinking schools should however be ignored and the physiological and ecological mechanisms behind the terminological struggle still have the same importance.

Finally, in a recent experiment I showed that delayed hatching egg production in some *A. tonsa* populations could be triggered by the low food availability even when copepods were raised under optimal temperature and light conditions (Figure 13) and that delayed hatching eggs could be cold stored with a very high hatching success for many months.

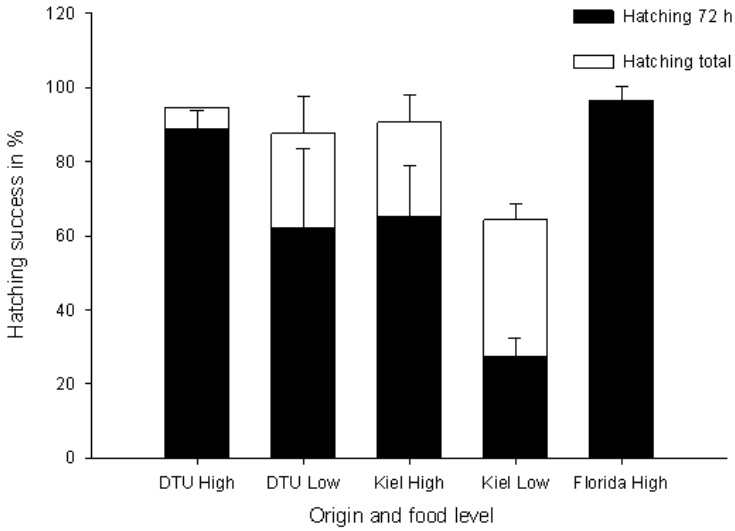

Figure 13: *Final hatching success after 72 hours hatching (subitaneous eggs) and total hatching after 1 month (Delayed Hatching Eggs + Subitaneous eggs) in three populations of Acartia tonsa raised under different food conditions (low: 2,000 cell.ml-1 and high: 40,000 cell.ml-1 of Rhodomonas salina) in a common garden experiment. (Drillet et al. unpublished data)*

7. Conclusions and future….

In the last 6 years I have been focusing on copepod and their value for the aquaculture sector. I have studied intensively the resting eggs stages which can be stored for two reasons, the main one is that storage systems for copepod eggs have to be developed in order to render the use of copepods as live feeds possible and easy in the short term. This is because continuous cultures of organisms require much maintenance. The second reason directly runs from the first one but is linked to the potential selection of populations of copepod that may have useful life history traits for the industry. Keeping embryos of valuable populations may then be of central interest.

Before we see large investments in the private sector more public research has to be pursued in research centers and universities to consolidate the development of intensive productions of copepods worldwide, and this development may take a decade. But without any doubt, this development will necessitate the transfer of technologies and knowledge from one field to another, and will be based on a multidisciplinary approach involving public and private partners with very different expertise. This was actually initiated in a recent submitted manuscript where partners from different horizons shared their knowledge to point out a few relevant areas where efforts should be put in the coming future (See manuscript 6). One of our recommendations was to transfer knowledge from shrimp and fish farming to copepods in the field of probiotic science. This was done last year with success in our laboratory where we increased the productivity of copepod females using preparation known to have probiotic effects (Manuscript 7). This was a first success in going through new tracks but much more should and will be achieved in the future.

Copepods are the most numerous metazoans on the planet and some species have optimal life history traits which could be used for improving the use of live prey. However, because the research in this field is spread over so many species and because the results may not be transferable between species (even not within a species, see Drillet 2008ab), it may take more time before we observe a fast development of the copepod businesses.

During my Ph.D. I had to chance to present some of my results to different audiences going from plankton ecologists to business people. A good proportion of ecologists I met seemed disturbed by my work as I approached the problems encountered in my research in the same way as they do, using appropriate small scale experimental setups, but trying to reach very different objectives. If their work consists in understanding ecological processes to explain the interactions between organisms, mine resides in using or forcing these processes in order to innovate copepod production systems and increase productivity and practicality. I believe that there are not two fields of sciences, Applied vs. Fundamental as some tend to claim but merely a conflict between researcher's interests which slow down the advancement of research as a whole; "fundamental researchers" do not read enough about advancement in applied research and vice versa while the knowledge is there and do not need to be rediscovered.

On the other hand, discussing my research with private partners, I really met two kinds of people: those who look at the economical potential of copepods today and claim that there is no future for it, and those who wish to develop the aquaculture and aquarium trade sector by rearing new species and are very interested in hearing about my projects. As in other fields, some people are interested in the challenges that new entrepreneurship can bring and others only think about the return on their investments which has to be always bigger.

One way to change this balance is to make a proper research study dealing with the cost of production and the price of the gain of productivity in a fish farm using copepods as live prey. Raising new species could also have other impacts than just monetary, as proposed earlier, this could be a tool to protect pristine environment like coral reefs where ornamental fishes are captured with very environmentally damaging methods. There again, a proper investigation should be launched.

I hope that my research will be useful to scientists interested in copepods, their resting eggs and aquaculture in general. It has been an intensive work which I carried out with enthusiasm even when sometimes everything seemed hopeless. I have achieved some understanding of copepod ecology and life history and have applied this to the development of new live prey which is a necessity in aquaculture. This period is over and I am ready to move on into new challenging projects where I hope to learn, innovate, develop and share my finding to a wider public.

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Manuscript 2:
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Manuscript 5:
Drillet, G., Jepsen, P.M., Højgaard, J.K., Jørgensen, N.O.G. & Hansen, B.W. (2008b) Strain-specific vital rates in four Acartia tonsa cultures II: Life history traits and biochemical contents of eggs and adults. Aquaculture, 279, 47-54.

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MANUSCRIPT 1

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Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring

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Abstract

For most fish species raised in marine aquaculture, the use of live feeds cannot be replaced by formulated diets. *Artemia* nauplii and rotifers are still the most commonly used live feeds. A good alternative lies in the use of copepods which could lead to the cultivation of new fish species. Cold stored subitaneous eggs from the continuously cultured calanoid copepod *Acartia tonsa* were used to investigate the effect of storage upon the viability of the eggs, the development of the copepod community originating from the cold stored eggs. Finally a 3 days snapshot of the egg production of the first generation of females was followed. This was done in order to develop a database usable within copepod dependent hatcheries. The viability of cold stored *A. tonsa* eggs remained high (>70% hatching rate) for 11 months of storage. Generally, the period of storage was observed to decrease the viability (hatching rate) of the eggs and no hatching was observed after twenty months of cold storage. Hatched populations of copepods experienced increased mortality rate with longer storage of the eggs from which they originated. This mortality ranged from 0.035 to 0.13 d^{-1} for non-stored (fresh) and 12 months stored eggs, respectively. However, all copepod communities originating from fresh to 12 months stored eggs reached adulthood. Additionally, the egg production from the stored generation was apparently normal and the viability of their eggs was not statistically different when compared to productions from non-stored communities. Contents of total fatty acids decreased during the storage period. Contents of free amino acids were not statistically different for eggs cold stored up to 12 months, but had decreased severely by 20 months. In conclusion, we consider it safe to store the eggs for up to one year at 2–3 °C during which the eggs retain their viability and biochemical composition. Cold storage of calanoid copepod eggs is relevant for aquaculture as inoculum for culturing live food.

Keywords: Copepod eggs; Cold storage; Viability; Inoculum; Amino-acid; Fatty acid; Aquaculture

1. Introduction

Most of the cultivated marine fish species cannot grow if fed only with formulated diets during their first developmental stages although some references show encouraging results with dry food only (Fernández-Díaz and Yúfera, 1997; Cahu and Infante, 2001). Until now, most of the hatcheries have been using the brine shrimp *Artemia* spp. and rotifers as live food. Since *Artemia* spp. has a low content of essential fatty acids, an enrichment is necessary before use in first feeding (Støttrup et al., 1986; Barclay and Zeller, 1996;

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Rodríquez et al., 1996; Rainuzzo et al., 1997; Sargent et al., 1997). Ninety percent of the world *Artemia* cyst harvest comes from the Great Salt Lakes (Utah, USA) where climatic changes may generate unforeseen lack of live food (Lavens and Sorgeloos, 2000) inducing economic inflation.

It is well accepted that many copepods are valuable source of food for fish larval rearing and although it is not often used in aquaculture industry, many results considering biochemical composition beside the size of the copepods suggest that the use of copepods will be established in the close future. Støttrup (2000) and Payne et al. (2001) strongly suggest that the inclusion of copepods in the aquaculture industry may increase the number of successfully reared fish species. Copepods have a slower passage through the gut of the fish larvae than *Artemia* spp. which leads to a more complete digestion and more efficient nutrient uptake (Pedersen, 1984). Today, most of the fish farms using copepods generally harvest them in situ or let them grow semi-extensively in outdoor tanks under semi-controlled conditions (Toledo et al., 1999; Engell-Sørensen et al., 2004). A list of more than 60 copepod species have been laboratory raised (Mauchline, 1998) but the copepod industry is still not fully developed primarily due to the lack of knowledge about large scale cultivation. However, Sun and Fleeger (1995), Schipp et al. (1999) and Payne and Rippingale (2001a) achieved simple and effective methods to produce a large number of copepods (*Amphiascoides atopus*, *Acartia* spp. and *Gladioferens imparites*, respectively) that could probably be adapted to the need of a potential copepod industry. Støttrup and Norsker (1997) presented a continuous culture of the harpacticoid copepod *Tisbe holothuriae* for raising turbot larvae and took into consideration the daily effort (man hours) which is very relevant in an aquaculture context. According to Payne and Rippingale (2001a), harpacticoid copepod production is clearly of value for larviculture. However, they consider that particular benefits can be obtained with calanoid copepods for which all development stages are present in the water column. McKinnon et al. (2003) suggest the smallest size of paracalanid copepods as a particular advantage whereas Shansudin et al. (1997) emphasize the potential value of in situ harvested copepods covering a broad range of prey sizes. In the present study, eggs from a continuous culture of the calanoid copepod *Acartia tonsa* were tested. The objective was to investigate the effect of storage period at 2–3 °C upon the viability, biochemical composition of the eggs and the fitness of the upcoming generation of copepods.

2. Material and methods

Two experiments were carried out using cold stored subitaneous *Acartia tonsa* eggs from the Danish Institute for Fisheries Research (DIFRES). The copepod culture was based on wild caught individuals isolated from net samples collected in 1981 from Øresund, the Sound between Denmark and Sweden, and assigned the identification code DFH-AT1 (Støttrup et al., 1986). These eggs had been produced in 200L dark tanks in 0.22 μm filtered seawater (30 psu) at 18–19 °C. The eggs (<24 h old) had been harvested, cleaned and stored in pasteurized filtered seawater in closed test tubes at 2–3 °C turning anoxic within 3–4 weeks (M. Olesen pers. comm.). The viability of the eggs stored this way for: 0, 3 and 6 months (experiment 1) and 0, 12 and 20 months (experiment 2), was tested for hatching success (hatching rates). The hatched copepods were tested for instantaneous mortality, stage development time, fecundity and hatching success of the second generation of eggs. Since experiments were not performed simultaneously, a control group with fresh or 1 week cold stored eggs (presented as non-stored eggs), acted as a reference for viability for all batches. Our experience shows that effects on viability of cold storage are negligible during the first 2 weeks. Hence, it is safe to use these eggs as fresh eggs. Samples of cold stored eggs were taken for biochemical analysis of free amino acids (stored in MilliQ water) and total fatty acid (FA) contents (stored in 2 : 1 Chloroform : Methanol) for each age group and kept at −80 °C until they were processed. Furthermore, dry weight and carbon content were measured on fresh eggs.

2.1. Egg viability

The batches of stored eggs were rinsed with GF/F filtered seawater (Whatman filters) and cleaned successively through different NITEX screens (90 and 70 μm mesh sizes, respectively) in order to remove decomposed particles present in the storage tubes. A hatching experiment (10 replicates) was carried out in Petri dishes containing 150–280 eggs in each and filled with 10 ml of aerated GF/F filtered seawater (Whatman filters). The exact number of viable (dark pigmented) eggs was counted and incubated at room temperature (23 °C) with the 8 μm ESD chryptophyte *Rhodomonas salina* (ESD = Equivalent Spherical Diameter). After 4 days, the number of eggs left was counted and the missing eggs were considered as hatched. A hatching rate (HR) was calculated. To substantiate the egg data an additional batch of 11 months stored eggs was tested.

2.2. Development of copepod communities

Between 8000 and 20,000 dark pigmented eggs of each age group (0, 3, 6, 12 and 20 months) were incubated in duplicate 2.3 l acid-washed Nalgene® bottles filled with GF/F filtered seawater (30 psu). Number of eggs available from the egg bank in the refrigerator initially: 0 months 78,000 eggs; 3 months 50,000 eggs; 6 months 77,000 eggs, 11 months 76,000 eggs, 12 months 15,800 eggs, 20 months 5019. These eggs were typically from 1–3 test tubes per storage time, and directly from the refrigerator divided into the duplicate incubation bottles. In each bottle 20,000 cell/ml of *R. salina* was added to ensure excess food (Kiørboe et al., 1985; Berggreen et al., 1988) together with 10 ml of phytoplankton culture media B1 (described by Hansen, 1989) to avoid the wilt of the algae. The phytoplankton culture was grown under constant light 150±5 µmol photons m^{-2} s^{-1} (Licor-198 with Li-192 SR. Nr. UWQ 4294 sensor). The copepod incubation bottles were completely filled to avoid bubbles of air inside and fixed to a 75 cm diameter plankton wheel (1 rpm) in a 17 °C walk-in climate room under constant dim light conditions.

Every two/three days during a period of 3 weeks, two 25 ml sub-samples were taken with a kip automat NS 29.2/32 (Witeg, Germany) from each bottle and fixed in acid Lugol solution in order to determine the abundance and the relation between eggs, nauplii, copepodites and adults. The O_2 concentration in the incubation bottles was measured with a UNISENSE oxygen microsensor (OX25) adapted to a picoamperimeter (PA2000), and the phytoplankton concentration monitored by an electronic particle counter (Z2 COULTER Counter) after each sub-sampling. Afterwards, the incubation bottles were 9/10 emptied by inverse filtration with a 45 µm mesh-end siphon. The bottles were gently refilled with clean GF/F filtered and oxygen saturated seawater (30 psu) containing the appropriate algal concentration and 10 ml of media as it was done at the start of the experiment. In comparison to the method used here, Takahashi and Onho (1996) changed 2/3 of the water in their bottles every fifth days, but just worked with an egg density of 150 l^{-1}. The abundance and development stage composition of *A. tonsa* in the bottles were determined by counting the copepods in whole sub-samples under a dissection microscope (Olympus SZ40). In order to follow the algal production, two bottles without eggs were given the same treatments. At the termination of the experiment, when all the incubation bottles contained adults in large proportion, the gender ratio was determined for each copepod community.

2.3. Mortality rates of copepod communities

The mortality rates (Z) (natural + sub-sample mortality) of the copepod communities were calculated from the reduction of the total number of organisms alive during the experiments (eggs, nauplii, copepodites and adults) until adulthood. The sub-sampling effect on the copepod communities was neglected (2.22% each day of sub-sampling), but the dilution factor generated when the incubation bottles were refilled after the sub-sampling was taken into account. The actual initial number of viable eggs was calculated by multiplying the number of eggs incubated with the hatching rate. It was assumed that the mortality rates were constant from the egg to the adult stages and density independent. The mortality rates were thus assumed to follow:

$$N_t = N_0 e^{-Zt}$$

Where N_t is the number of organisms at the time t. N_0 is the number of organisms initially, Z is the total mortality coefficient and t is the time in days.

2.4. Performance of females

When adult females were present in all the copepod communities, 10–20 females (0–5 days old) were isolated and incubated individually in 600 ml acid washed polycarbonate Nalgene® bottles with phytoplankton (*R. salina* >40,000 cells ml^{-1}) in order to monitor the egg production. The incubation bottles were fixed on a plankton wheel for 2 or 3 days and the number of produced eggs was counted daily. The entire content of each of the incubation bottles were gently filtered through 2 different meshes (180 and 45 µm) plugged on top of each other. The females were re-incubated in fresh food suspensions. The spawned eggs were counted, incubated in small Petri dishes with *R. salina* and were allowed to hatch for 4 days before they were fixed with acid Lugol. The disappeared eggs were considered as hatched and the HR was calculated. Following the incubation, the females were fixed in acid Lugol, their cephalothorax length was measured and their carbon content calculated according to Berggreen et al. (1988):

$$W = 1.11 \times 10^{-5} L^{2.92}$$

Where W is the female body weight (ng Carbon) and L the prosome length in micrometer.

The specific egg production (SEP) per day was calculated. This represents the quantity of carbon used

per day for egg production (see below) divided with the carbon weight of the specific female. A set of data was only used if the female was alive at the end of the incubation.

All results in egg production and hatching rate of the eggs were compared statistically with a Kruskal–Wallis One Way Analysis of Variance on Ranks and the Students *t*-test.

2.5. Carbon content and dry weight of fresh copepod eggs

Two replicates of 10^4 fresh eggs were rinsed and cleaned and transferred to individual precombusted GF/F filters (1.28 cm diameter). The filters with eggs were washed with 20 ml MilliQ water to remove salt particles. The filters were dried in NUCLON® Multi wells at 50 °C for 24 h and weighed on a METTLER TOLEDO MT5 balance (±1 μg) to a constant dry weight. Ten replicates of 2570 fresh eggs were cleaned and transferred to individual precombusted GF/F filters and MilliQ rinsed. Afterward, they were transferred to individual capsules of tinfoil, dried 24 h at 50 °C and stored in a dessicator. The following day the capsules were closed and processed for determination of carbon and nitrogen content in an elementar analyzer (CE instruments EA1110 CHNS).

2.6. Fatty acid analysis of cold stored copepod eggs

The fatty acid composition of eggs with different storage ages (0, 3, 6, 12 and 20 months) were determined on 2–21 replicate samples by extraction of the lipids by a chloroform methanol mixture (2:1) followed by trans esterification of the egg lipids by acetyl chloride in methanol. The fatty acid methyl esters were analyzed by gas chromatography–mass spectrometry (GC–MS).

A 2:1 chloroform methanol mixture (1 ml) was transferred to a vial with 2000–2500 eggs followed by the addition of 40 μl of a 46 μg ml^{-1} intern standard solution of heptadecanoic acid methyl ester in methanol. The vial was sonicated at 0 °C for 2 h followed by evaporation of the solvent at 60 °C by applying a flow of nitrogen. Reagent solution (1 ml) composed of a mixture of toluene, methanol and acetyl chloride (40:50:10) was added and the vial was heated for 2 h at 90 °C. Aqueous $NaHCO_3$ (5% by weight, 1 ml) was added to the vial and after vigorous shaking the upper layer containing the fatty acids methyl-esters was removed. The water phase was extracted twice with heptane (1 ml) and the combined organic layers were collected and evaporated at 60 °C by a gentle stream of nitrogen. Finally, the methyl esters were re-suspended in chloroform (0.5 ml) and an aliquot of the sample (5 μl) was analysed by GC–MS. The GC–MS instrument was composed of an Agilent 6890 series gaschromatograph equipped with a PTV inlet and an Agilent 5973 mass selective detector. The column was a 60 m Agilent DB23 with an i.d. of 250 μm and a film thickness of 0.3 μm. The carrier gas was Helium at a constant flow rate of 1 ml min^{-1}. The oven temperature program was starting at 60 °C with a temperature ramp of 24.6 °C min^{-1} until 200 °C kept for 10 min followed by a second temperature ramp of 5 °C min^{-1} until 250 °C kept for 3 min. The mass spectrometer was run in selective ion monitoring mode (SIM) by application of the masses m/z=55, 74, 79 and 81. The PTV inlet (Programmable temperature vaporization) was operated in split less mode and with the evaporation program going from 60 to 300 °C with a temperature ramp of 720 °C min^{-1} and kept for 2 min.

2.7. Amino acids analysis of cold stored copepod eggs

For analysis of free amino acids, known numbers of copepod eggs (from 1000 to 1250) were transferred to 1.5 ml plastic vials with 200 or 400 μl MilliQ water and were subsequently stored at −80 °C. Free amino acids in the eggs were extracted on 3–8 replicate samples by heating the samples to 95 °C for 5 min after which the samples were filtered through 13 mm 0.2 μm pore size GHP polypropylene membrane filters (Waters Corporation). Ten microliters of the filtrates were transferred to a standard 1.5 ml HPLC vial with 70 μl 10 mM borate buffer at pH 8.8. Primary and secondary amines in the sample were derivatized with 20 μl 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Yu et al., 1994). An AccQ·Tag kit (Waters Corp.) was used for the derivatization. The derivatives were heated to 55 °C for 10 min to degrade a tyrosine side product that interferes with the chromatographic separation of the derivatized amino acids. The amino acid derivatives were separated on a Waters Alliance 2695 separation module with a 3.9×150 mm Nova-Pak C-18 column. Solvents for the separation were (A) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 5.70, (B) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 6.80 (both solvents were adjusted with H_3PO_4), (C) acetonitrile and (D) water. Solvent composition for the separation of the amino acids was provided by Waters Corp.

The separated amino acid derivatives were quantified by fluorescence (250 nm excitation and 395 nm emission) using a Water 474 scanning fluorescence

detector. In addition to standard protein amino acids, the analysis included α -aminobutyric acid (α-ABA), γ-aminobutyric acid (γ-ABA), ornithine and taurine. Detection limit of the method was about 1 pmol of each amino acid.

3. Results

3.1. Egg viability of cold stored copepod eggs

The HR of the copepod eggs decreased with the time of storage (Fig. 1). The maximum HR (mean±s.d) occurred with fresh eggs (90.3%±3.2%) and showed a decrease for stored eggs (69.9%±2.4%, 78.4%±4.2% and 73.9%±4.2% for 3, 6 and 11 months, respectively). After 12 months of cold storage, the HR decreased to 37%±5.9%, and no eggs hatched after 20 months. Statistical analysis showed that the HR of fresh eggs was significantly different from the HR of all the stored eggs ($P<0.001$; *t*-test). HR of the 12 months stored eggs was significantly different from the HR of all the other groups (3 and 6 months vs. 12 months; *t*-test; $P<0.001$/ 11 vs.12 months; Mann–Whitney $P<0.001$). No significant difference was observed between 3 and 11 months stored eggs ($P>0.1$; Mann–Whitney). The HR of 6 and 11 months stored eggs were not different (*t*-test; $P>0.05$).

3.2. Phytoplankton and oxygen concentrations during incubations

Due to grazing of the copepod communities and growth of the algae, variations in concentration of *R. salina* appeared. Fig. 2A shows a representative evolution of algal concentration in a set of incubation bottles containing a high density of copepods (fresh eggs) besides in control bottles (without copepods). After day 5, and throughout the experiment, the copepod community was grazing a large amount of phytoplankton, forcing the algal concentration to decline to low levels (from >20,000 cells ml^{-1} to <900 cells ml^{-1} within 2 days). When the water was changed it restored the algal concentrations to >20,000 cells ml^{-1}. In contrast, in the control bottles, the algal concentration increased almost twofold in the period between the subsamples suggesting adequate algal growth conditions. By adding phytoplankton every 2 days, starvation was to some extent prevented and competition for food lowered. Fig. 2B shows an average concentration of algae in between two days of sub-sampling in the different experiments. On average, the concentration of *R. salina* never decreased below 10,000 cells ml^{-1} while it reached average concentrations >40,000 cells ml^{-1} when no grazing occurred.

The oxygen concentration also decreased strongly with the increasing concentration of copepods in the incubation bottles but was always >2 mg of O_2 l^{-1} (1.4 ml l^{-1}) (Fig. 3). During the individual female incubations for egg production, the water was 100% O_2 saturated and the algal concentrations >40,000 cells ml^{-1}.

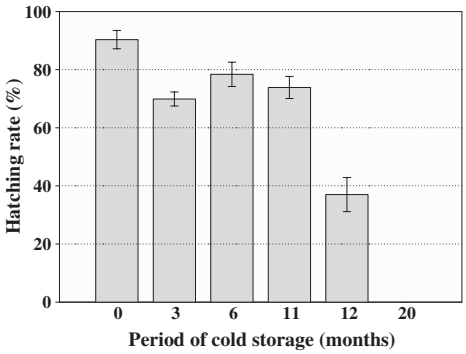

Fig. 1. *Acartia tonsa*. Hatching rate of subitaneous eggs stored at 2–3 °C for different periods of time (mean±s.d.). No hatching of the eggs occurred after 20 months.

3.3. Development of the copepod communities originating from cold stored eggs

The succession of the different development stages was observed in all the incubation bottles except the ones containing the 20 months stored eggs (Fig. 4). Nauplii were present on day 3 and copepodites were found in the sub-samples on day 10, after the nauplii peaked in all age groups. However, adults did not appear at the same time in the different copepod communities. Development times were found to be: 10 days for the fresh eggs, 12 days for 3 and 6 months cold stored eggs and 14 days for the 12 months cold stored eggs. When adults appeared, they immediately initiated reproduction and small peaks of newly spawned eggs/nauplii were observed. Since no hatching occurred from the 20 months stored eggs, neither nauplii nor other development stages were observed but the egg degradation was evident. At the end of the experiments the gender ratio (female/male) were 61%, 61%, 63%, 51% for copepod communities originating from the fresh, 3, 6 and 12 months cold stored eggs, respectively.

Since the number of total organisms (eggs, nauplii and copepodites) counted on day 3 for fresh, 3 and 6

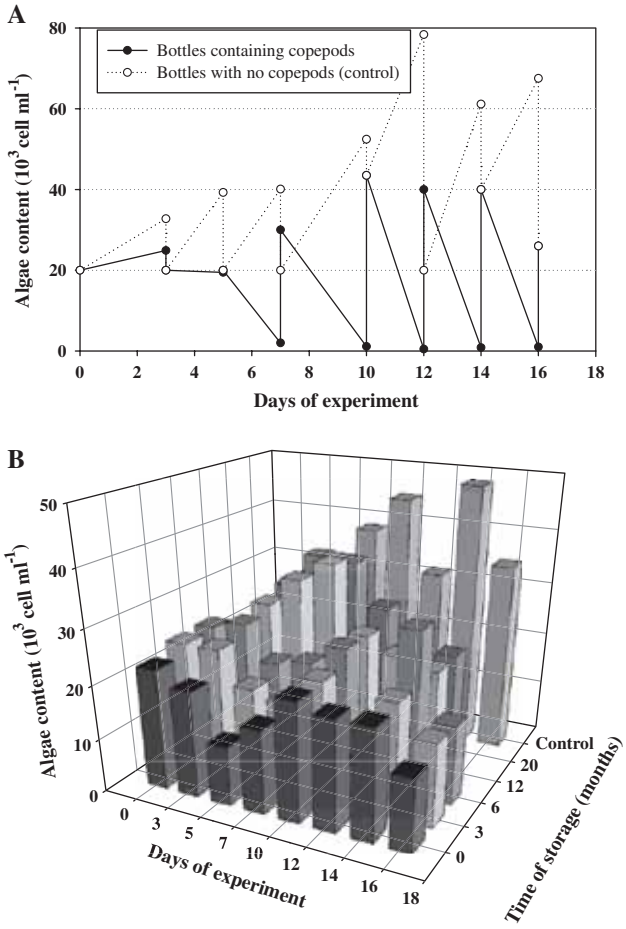

Fig. 2. A. Mean development of the food algae, the 8 μm ESD chryptophyte *Rhodomonas salina* concentration in a set of incubation bottles containing a high density of copepods (originating from fresh eggs, for densities, see Fig. 4) and in control bottles (without copepods). B. Average concentration of *R. salina* in between two days of sub-sampling in the different sets of incubation bottles (duplicates) during the experiment.

months cold stored eggs was very low compared to the following samples, this data was concluded as erroneous and therefore neglected in the calculations of the average mortality rates. *Z* increased continuously with the increasing period of cold storage from 0.035 d^{-1} for fresh eggs up to 0.13 d^{-1} for 12 months cold stored eggs no matter initial organism density (Fig. 5).

3.4. Performance of females originating from cold stored eggs

Acartia tonsa females were incubated for egg production for each cold storage period. During the first experiment, the females produced 10±7.1 egg. $female^{-1}d^{-1}$ for the fresh egg batch, 7.4±4.6 egg. $female^{-1}d^{-1}$ for the 3 months cold stored egg batch and 11.6±7.4 egg.$female^{-1}d^{-1}$ for the 6 months cold stored egg batch. During this experiment, the female incubation bottles were contaminated by histophageous ciliates. During the second experiment however, females from the fresh egg batch produced 28.5±9.2 egg. $female^{-1}d^{-1}$ and females from the 12 months cold stored egg batch produced 32.2±17.8 egg.$female^{-1}d^{-1}$. Fresh eggs from our culture (mean±s.d. diameter: 80.95±4 μm) had a dry weight of 117±14.9 ng egg^{-1}, a carbon content of 58.38±5.3 ng egg^{-1} and a nitrogen

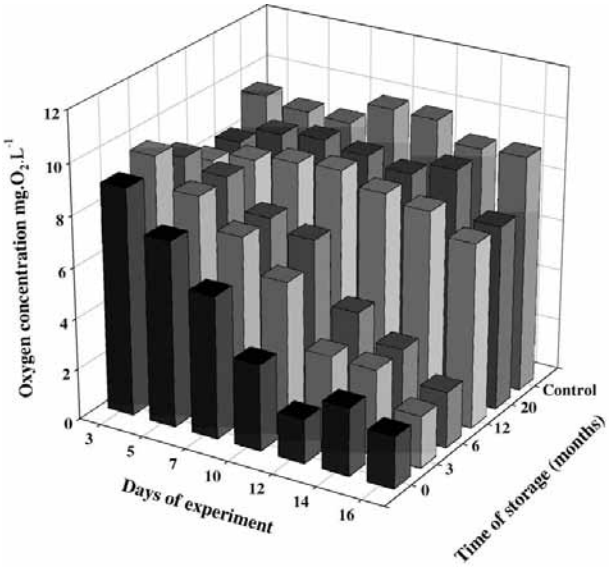

Fig. 3. Oxygen concentrations in the incubation bottles after 2–3 days of incubation. After each sub-sample, the bottles were 9/10 emptied and refilled with 100% O_2 saturated clean water with *R. salina*.

content of 12.05±0.83 ng egg^{-1}. The specific carbon value was used for the calculation of the SEP for each female. For each age group, an average SEP was calculated and rescaled to the control SEP for each experiment (Fig. 6). The rescale operation was necessary to compare results from the two different experiments. There were no significant differences between the SEP calculated from the different cold storage periods (Kruskal–Wallis: $p<0.05$).

The average hatching rates of the produced eggs are presented in Fig. 7. The hatching rate of each batch of eggs was statistically compared to their control (fresh eggs batch) and no differences between batches of eggs of different cold storage periods were found. (Kruskal–Wallis: $p<0.05$; Mann–Whitney: $p<0.05$).

3.5. Biochemical characteristics of the cold stored copepod eggs

The fatty acid analysis was focused on the absolute value (nanogram of fatty acid egg^{-1}) of 20 important unsaturated fatty acids listed in Table 1. The total quantity of the studied fatty acids tended to decrease with the period of cold storage (Fig. 8) decreasing from 5.99 ng egg^{-1} for the fresh eggs to 3.19 ng egg^{-1} for the 20 months old cold stored eggs. An unexplainable high value was found for the 12 months old cold stored eggs (5.18 ng egg^{-1}). Most of the studied fatty acids showed a decreasing pattern during the cold storage. However, an increase of palmitoleic acid (16:1(*n*7)) and arachidonic acid (ARA; 20:4(*n*6)) appeared after 3 months of storage and decreased again until the last tested storage period. Oleic acid contents (18:1(*n*9)) were found in higher amount after 6 and 11 months of cold storage than in eggs stored less than 6 months increasing from 0.19 ng egg^{-1} after 3 months of cold storage to 0.47 ng egg^{-1} after 6 months of cold storage. Some of the unsaturated fatty acids were present in very low concentrations and tended to stay stable or decreased slightly during the storage period.

Six major fatty acids were present in the cold stored egg samples and followed the general decreasing pattern. These 6 fatty acids represented together 80% of the total unsaturated fatty acids and DHA and EPA alone were responsible for half of this. The three quantitatively most important of these were alpha-linoleic acid (18:3(*n*3)), EPA (20:5(*n*3)), and DHA (22:6(*n*3)) and they represented in average, 18%±4%, 21%±5% and 18%±3% of the total amount of unsaturated fatty acids along the storage period. The three other important fatty acids, 18:4(*n*3), 18:2(*n*6), 18:1(*n*7) represented, respectively, 11%±3%, 5%±

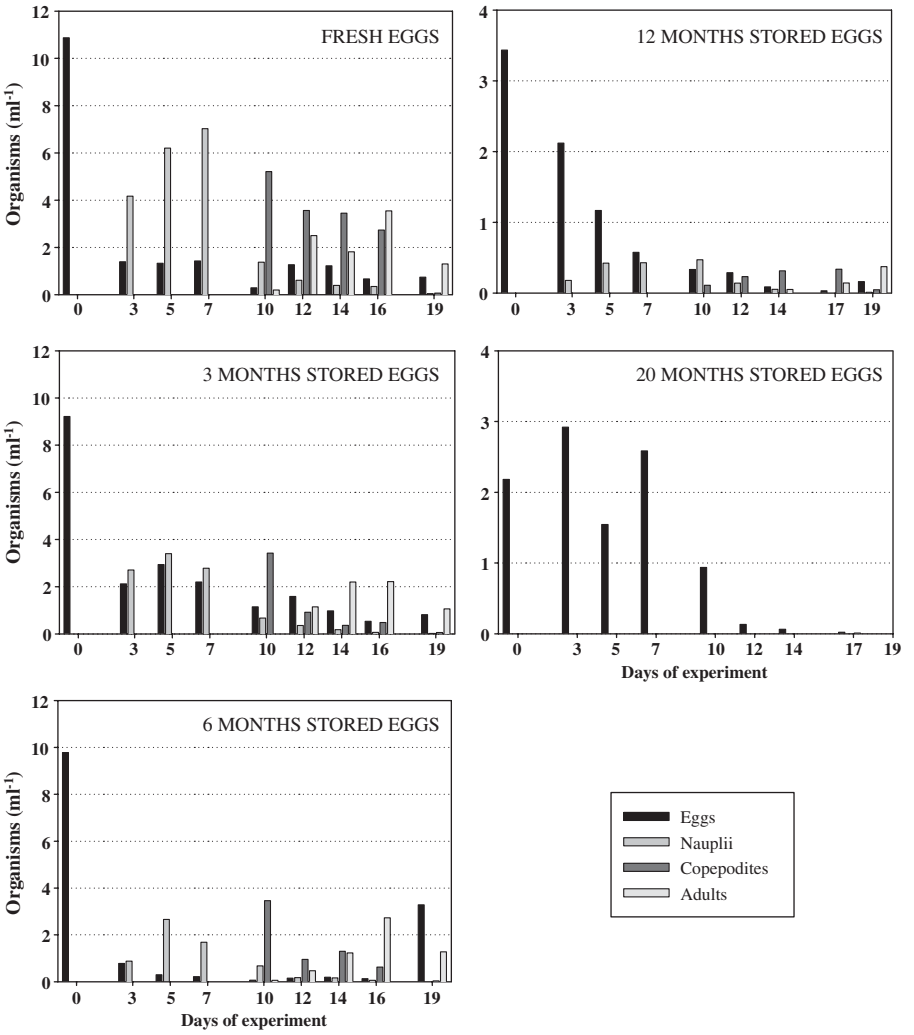

Fig. 4. *Acartia tonsa*. Development of the copepod communities originating from eggs cold stored at 2–3 °C for different periods. All results are mean of duplicate incubations.

2%, 8%±4% of the total amount of unsaturated fatty acids. ARA was not present in high quantity in the eggs but it seemed that its amount increased between 0 and 3 months of storage. In average, during the storage period, 70% of the total unsaturated fatty acids was represented by $n-3$ polyunsaturated fatty acid (PUFA) while monoemes and $n-6$ unsaturated fatty acids represented, respectively, 20% and 10% of the total amount. Statistical differences existed between the total content of fatty acid in the cold stored eggs. The differences existed between 0 and 3 months (Mann–Whitney Rank Sum Test: $P<0.05$), 0 and 6 months (Mann–Whitney Rank Sum Test: $P<0.05$), 0 and 12 months (Mann–Whitney Rank Sum Test: $P<0.05$) and between 0 and 20 months (Mann–Whitney Rank Sum Test: $P<0.05$). Since only 2 data were present for the 11 months batch of eggs, no statistical analyses were made.

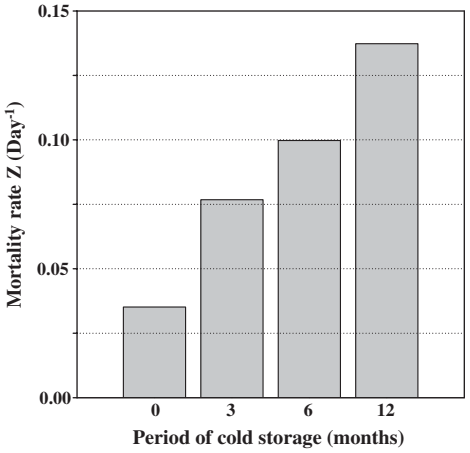

Fig. 5. *Acartia tonsa*. Average mortality rates (*Z*) of the copepod communities in the incubation bottles after different periods of cold storage at 2–3 °C of the eggs. No error bars are present since *Z* results are a mean of duplicate incubations.

The total amount of free amino acids in the eggs was stable between 51 and 62 pmol egg^{-1} during the first 12 months of cold storage and decreased considerably afterwards to reach 13 pmol egg^{-1} after 20 months (Fig. 9). These results were equivalent to a concentration of free amino acids ranging from 436 to

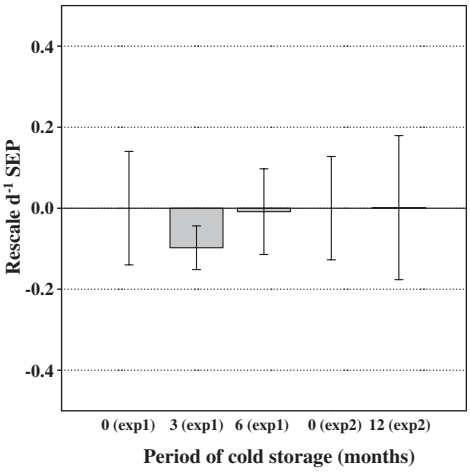

Fig. 6. *Acartia tonsa*. Mean±s.d. rescaled Specific Egg Production of females originating from eggs cold stored at 2–3 °C for different periods. All data from the 2nd experiments have been rescaled based on respective batches of fresh eggs (control) so that all data can be compared.

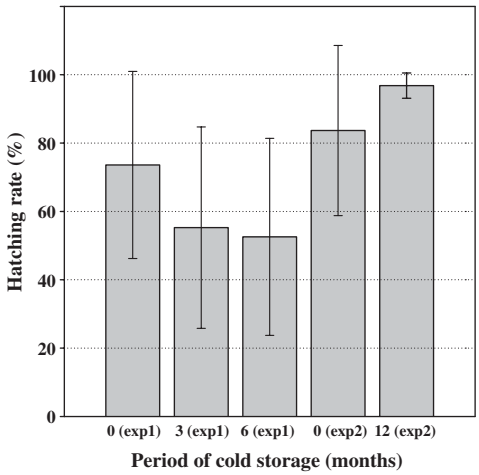

Fig. 7. *Acartia tonsa*. Mean±s.d. hatching rates of eggs produced by females originating from eggs cold stored at 2–3 °C for different periods. Females grown from cold stored eggs have been separated into two experiments. The hatching success of the newly produced eggs needs to be compared to the fresh control batch from the same experiment.

530 μmol g^{-1} (DW) for the 0–12 months cold stored eggs and 111 μmol g^{-1} (DW) for the 20 months cold stored eggs. However, no statistical differences in the total content of amino acid were observed between fresh eggs and eggs cold stored for 6, 11 and 12 months. Only the 3 and 20 months cold stored eggs contained quantities statistically different from the non-stored eggs (0–3 months: Student's *t*-test: $P<0.05$; 0–20 months: Student's *t*-test: $P<0.01$). Table 2 shows a list of the free amino acids present in the samples and their proportions. From the fresh eggs to the 12 months old cold stored eggs, the amino acids present in highest amount were (from the highest proportion to the lowest) Pro, Ala, Tau, Glu, Gly, Arg, Lys. Each of these represented more than 3% of the total amino acids present in the eggs. Pro alone represented up to 30%±2% mean±s.d. of the total amino acids. Only Lys quantities were <3% for the 6 and 12 months old cold stored eggs. Occasionally some other amino acids represented >3% of the total amount but were still representing less than 3% in average along the egg storage period (e.g. His, Ser, Asp). The pattern was different for the 20 months old cold stored eggs where the free amino acids present in highest amount were Asn, Glu, Pro, Lys, Ala, Leu, Arg, Ser, Val and each of these represented more than 3% of the total free amino acids. However, the standard deviations were high for

Table 1
Acartia tonsa. Unsaturated fatty acid composition of eggs after different periods of cold storage at 2–3 °C. Results are presented as mean±s.d. in ng egg^{-1}

Fatty acids	Fresh	3 months	6 months	11 months	12 months	20 months
No of samples	21	4	6	2	3	3
14:1	0.05±0.03	0.07	0.05±0.02	0.06	0.03±0.02	0.00
16:1 *n*−7	0.14±0.08	0.37±0.34	0.17±0.07	0.10	0.25±0.21	0.02
18:1 *n*−9	0.35±0.39	0.19±0.10	0.47±0.19	0.39	0.16±0.14	0.07±0.01
18:1 *n*−7	0.33±0.08	0.30±0.11	0.25±0.10	0.22	0.41±0.34	0.54±0.05
20:1 *n*−9	0.03±0.02	0.01	0.02±0.01	0.01	0.02±0.02	0.02
22:1 *n*−11	0.04±0.03	0.03±0.01	0.05±0.02	0.03	0.02±0.01	0.03±0.01
24:1	0.09±0.15	0.04±0.01	0.03±0.01	0.01	0.05±0.05	0.06±0.01
Total monoemes	*1.02*	*1.01*	*1.05*	*0.82*	*0.94*	*0.74*
18:3 *n*−3	1.26±0.48	0.65±0.08	0.85±0.62	0.43	1.17±0.96	0.50±0.04
18:4 *n*−3	0.77±0.35	0.46±0.07	0.34±0.24	0.46	0.43±0.35	0.32±0.02
20:3 *n*−3	0.05±0.01	0.03±0.01	0.04±0.01	0.05	0.03±0.02	0.06±0.01
20:4 *n*−3	0.05±0.01	0.04±0.01	0.03±0.01	0.03	0.02±0.02	0.03
20:5 *n*−3	1.19±0.54	1.54±1.10	0.79±0.29	0.51	0.92±0.76	0.61±0.07
22:5 *n*−3	0.07±0.02	0.12±0.06	0.08±0.01	0.07	0.06±0.05	0.08
22:6 *n*−3	1.09±0.32	0.84±0.16	0.84±0.34	0.42	1.03±0.84	0.64±0.07
Total n−3	*4.48*	*3.68*	*2.97*	*1.97*	*3.67*	*2.24*
18:2 *n*−6	0.31±0.24	0.26±0.06	0.31±0.20	0.11	0.38±0.31	0.09±0.01
18:3 *n*−6	0.02±0.01	0.03±0.01	0.02±0.02	0.01	0.03±0.02	0.00
20:2 *n*−6	0.06±0.02	0.06±0.02	0.07±0.02	0.03	0.07±0.06	0.06±0.01
20:3 *n*−6	0.00	0.01	0.00	0.00	0.01±0.01	0.00
20:4 *n*−6	0.07±0.02	0.08±0.02	0.05±0.02	0.02	0.06±0.05	0.03
22:5 *n*−6	0.03±0.01	0.05±0.01	0.03±0.01	0.02	0.03±0.02	0.02
Total n−6	*0.49*	*0.49*	*0.47*	*0.20*	*0.57*	*0.21*
Total poly-unsaturated	*4.97*	*4.17*	*3.45*	*2.16*	*4.24*	*2.45*
DHA/EPA	0.91	0.55	1.06	0.83	1.12	1.04
EPA/AA	17.5	19.1	17.1	21.3	14.7	18.0

some of the amino acids in the different groups of cold stored eggs rendering a direct comparison of the amino acids present in low quantities impossible.

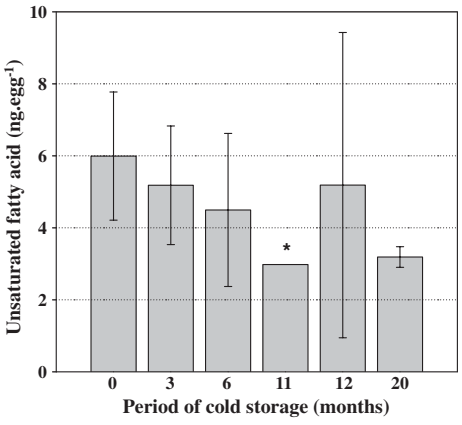

Fig. 8. *Acartia tonsa*. Total unsaturated fatty acid contents in ng egg^{-1} after different periods of cold storage at 2–3 °C (mean±s.d.). *No standard deviation was calculated for the 11 months cold stored eggs since 2 samples were processed.

4. Discussion

The experiments were performed to understand the effects of cold storage on eggs of *Acartia tonsa*. The hatching rate of the cold stored copepod eggs decreased as the period of cold storage increased. After a storage period of 20 months, *A. tonsa* eggs did not hatch. However, no eggs stored for periods between 12 and 20 months were tested. Therefore we cannot exclude that any eggs can hatch after 12 months of cold storage. Payne and Rippingale (2001b) stored fresh nauplii of *Gladioferens imparites* for up to 12 days at 8 °C (survival 99%) but mortality was almost total after 36 days and therefore it seems more interesting to store eggs than their nauplii for long period. Other reports regarding the effect of copepod eggs storage upon their viability exist, but none of them described the effects over the first generation and further on its offspring. Marcus and Murray (2001) tested the viability of diapause eggs of *Centropages hamatus* after a period of storage under anoxia. More than 80% of the eggs hatched when stored for 4–17 months at 25 °C. Only 44 copepods species belonging to Acartiatiidae, Centropagidae, Pontellidae and Temoridae have been described

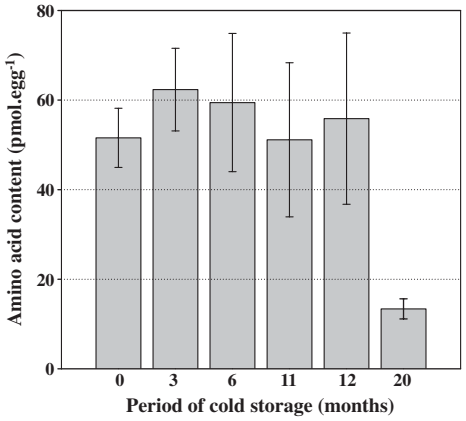

Fig. 9. *Acartia tonsa*. Total amount of free amino acids in pmol egg^{-1} after different periods of cold storage at 2–3 °C (mean±s.d.).

to produce diapause eggs (Mauchline, 1998). However, there are a lot of difficulties regarding forced induction of the production of diapause eggs. In the present experiments, we stored subitaneous eggs at low temperature at which they switch to a quiescent state due to suboptimal environmental conditions. Thus, it is shown in the present study that cold stored egg viability is kept high for 12 months. However, it is possible that the findings for the investigated culture of *A. tonsa* cannot be extrapolated to other populations of the same species since genetic variations have been shown for geographically distinct *A. tonsa* populations (Caudill and Bucklin, 2004). Also, the long term cultivation under constant conditions is likely to cause genetic drift.

It seems obvious that the embryon still develops or retains a physiological activity during cold storage indicated by biochemical changes of the eggs. Støttrup et al. (1999) showed that the fatty acid composition of *A. tonsa* nauplii hatched from cold stored eggs changed over time in a similar manner to the eggs in the present study. These changes possibly reflect a slow and partly completed fatty acid metabolism by the embryon. Recently we have documented heat flux, i.e. metabolism in eggs under anoxia by isothermal microcalorimetry (Nielsen et al., submitted for publication). The resulted changes in biochemical composition during cold storage obviously decrease the egg viability. After long storage

Table 2
Acartia tonsa. Free amino acid composition of the eggs after different periods of cold storage at 2–3 °C. Results are presented in mean±s.d. percentage of each amino acid compared to the total pool (100%)

Amino acids (percentage of total AA)	Fresh	3 months	6 months	11 months	12 months	20 months
No of samples	8	8	6	3	6	5
Asp	1.34±0.16	1.68±0.22	1.31±0.27	1.45±0.09	1.18±0.29	2.45±0.28
Glu	10.43±0.95	9.49±1.5	7.49±1.59	3.99±0.59	7.32±0.61	13.29±1.41
Ser	3.41±0.59	3.66±0.89	2.63±0.66	2.17±2.27	2.9±40.88	4.18±3.72
Asn	0.76±0.09	3.80±1.52	1.79±1.56	1.53±2.08	5.62±0.85	14.89±2.57
AMQ	2.87±0.37	2.15±2.35	3.09±2.44	6.75±1.72	0.77±0.16	2.23±0.62
Gly	4.37±0.39	3.59±0.49	4.12±0.80	9.03±0.75	4.61±0.49	2.68±0.31
Gln	3.54±0.25	2.35±0.14	6.02±0.44	0.94±0.82	1.64±0.28	1.28±0.15
His	4.14±0.22	1.21±0.79	1.93±1.24	3.46±2.36	0.96±0.07	1.25±0.67
NH3	3.83±2.13	4.71±0.5	3.92±1.05	5.42±0.76	7.02±2.41	12.91±1.38
Tau	7.91±0.56	9.31±0.57	7.67±1.07	13.10±1.4	6.19±0.43	2.25±0.36
Thr	2.13±0.11	1.58±0.26	1.86±0.42	3.26±0.15	2.18±0.22	2.31±0.59
Arg	4.37±1.13	3.86±0.48	3.57±0.61	5.1±10.40	4.20±0.23	4.26±0.34
Ala	6.52±3.15	9.95±0.31	10.97±0.26	7.16±1.25	13.68±0.78	5.17±0.38
Gaba	0.00	0.40±0.38	0.4±50.01	0.20±0.35	0.39±0.09	0.00
Pro	36.59±2.41	30.44±1.07	35.65±1.63	22.05±2	28.79±2.25	8.41±1.09
Aaba	0.10	0.00	0.05±0.01	0.00	0.06±0.04	0.00
Tyr	1.06±0.05	0.99±0.06	0.94±0.18	1.79±0.56	0.97±0.04	1.75±0.16
Val	1.54±0.07	1.97±0.15	1.77±0.19	2.35±0.09	2.48±0.09	3.85±0.55
Met	0.77±0.04	0.76±0.05	0.75±0.04	0.88±0.09	0.72±0.05	1.20±0.10
Ile	1.08±0.05	0.91±0.22	1.01±0.26	1.69±0.11	1.05±0.17	1.84±0.36
Orn	0.22±0.13	0.89±0.35	0.75±0.3	0.37±0.22	1.45±0.17	1.76±0.28
Leu	1.56±0.07	1.73±0.11	1.56±0.05	2.24±0.12	2.00±0.09	4.48±0.74
Lys	4.47±0.31	3.97±0.51	2.70±0.5	3.73±0.75	2.77±0.1	5.80±0.61
Phe	1.03±0.04	0.98±0.07	1.03±0.21	1.43±0.24	1.05±0.05	1.78±0.31
Trp	0.00	0.00	0.00	0.00	0.00	0.00

periods, the quiescent eggs may not be able to hatch due to the lack of exploitable energy.

4.1. Incubation of cold stored eggs

The development time to reach adulthood was normal during our experiments (Fig. 4). According to Berggreen et al. (1988) optimum development rate for *A. tonsa* fed *R. baltica* led to adulthood after 12.5 days and Medina and Barata (2004) also observed arrival of adults at day 12 in their cultures at comparable temperatures (20 °C). This confirms that no adverse conditions like severe starvation occurred during our incubation experiments. Furthermore, the fastest development time (10 days to reach adulthood) occurred in bottles with the highest initial organism density. This differs from the results found by Medina and Barata (2004) where a density dependent decrease of egg production and a slower development time was observed when increasing the copepod density from 500 to 2000 adult copepods l^{-1}, supporting sufficient conditions in our copepod incubations.

After 2 days of incubation, the most concentrated bottles containing developed copepods reached hypoxic conditions (2 mg O_2 L^{-1}) due to high density of copepods and degradation of faecal-pellets. These short time hypoxic conditions are not considered to interfere with the development of the copepod communities since Marcus et al. (2004) observed that 1.4 ml l^{-1} O_2 (2 mg O_2 l^{-1}) did not increase the mortality but resulted in lower fertility among *A. tonsa*. At 0.7 ml O_2 l^{-1} (1 mg O_2 l^{-1}) both fertility and mortality were affected. Furthermore the low oxygen concentration was only observed in the copepod communities originating from one-week old eggs which experienced the lowest mortality rate. Though it was shown by Lutz et al. (1994) that low oxygen concentrations decreased the viability of the eggs and increased the mortality of copepods, we can exclude the problem in the present study.

The incubation bottles were not strongly shaken before sub-sampling. This was decided on in order to avoid stressing the copepods. However, cluster formations of the eggs occurred limiting the accuracy of the sub-sampling method.

Adults occurred already at day 10 in the copepod community originating from non-stored eggs and appeared at day 14 for the 12 months cold stored eggs. Very old eggs (20 months cold stored eggs) only had a minor part of organic matter visible in the middle of the eggs (visual inspection in microscope). Generally they did not hatch but their degradation was delayed, and it could take up to 10 days before they started to degrade. In comparison, nonviable eggs freshly produced started their degradation during the first day of incubation. When eggs are disintegrating, we can observe a halo of filamentous bacteria growing around the shell of the eggs; the shell looks then more brownish and a bit opaque. When the eggs hatch, the remaining egg shell does not present these bacteria and the shell is broken. Therefore, it is relatively easy, just by thorough observation to see. With the coloration of Lugol's these differences appear even more clearly. In our experience, disintegrating eggs are more easily observable than disintegrating nauplii, which disappear faster. The nauplii hatched from fresh eggs might develop faster than the nauplii hatched from cold stored eggs. This was not observed before the copepod communities reached the adult stages since the development stage distinction was only made between eggs, nauplii, copepodites and adults. Generally, a slower stage development was observed among the copepods originating from eggs with an increased cold storage period. The nauplii hatching from the cold stored eggs probably need to build up new reserves as soon as they start feeding as a prerequisite condition for continuing stage shifts. The nauplii hatching from fresh eggs are suggested to have more energy left from the yolk sac and therefore need less time to rebuild these reserves before they molt to the next nauplii stage. But this theory needs further investigations.

4.2. Mortality of the copepod communities originating from cold stored eggs

High mortalities are commonly observed in copepod cultures probably attributed to the handling of the copepods. Medina and Barata (2004) showed mortality rates varying from 0.07 to 0.0975 d^{-1} and Kiørboe et al. (1985) described mortality rates below 0.1 d^{-1} when females of *A. tonsa* were at a density of 25 l^{-1} while Berggreen et al. (1988) observed mortality rates up to 0.59 d^{-1} when starving *A. tonsa*. It was observed that the copepod community originating from the 12 months cold stored eggs experienced a high mortality when compared to those originating from fresh eggs. Mortalities found in our experiments (0.035–0.130 d^{-1}) were however within the range of reported mortalities. Since physical–chemical–biological conditions among the incubation bottles were quite similar and since the organism densities in the copepod communities originating from fresh eggs were the highest, the density effect can be excluded to explain the observed differences in mortality. The explanation for the higher

post-hatching mortalities among copepod communities originating from the cold stored eggs must be ascribed to the biochemical changes occurring during cold storage. This could result in higher mortality of the nauplii, before these can feed since copepods go through their first nauplii stages without eating (e.g. Berggreen et al., 1988), but spend a lot of energy to molt (Mauchline, 1998). Calculating the post-NIII mortality could have been informative to determine the cold storage effect on mortality after the nauplii become depending on external food, but was unfortunately not possible in the present experiment. We hypothesize that when nauplii start feeding no difference in mortality will be observed, but this remains to be documented.

4.3. Egg production and viability of the eggs—the fitness of the next generation

No statistical differences were observed in fertility and egg viability among the females originating from cold stored eggs of different storage periods. The egg production results from the first experiment were very low, probably due to the presence of ciliates in the bottles. Low egg production is commonly observed in our 70 l copepod culture tanks when ciliates are present in large densities (Personal observation). If this is due to direct food competition or some direct effect on the copepod community itself remains unknown. The egg production of the second experiment however was higher and comparable to what is reported in the literature. According to Tiselius et al. (1995), cultivated *A. tonsa* produced between 20 and 30 egg female^{-1} d^{-1} when fed *Thalassiosira weissflogii,* and Medina and Barata (2004) showed a maximum egg production of 32 eggs female d^{-1} when fed with a mixture 1:1 of *Isochrisis galbana* and *Rhinomonas reticulata*.

In our first experiment, SEP was only 0.15, 0.076 and 0.15 d^{-1} for females originating from 0, 3 and 6 months cold stored eggs, respectively. Results from the second experiment (0.34 and 0.34 d^{-1} for 0 and 12 months cold stored eggs) comparable to Berggreen et al. (1988) reporting a maximum SEP of 0.44 d^{-1}, clearly suggest that the cold storage of the eggs do not have any effects on the resultant egg production by the 2nd generation females. However, the present study followed the SEP for a period of 3 days in the beginning of the female production because the potential effects of cold storage are likely to appear from the beginning of the reproduction period. Some females appeared earlier in some of the incubation bottles than in the others. Therefore these females have produced eggs in the incubation bottles prior to the egg production experiment. This is not likely to influence the results since egg production is known to reflect mainly the food availability which is similar in the different groups (Mauchline, 1998).

However, from a cultivation point of view, the food supply needs to be adjusted depending on species, development stage, copepod density and all environmental conditions in order to optimize the egg production and the somatic growth and thereby achieving a culture which is commercially interesting (e.g. Berggreen et al., 1988; Kleppel and Burkart, 1995; Jonasdottir and Kiørboe, 1996; Huskin et al., 2000; Murray and Marcus, 2002; McKinnon et al., 2003).

4.4. Hatching and nutritional value of copepod eggs

The hatching rate of the second generation eggs ranged from 52.6% (6 months cold storage) to 97.4% (12 months cold storage) and no statistical differences were detected. The high HR furthermore indicated that *A. tonsa* received appropriate food quantity and food quality since these parameters are crucial for the HR (Tang and Dam, 2001).

Støttrup (2000) and Støttrup and McEvoy (2003) reviewed the advances in copepod production systems and explained the role of their nutritional value especially the lipid content of the copepods. Many studies on larval pigments in reared fish showed that ingestion of copepods led to lower incident of malpigmentation than when the fish were reared on the traditional live food (McEvoy et al., 1998). The same was shown for wild harvested plankton (Næss et al., 1995). PUFA and the ratio between essential fatty acids (e.g. DHA/EPA) are very important for larval development. It has been proven that copepods contain more PUFA and a far better ratio of essential fatty acids than other live prey organisms even when those are enriched (e.g. Støttrup et al., 1999; Toledo et al., 1999; Payne et al., 2001).

Linoleic acid (18:2($n-6$)) and alpha-linolenic acid (18:3($n-3$)), which are synthesized by plants, are the precursor of all $n-6$ and $n-3$ PUFA. Since $n-6$ and $n-3$ PUFA are very important for fish larvae they need, to be ingested though the diet. Marine fish have been reported unable to biosynthesize DHA de novo from shorter chain precursors such as 18:3($n-3$) (review by Sargent et al., 1999). Therefore, the contents of EPA and DHA in the food are essential for fish development and appear to have a special function in the phospholipids of the nervous system and in the eye (Christie, 2003). EPA and DHA in our egg samples were found in very high proportions as compared to the total amount of

unsaturated fatty acids as reported for adult *Eurytemora* sp. and *Temora longicornis* (Evjemo and Olsen, 1997). In our samples, $n-3$ PUFA represented on average 70% of the unsaturated fatty acids. High proportions of $n-3$ FA especially EPA and DHA are common in copepods and wild zooplankton (Næss et al., 1995; Evjemo and Olsen, 1997; Støttrup et al., 1999). Therefore copepods are recommended for first feeding of fish in farming industries (Støttrup et al., 1999; Støttrup and McEvoy, 2003; Engell-Sørensen et al., 2004). Moreover, the studied copepod eggs were also quite rich in linoleic acid which is also an essential fatty acid in marine fish larvae. ARA which is known to be crucial since it is an essential component of the membrane phospholipids was not present in great amount in the present eggs in comparison to the other PUFAs. However, ARA contents of the eggs increased after 3 months. This is comparable to the only other published study of biochemical consequences of cold storage of *Acartia tonsa* eggs by Støttrup et al. (1999), who found a similar pattern working on nauplii which originated from cold stored eggs from the same culture.

The pool of free amino acids was stable during cold storage as long as the eggs were able to hatch, and decreased thereafter, revealing that eggs do not primarily use amino acids as an energy source. Despite that the hatching rate was decreased after 12 months, the un-hatched eggs might still be alive, but might lack energy to hatch. Live eggs might protect themselves from degradation and therefore from depletion of their free amino acid contents. This protection will last until the increased metabolism due to the increase of temperature kills the eggs. This could explain why un-hatched eggs are degraded relatively slowly as observed during our viability experiments.

The main free amino acids present in our samples are the same as the quantitatively most important ones reviewed by Båmstedt (1986) for 6 calanoid copepod species. Also, the total free amino acid contents per gram (DW) in our samples are comparable to the *Acartia tonsa* contents presented by Båmstedt (1986). However, the present investigation also revealed a high proportion of glutamate which has not been described before as a quantitatively important amino acid in calanoid copepods. Presence of high levels of taurine was also detected. Taurine is known to stimulate growth rates in turbot, *Scophthalmus maximus* (Conceição et al., 1997) and might also be an advantage or requirement for other fish species that are unable to synthesize it themselves (Takeuchi, 2001). For the 20 months old cold stored eggs, the pattern of the most important amino acids is different probably due to propagation of the degradation process during storage. However, since no hatching occurred after this period of cold storage, data are not relevant for an aquaculture point of view. Several amino acids have been described as feeding stimulants for fish larvae and Helland et al. (2003) showed that the amount of proteins and free amino acids in wild zooplankton (mainly copepods) was higher than in enriched *Artemia*. In this study, the origins of the amino acids were not investigated but the pool of free amino acid is likely to be different from other copepod species since it seems to follow a species specific pattern (Helland et al., 2003).

No complete sets of biochemical data are available on copepod eggs in the literature. This is presumably due to difficulties in producing them in large amounts necessary for the analysis. Since it is the nauplii that are used as live feed, a knowledge about biochemical composition of the nauplii is needed. Fatty acid and amino acid patterns like other reserves and compounds are likely to be different in the nauplii as compared to the eggs since the egg shells are lost during hatching and because reserves are used or transformed during the early development of the nauplii. This issue will be investigated in future studies.

5. Conclusions

There is still a need for live prey in aquaculture and copepods are good candidates. The superior nutritional value of the eggs and the nauplii is well documented. Additionally the subitaneous eggs tolerate cold storage for up to 12 months. These observations are encouraging and hopefully will promote research projects that aim to develop technological improvements for the culture of copepods in aquaculture.

By decreasing the temperature, the instantaneous hatching of newly spawned eggs decrease or stop and eggs switch to a quiescent stage in which they can stay until the environmental conditions become favourable. However, eggs undergo some biochemical changes that decrease their viability to hatch and might increase the mortality of the succeeding non-feeding nauplii. These effects of cold storage might change depending on other factors like salinity, light, oxic conditions and presence of organic compounds in the storage media. These storage conditions should be investigated in order to further improve egg storage and thereby the development of copepod use in aquaculture. The quality of viable eggs decreases during the period of storage and might create a problem for its use as live food after several months of storage. However, storage of the eggs is revealed to be an excellent way to keep inoculae of copepods to initiate a new lagoon producing fresh eggs/

nauplii in great amounts to feed fish larvae. The cost of copepod production remains high because of the lack of knowledge in copepod culture engineering. Nevertheless, it has often been suggested that using copepods as live feeds might be a durable strategy for raising small-mouthed fish species which experience high larval mortalities.

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MANUSCRIPT 2

Biochemical and technical observations supporting the use of copepods as live feed organisms in marine larviculture

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Abstract

The use of live prey is still necessary for a large number of raised fish species. Small sized rotifers are usually used as live preys during the first days of feeding in small mouth fish. An alternative to this is the use of copepods as prey for first feeding. In this study, the sizes, weight and biochemical contents of two copepods and one rotifer species raised on similar algal food conditions were compared. Rotifers contained a higher proportion of essential amino acids in the free amino acid (FAA) fraction (43%) than copepods (30–32%). However, rotifers had lower levels of important fatty acids like DHA (7% compared with 23–32% in copepods) and their DHA/EPA ratio was lower than that in copepods (0.54 compared with 1.35–1.63 in copepods). The FAA pattern of the preys tended to be species-specific and its implications from an aquaculture point of view is discussed. In contrast, the-protein bound amino acids tended to be very conservative among the studied organisms. The second part of the work is focused on 'the price' of hatching in *Acartia tonsa* eggs before or after cold storage at 3 °C. The fatty acid contents in *A. tonsa* tended to decrease with the storage time. It also decreased with hatching of the nauplii, but its proportion compared with the dry weight remained constant.

Keywords: copepod, rotifer, *Acartia*, *Tisbe*, *Brachionus*, fatty acid, amino acid

Introduction

The results concerning first feeding of commercially important fish on dry food organisms are encouraging (Fernández-Díaz & Yúfera 1997; Cahu & Infante 2001). However, live feeds cannot always be substituted because of biochemical and behavioral constraints of the fish larvae. Much work on the subject has recently been reviewed by Støttrup (2003). Raised copepods as well as harvested zooplankton contain biochemical characteristics that make them a good alternative or supplement live food for larval rearing (Næss, Germain-Henry & Naas 1995; Shansudin, Yusof, Azis & Shukri 1997; Støttrup & Norsker 1997; McEvoy, Næss, Bell & Lie 1998; Rønnestad, Helland & Lie 1998; Payne & Rippingale 2000; Støttrup 2000; Payne, Rippingale & Cleary 2001; Evjemo, Reitan & Olsen 2003). Copepods are already used semi-extensively on an industrial scale (Toledo, Golez, Doi & Ohno 1999; Engell-Sørensen, Støttrup & Holmstrup 2004). They are also part of the natural fish preys present in aquaculture ponds (Sipaùba-Tavares, Bachion & Braga 2001), and promising results from some sustainable intensive cultures have been reported (Sun & Fleeger 1995; Schipp, Bosmans & Marshall 1999; Payne & Rippingale 2001b). Despite the fact that good advances have been achieved in copepod production methods, the storage still remains an important challenge to be investigated.

Gladioferens imparites nauplii have been shown to survive cold storage (8 °C) for up to 12 days with almost no mortality (Payne & Rippingale 2001a). Another solution resides in copepod diapause eggs that can be stored for long periods of time and shipped anywhere at room temperature and used as inocula for cultures (Marcus & Murray 2001). However, it is also possible and presumably easier to store subitaneous copepod eggs at cold temperature and still retain their viability and biochemical quality

(Drillet, Iversen, Sørensen, Ramløv, Lund & Hansen 2006).

As has been shown for the amino acid and protein content in *Artemia* spp. (Helland, Triantaphyllidis, Fyhn, Evjen, Lavens & Sorgeloos 2000), the contents of these substances in copepods are likely to vary depending on species and their origin, and also on the culture methods in use. More work needs to be done in this direction in order to identify the right live feed candidates to offer choices to the fish farmers depending on their specific needs for fry. If we seriously aim at utilizing copepods in fish farming, we need to explore the potential of copepod nauplii, qualitatively and quantitatively, and compare it with other live feeds of which we have proper knowledge. Some progress has been made but most of it has been focused either on wild zooplankton and compared with *Artemia* or on a single species of cultivated copepod compared with a single strain of cultivated rotifer. For many purposes in larviculture, *Artemia* remains too large a prey for the first days of larval feeding, and so the quality of the live prey needs to be compared with relatively similar-sized preys, i.e. copepod nauplii and rotifers. In the present study, we analysed and compared selected biochemical traits of two cultured copepods (*Acartia tonsa* (Dana) and *Tisbe holothuriae* (Humes)) with the rotifer *Brachionus plicatilis*, all cultured on the same unialgal diet and processed with the same method. From earlier work, we knew the fatty acid pattern of *A. tonsa* nauplii from the same continuous copepod culture used in this study (Støttrup, Bell & Sargent 1999) and we knew the biochemical quality of eggs, also from the same culture (Drillet *et al.* 2006). But to our knowledge, only a few authors have described the biochemical changes occurring when the embryo develops in the egg and finally hatches to nauplii. The eggs and nauplii from *Calanus finmarchicus* and *Calanus helgolandicus* have been studied by Laabir, Poulet, Cueff and Ianora (1999) and Helland, Nejstgaard, Humlen, Fyhn and Båmstedt (2003a) with respect to the amino acid fraction. In the present study, we describe the changes in the pattern of amino acids and fatty acids (FA) before and after the hatching process of fresh eggs and up to 9 months cold-stored *A. tonsa* eggs. We discuss the biochemical consequences of the cold storage process of copepod eggs that could be used in larviculture, either as an inoculum or for direct use as live feed.

Material and methods

Algae culture

The 8 μm ESD chryptophyte *Rhodomonas salina* culture was grown under constant light 150 ± 5 μmol photons $m^{-2} s^{-1}$ (Licor-198 with Li-192 SR.Nr. UWQ 4294 sensor, Li-Cor, Lincoln, NE, USA) using 500 mL to 6 L autoclaved glass balloons filled with B1 phytoplankton culture media (Hansen 1989). The phytoplankton was grown at 17 °C in a walk-in climate room and the concentration was monitored with an electronic particle counter (Z2 COULTER Counter, Beckman Coulter, Miami, FL, USA).

Live feeds involved

Copepod eggs (*A. tonsa*) from the Danish Institute for Fisheries Research (DIFRES) in Charlottenlund (Denmark-EU) were used for the experiments. These eggs had been produced by a 20-year-old indoor *A. tonsa* culture fed *R. salina*. These copepod cultures were held in cylindrical tanks, in the dark at 19 °C, in 0.2 μm filtered sea water (30 g L^{-1}) with gentle bubbling. The daily produced eggs were rinsed with pasteurized sea water and stored in closed test tubes in the dark at 2–3 °C for different periods of time (non-stored, 5 months and 9 months cold-stored). The test tubes were totally filled with 0.2 μm filtered sea water (30 g L^{-1}) and closed with nonpermeable lids. The rotifer culture (*B. plicatilis*, medium strain) was kept at Roskilde University (RUC) in 6 L round-bottom glass balloons with heavy bubbling. The cultures were kept at 25 °C in 0.2 μm filtered sea water (30 g L^{-1}) and fed *R. salina* in excess. Before the analysis, the entire culture was filtered through two NITEX mesh screens (90 and 70 μm) in order to separate the required fraction of rotifers for which the size was comparable with the size of the *A. tonsa* nauplii. The *T. holothuriae* culture was kept in a 15 L tank filled with 0.2 μm filtered sea water (30 g L^{-1}), and the tank contained broken glass pipettes (3–5 cm) in order to increase the growing surface for the copepods. The culture was kept in a walk-in climate room at 15 °C. The nauplii were separated from the adults by successive filtrations through 90 and 70 μm NITEX mesh screens as for the rotifers.

The body sizes of all the organisms were measured under an Olympus SZ40 dissection microscope (Olympus Optical (Europa) GmBH, Hamburg, Germany) at × 80 magnification. Copepod eggs, nauplii and rotifers were transferred into holding containers.

The container was a 2.3 L acid-washed polycarbonate Nalgene® bottle (Nalge Company, Rochester, NY, USA) filled with GF/F filtered seawater (30 g L^{-1}). From the holding container, subsamples were taken using a 10 mL kip automat NS 29.2/32 (Buch & Holm, Witeg, Germany) in order to count the exact number of items used for any further analysis.

Dry weight (DW) and carbon weight (CW)

Two replicates of 10^4 fresh *A. tonsa* eggs (nonstored) were cleaned and transferred to individual precombusted GF/F filters (1.28 cm diameter). The filter with eggs was washed with 20 mL MilliQ water to remove the salt. The filters were dried in NUCLON® Multi wells at 50 °C for 24 h and weighed before and after adding eggs on a METTLER TOLEDO MT5 balance ($\pm$ 1 μg) (Buch & Holm).

The CW of the *A. tonsa* nauplii was calculated from the (length–weight) regression curve developed on the very same culture by Berggreen, Hansen and Kiørboe (1988). Their DW was estimated using the CW–DW conversion proposed by Båmstedt (1986): DW = CW/0.447 (this average conversion factor was calculated from measurements on 63 species of copepods). The CW of *T. holothuriae* was calculated from the regression curve presented by Uye, Aoto and Onbe (2002) for the small Harpacticoid *Microstella norvegica*, and their DW was calculated in the same way as for *A. tonsa*, using the conversion from Båmstedt (1986). The rotifer CW was calculated using the mean lorica length/CW regression curve developed by Hansen, Wernberg-Møller and Wittrup (1997), and their DW calculated using the mean CW–DW conversion ratio predicted for the same rotifer species by Yùfera, Parra and Pascual (1997).

Fatty acid and amino acid samples

The copepod eggs, nauplii and rotifers FA samples were prepared by subsampling the organisms from the holding container. A known number, between 2000 and 3000 organisms, was concentrated on a 45 μm mesh from which they were gently removed and transferred into GC glass vials containing a chloroform: methanol mixture (2:1 vol:vol). It was not always possible to prepare numerous samples because of the sometimes limited size of the stored egg samples used and their hatching success (the number of samples varied from one to four). The amino acid samples were prepared using the same subsampling technique and transferred into small plastic containers containing MilliQ water. Several 5 mL samples of the *R. salina* culture (approximately 10^6 cells mL^{-1}) were filtered through GF/F glass fiber filters (Whatman), which were processed in the same way as the prey organisms. In all the cases, the samples were immediately stored at -80 °C after they were prepared and kept at this temperature until they were processed.

Hatching success (HS) of cold-stored eggs (3 °C)

A known number of eggs ranging from 200 to 400 were placed in small Petri dishes (35 mm) and left to hatch for 4 days at 17 °C. At day 2, a few drops of *R. salina* were added. The replicates were finally fixed with acid Lugol's solution and counted under a dissection microscope. The unhatched eggs were counted and the missing ones were considered as hatched.

Fatty acids analysis

The fatty acid composition of the different prey organisms was determined by the extraction of the lipids by a chloroform:methanol mixture (2:1 vol:vol) followed by trans esterification of the lipids by acetyl chloride in methanol. The fatty acid methyl esters were analysed by gas chromatography-mass spectrometry (GC-MS).

Before the extraction started, 40 μL of a 46 ng mL^{-1} internal standard solution of heptadecanoic acid methyl ester in methanol was added to each vial. The vials were sonicated at 0 °C for 2 h followed by evaporation of the solvent at 60 °C under a flow of nitrogen. Reagent solution (1 mL) composed of a mixture of toluene, methanol and acetyl chloride (40:50:10) was added and the vials were heated for 2 h at 90 °C. Aqueous $NaHCO_3$ (5% by weight, 1 mL) was added to the vials and after vigorous shaking the upper layer containing the fatty acid methyl esters was removed. The water phase was extracted twice with Heptane (1 mL) and the combined organic layers were collected and evaporated at 60 °C under a gentle stream of nitrogen. Finally, the methyl esters were resuspended in chloroform (0.5 mL) and an aliquot of the sample (5 μL) was analyzed by GC-MS. The GC-MS instrument was composed of an Agilent 6890 series gaschromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a PTV inlet and an Agi-

lent 5973 mass selective detector. The column was a 60 m Agilent DB23 with an i.d. of 250 μm and a film thickness of 0.3 μm. The carrier gas was helium at a constant flow rate of 1 mL min^{-1}. The oven temperature programme was initially at 60 °C with a temperature ramp of 24.6 °C min^{-1} until 200 °C, which was maintained for 10 min followed by a second temperature ramp of 5 °C min^{-1} until 250 °C, which was maintained for 3 min. The mass spectrometer was run in selective ion monitoring mode (SIM) by the application of the masses $m/z = 55$, 74, 79 and 81. The PTV inlet (programmable temperature vaporization) was operated in split-less mode and with the evaporation program going from 60 to 300 °C with a temperature ramp of 720 °C min^{-1} and maintained for 2 min.

Amino acids analysis

For analysis of free, intracellular amino acids, samples of eggs, nauplii, rotifers and algae were transferred to 1.5 mL plastic vials with 200 or 400 μL of Milli-Q water and were subsequently stored at − 80 °C. Free amino acids (FAA) in the organisms were extracted by heating the samples to 95 °C for 5 min after which the samples were centrifuged at 4000 *g* for 5 min. The supernatants were filtered through 13 mm 0.2 μm pore size GHP polypropylene membrane filters and were assumed to include the intracellular pool of FAA. The FAA were quantified directly as indicated below. For analysis of the total content of combined amino acids in the organisms, most likely bound in proteins, the precipitated material was freeze-dried and hydrolysed to FAA using a microwave-assisted vapour-phase technique (Jørgensen & Jensen 1997). The dried, hydrolysed samples were redissolved in 400 mM borate buffer at pH 12.0, sonicated for 1 min and filtered through 0.2 μm filters.

Ten microliters of the nonhydrolyzed filtrates and the hydrolysed samples were transferred to 1.5 mL HPLC vials with 70 μL 10 mM borate buffer at pH 8.8. Primary and secondary amines in the sample were derivatized with 20 μL 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Yu, Li, Krull & Cohen 1994). The commercial AccQ*Tag kit (Millipore, Milford, MA, USA) was used for the derivatization. The derivatives were heated to 55 °C for 10 min to degrade a tyrosine side product that interferes with the chromatographic separation of the derivatized amino acids. The amino acid derivatives were separated on a Waters Alliance 2695 separations module with a 3.9 × 150 mm Nova-Pak C-18 column. Solvents for the separation were (A) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 5.70, (B) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 6.80 (both solvents were adjusted with H_3PO_4), (C) acetonitrile and (D) water. The solvent composition was provided by Waters Corporation. The separated amino acid derivatives were quantified by fluorescence (250 nm excitation and 395 nm emission) using a Water 474 scanning fluorescence detector. In addition to standard protein amino acids, taurine (Tau), ornithine (Orn) and γ-aminobutyric acid (GABA) were included in the analysis. The proportion of each studied amino acid was calculated for both of the amino acid fractions: the FAA fraction and the protein bound amino acid fraction.

Results

Data on live prey organisms

Table 1 summarizes characteristics for the different live prey organisms. Hatching success (HS) of *A. tonsa* eggs was 62 ± 1%, 66 ± 8% and 50 ± 9% for the nonstored, 5 and 9 months stored eggs respectively. *Acartia tonsa* eggs did not shrink while being cold-stored and their diameter kept constant ranging from 81 ± 4 μm for the 5 months stored eggs to 83 ± 3 μm for the fresh eggs. The sizes of the copepod nauplii were similar to each other and smaller than those of the rotifer. The sizes varied from 116 ± 8, 110 ± 12 and 114 ± 9 μm for the nonstored, 5 and 9 months old *A. tonsa* nauplii, respectively, to 111 ± 17 μm for *T. holothuriae* and reached 143 ± 13 μm for the rotifers. The *A. tonsa* and *T. holothuriae* nauplii carbon contents (CW) were quite comparable (21.4 and 25.76 ng respectively) and also their calculated dry weight (DW) (47.9 ng and 57 ng respectively). However, the DW of the two copepod species were closer to each other than that of the rotifer. *Brachionus plicatilis* which weigh 163.3 ng DW and was therefore about three times heavier than the copepods.

Fatty acids

Table 2 presents the absolute quantities of different groups of FA and the quantities of FA DW^{-1} per studied food organism. The table also contains the ratio docosahexaenoic acid (DHA)/eicosabentaenoic acid (EPA) and (DHA+EPA)/Total FA in the organisms as well as their proportion of essential amino acids (EAA) in the FAA pool. In terms of absolute amounts of FA per prey organism (ng $organism^{-1}$), the rotifer

Table 1 Characteristics of the different live prey organisms measured during the experiments ± standard deviation

	Acartia tonsa: non-stored eggs	*Acartia tonsa*: 5 months stored eggs	*Acartia tonsa*: 9 months stored eggs	*Acartia tonsa*: fresh nauplii	*Acartia tonsa*: 5 months stored nauplii	*Acartia tonsa*: 9 months stored nauplii	*Brachionus plicatilis*: medium strain	*Tisbe holothuriae*:nauplii
Individual size (length µm)	83 ± 3	81 ± 4	82 ± 3	116 ± 8	110 ± 12	114 ± 9	143 ± 13	111 ± 17
Carbon weight (ng individual^{-1})	58.4 ± 5.3	No data	No data	21.4	No data	No data	84.6	25.8
Dry weight (ng individual^{-1})	117 ± 14.9	No data	No data	47.9	No data	No data	163.3	57.0
Hatching success	62 ± 1%	66 ± 8%	50 ± 9%				No data	No data

species was the richest prey. Only *T. holothuriae* was richer when it came to the DHA and EPA contents. However, the rotifer species represented the biggest prey in term of body size, CW and DW, and the results were totally the opposite when the amount of FA DW^{-1} was calculated. In this case, *T. holothuriae* contained 18% of FA DW^{-1} while *A. tonsa* and *B. plicatilis* contained only 8% and 9% FA DW^{-1} respectively. Also, *T. holothuriae* contained more n-3 FA DW^{-1} than the other prey organisms, followed by *A. tonsa* and *B. plicatilis*.

The ratio DHA/EPA was 1.35 for *A. tonsa* nauplii originating from non-stored eggs and reached 1.63 for *T. holothuriae*, but was very low for the rotifer (0.54). The sum of EPA and DHA represented 40% to 52% of the total fatty acid content for the copepods but was low for the rotifers (19%).

The percentage of each studied FA compared with the total FA amount in the different prey organisms and in the food algae is presented in Table 3. Some of the FA were found in very low or undetectable proportions and are as a consequence representing 0% of the total FA. *A. tonsa* nauplii contained the highest proportion of saturated FA (26%) compared with the other preys and preferentially more 16:0. The proportion of saturated FA was 17% for *B. plicatilis* and reached 22% for *T. holothuriae*. *Brachionus plicatilis* tended to contain more monoems than the other live prey (13% as opposed to 8% and 11% in the copepod preys). The rotifer was also characterized by a higher proportion of the 18:2 n-6, 18:4 n-3, 20:4 n-3, 20:4 n-6 FA than the copepods. Arachidonic acid (ARA) (20:4 n-6) represented 3% of the total FA in the rotifers while it was only found in low or trace amounts in the copepods (Table 3). The ratio EPA/ARA was 4.3 for the rotifers, 20 for *T. holothuriae* and >17 for *A. tonsa* (data not shown).

The proportion of the n-6 FA was the highest in the rotifer and reached 16% compared with 4–6% in the copepods. However, the proportion of n-3 FA in the rotifer was slightly lower, but still comparable to what was found in the copepods. These proportions (n-3 FA) in all the organisms ranged from 55% to 62% of the total FA. However, the proportion of EPA and DHA was the lowest for the rotifer (13% and 7% respectively). The EPA proportion was 17% for *A. tonsa* nauplii and 20% for *T. holothuriae*. The DHA proportion was 32% and 23% for *T. holothuriae* and *A. tonsa* nauplii originating from nonstored eggs respectively (Fig. 1).

All the live feed organisms or their brood stock were fed *R. salina*, which was particularly rich in

Table 2 Absolute quantities of different fatty acids (FA ± SD) and groups of fatty acids per studied live feed organisms and their relative quantities compared with their dry weight (DW) shown in percentages

	Acartia tonsa: **non-stored nauplii**	***Brachionus plicatilis:*** **medium strain**	***Tisbe holoturidae*****: nauplii**
Total saturated FA content (ng individual^{-1})	1.06	2.60 ± 0.53	2.26
Total monoems FA content (ng individual^{-1})	0.32	2.03 ± 0.43	1.16
Total n-6 FA (ng individual^{-1})	0.16	2.40 ± 0.48	0.59
Total n-3 FA (ng individual^{-1})	2.49	8.47 ± 1.80	6.37
Total PUFA content (ng individual^{-1})	2.65	10.87 ± 2.33	6.96
Total FA content (ng individual^{-1})	4.03	15.50 ± 3.15	10.37
Total EPA content (ng individual^{-1})	0.68	1.95 ± 0.40	2.04
Total DHA content (ng individual^{-1})	0.92	1.05 ± 0.27	3.32
Ratio DHA/EPA	1.35	0.54	1.63
Total saturated FA/DW (%)	2	2	4
Total monoems/DW (%)	1	1	2
Total n-6/DW (%)	0	1	1
Total n-3/DW (%)	5	5	11
Total PUFA/DW (%)	6	7	12
Total FA/DW (%)	8	9	18
Ratio total (EPA+DHA)/total FA (%)	40	19	52
Proportion of essential amino acids in the free amino acid pool (%)	30	43	32

The table also presents the DHA/EPA ratio and the (EPA+DHA)/total FA ratio and, finally, the proportion of essential amino acids in the free amino acid pool for the different live feed organisms.
DW, dry weight; DHA, docosahexaenoic acid; EPA, eicosabentaenoic acid.

18:3 n-3 FA (17%), 18:4 n-3 FA (22%), 20:5 n-3 FA (EPA, 14%), but poor in 22:6 n-3 (DHA, 9%). The low proportion of DHA was characteristic for the food algae and for the rotifer. The DHA proportion in the algal food was 9% of the total FA. *Rhodomonas salina* contained 12:0, 14:0 and 16:0 FA in great proportions of its saturated FA pool, but this pattern was not always found in the studied live feed organisms, which tended to be richer in longer chained saturated FA.

Amino acids

Figure 2 represents the percentage of each FAA compared with the total amount of FAA in each of the studied organisms (see for abbreviations in Table 4). The rotifer contained a mixture of most of the FAA studied with no pronounced peaks. The highest concentration was found for Glu, which accounted for 10% of the total FAA. The copepod nauplii were characterized by the presence of a prominent FAA peak in each species. Gly represented 26% of the total FAA in *T. holothuriae* while Pro accounted for 22% of the total FAA in *A. tonsa*. *Brachionus plicatilis* and *T. holothuriae* were found to contain less Pro than *A. tonsa* and the rotifer also contained less Tau (2%) and Gly (6%) than the copepods. However, the rotifer tended to have higher levels of three essential FAA than the copepods: Thr, Ile and Phe that accounted for 4%, 3% and 4% of the total FAA, respectively, in *B. plicatilis*. When comparing the proportion of FAA in *R. salina* and in the studied animals, four major trends were identified. *Rhodomonas salina* contained more Glu, Asn and Arg than the studied live feed organisms (18%, 6.5%, 13.9% of the FAA respectively). Also, the EAA fraction in the algal FAA reached 32% (data not presented). The proportion of EAA in the FAA pool was high in the rotifer (43%) compared with the copepods in which the proportions were 30% (*A. tonsa*) and 32% (*T. holothuriae*) respectively (Table 2).

Differences were less obvious between the studied organisms with regard to the protein-bound amino acids (PAA) as the proportional patterns were very similar (Table 4). However, some PAA were present in higher proportion than others. Gly, Ala, Leu, Val were found to be the major PAA in the tissues of all the organisms with the exception of *T. holothuriae* that showed a lower proportion of Val. When comparing the proportions of each PAA between the live feed organisms and the algae, it was observed that Glu and Ser tended to be present in lower proportion in the algae than in the animals. The fraction of EAA in the PAA was higher than in the FAA but no particular

Table 3 Relative quantity of each of the studied fatty acids (FA) or groups of fatty acids compared with the total amount of fatty acid in the studied live feed organisms and in their food or maternal food (in percentage % ± SD)

	Acartia tonsa **nonstored nauplii**	***Brachionus plicatilis*** **medium strain**	***Tisbe holothuriae*** **nauplii**	***Rhodomonas salina***
12:0	0	0	0	3 ± 3
14:0	3	2 ± 1	0	6 ± 4
15:0	0	0	0	0
16:0	15	9 ± 2	9	8 ± 3
18:0	6	4 ± 1	8	1 ± 0
20:0	1	0	0	0
22:0	1	0	1	0
24:0	0	0	2	0
14:1	0	1 ± 0	1	0
16:1 n-7	1	2 ± 0	2	2 ± 0
18:1 n-9	1	2 ± 0	3	2 ± 1
18:1 n-7	5	6 ± 1	5	5 ± 2
20:1 n-9	0	2 ± 0	0	0
22:1 n-11	0	1 ± 0	0	0
24:1	1	1 ± 0	0	0
18:2 n-6	2	9 ± 2	2	7 ± 3
18:3 n-6	0	2 ± 0	0	2 ± 1
18:3 n-3	13	14 ± 3	5	17 ± 7
18:4 n-3	7	9 ± 2	3	22 ± 9
20:2 n-6	1	0	0	0
20:3 n-6	0	1 ± 0	0	0
20:4 n-6 (ARA)	0	3 ± 1	1	2 ± 1
20:3 n-3	1	1 ± 0	0	0
20:4 n-3	1	9 ± 2	1	1 ± 0
20:5 n-3 (EPA)	17	13 ± 3	20	14 ± 6
22:5 n-6	1	1 ± 0	1	0
22:5 n-3	1	2 ± 0	1	0
22:6 n-3 (DHA)	23	7 ± 2	32	9 ± 4
Total saturated	26	17	22	18
Total monoems	8	13	11	9
Total n-6	4	16	6	11
Total n-3	62	55	61	63
Total PUFA	66	70	67	74

trend between the studied live feed organisms was noticed. The proportions of EAA in the PAA ranged from 47% (rotifer) to 56% (5 months old *A. tonsa* eggs).

Hatching of *Acartia tonsa* eggs after months of cold storage at 2–3 °C

As biochemical analysis was carried out on *A. tonsa* eggs and their nauplii, it was possible to describe the biochemical contents of the eggs and the nauplii following hatching in both freshly produced and cold-stored eggs. It was important to note that the DW of the nonstored eggs was more than two times higher than that in the nauplii originating from the non-stored eggs (117 ng DW compared with 47.9 ng DW).

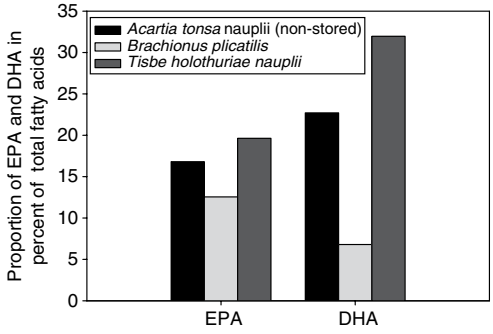

Figure 1 Eicosabentaenoic acid and docosahexaenoic acid content as a percentage of the total fatty acid content of the three live prey organisms studied.

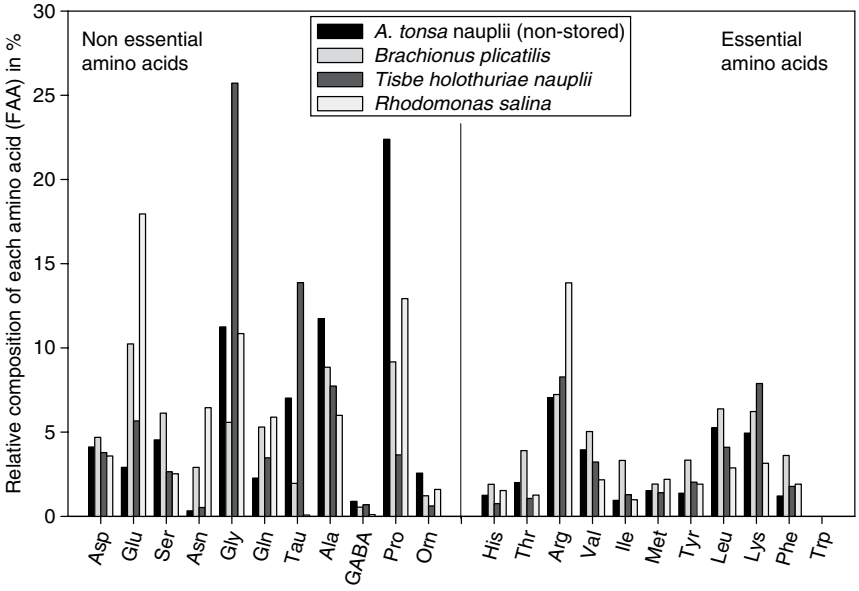

Figure 2 Proportion of the different free amino acids (FAA) in the studied live prey organisms and in the food algae *Rhodomonas salina*.

Similarly, the absolute content of total FA in the nonstored eggs was more than twice that in the hatched nauplii (10.43 ng and 4.03 ng respectively, Fig. 3). These relative contents were similar when compared with their DW (9% and 8% of FA DW^{-1} for the nonstored eggs and nauplii respectively). The total FA-content of the eggs tended to decrease when storage time increased ranging from 10.43 ng egg^{-1} in the nonstored eggs to 9.11 and 7.91 ng $eggs^{-1}$ in the 5 and 9 months cold-stored eggs respectively. On an average, the proportion of saturated FA increased when hatching occurred (Table 5) (in proportion to their total FA content). Thus, an increase of 16:0 and 18:0 between the eggs and their nauplii was observed. Also, the proportion of saturated FA in the nauplii increased with the storage time of the eggs they were hatching from. It was 26%, 30% and 33% for the nauplii hatching from nonstored, 5- and 9-month-old eggs respectively. The proportion of monoems was stable in the eggs during cold storage and also when the eggs hatched to nauplii. The total n-3 FA, and, therefore, the PUFA proportion tended to decrease when the eggs hatched to nauplii (PUFA: 71–77% for the eggs compared with 57–66% for the nauplii). This decrease was remarkable for the 18:4 n-3 and obvious for the 18:3 n-3, decreasing from 19% to 13% and from 13% to 7% respectively. However, the EPA and DHA proportion increased in all the nauplii compared with the eggs they were hatching from even after 9 months of cold storage.

The FAA proportions of *A. tonsa* also changed when hatching occurred (Fig. 4). The general pattern of the amino acid composition was constant. However, the Glu proportions had a tendency to decrease between the eggs (7–9% of total FAA) and the nauplii (3–4% of total FAA). The inverse tendency was observed for the Gly contents that increased from 3% to 4% of total FAA in the eggs to 11–13% in the nauplii. However, no changes were evident when comparing the proportion of PAA before and after hatching with the exception of a few weak trends. Gly tended to increase from 11% to 15% from the fresh eggs to the fresh nauplii. Val followed the same tendency, but this increase was not obvious. Glu proportions decreased from 10% to 3% when non-stored eggs hatched into nauplii.

Discussion

The hatching success of both nonstored and cold-stored subitaneous *A. tonsa* eggs was slightly lower than what has been previously observed in the laboratory by Drillet *et al.* (2006) even though no explanations were obvious. The dry weight of *B. plicatilis* could seem high compared with the copepods. how-

Table 4 Relative composition of the different protein bound amino acids (PAA) in percentage (%)

	Acartia tonsa: nonstored eggs	*Acartia tonsa*: 5 months stored eggs	*Acartia tonsa*: 9 months stored eggs	*Acartia tonsa*: fresh nauplii	*Acartia tonsa*: 5 months nauplii	*Acartia tonsa*: 9 months nauplii	*Brachionus plicatilis*: medium strain	*Tisbe holothuriae*: nauplii	*Rhodomonas*: *salina*
Non essential AA									
Asp: Aspartate	6	2	3	2	2	2	1	2	1
Glu: Glutamate	10	4	7	3	4	3	3	4	2
Ser: Serine	7	7	7	8	8	8	8	9	1
Asn: Asparagine	0	0	0	0	0	0	0	0	0
Gly: Glycine	11	11	11	15	11	12	10	11	13
Gln: Glutamine	3	5	2	5	4	5	4	4	6
Tau: Taurine	0	0	0	0	0	0	0	0	0
Ala: Alanine	8	10	8	10	11	11	11	15	17
GABA	0	0	0	0	0	0	0	0	0
Pro: Proline	4	5	5	4	5	5	6	7	7
Orn: Ornithine	1	0	2	1	1	1	0	1	2
Essential AA									
His: Histidine	3	3	3	4	3	3	3	3	1
Thr: Threonine	5	4	6	4	5	4	4	4	1
Arg: Arginine	5	6	6	6	6	5	6	5	7
Val: Valine	7	9	8	8	11	11	12	5	10
Ile: Isoleucine	4	5	5	4	4	4	4	4	5
Met: Methionine	2	3	2	2	2	2	2	2	1
Leu: Leucine	8	9	8	8	8	7	8	7	10
Tyr: Tyrosine	5	7	5	7	6	7	7	8	4
Lys: Lysine	5	3	8	2	4	1	1	2	2
Phe: Phenylalanine	5	7	5	6	5	7	8	7	7
Trp: Tryptophan	0	0	0	0	0	0	0	0	0
Sum EAA	49	56	55	51	54	53	55	47	49

The last line of the table shows the proportion of essential amino acids (EAA) in all the studied live food organisms. No standard deviation is presented because of the low number of samples (between 1 and 2).

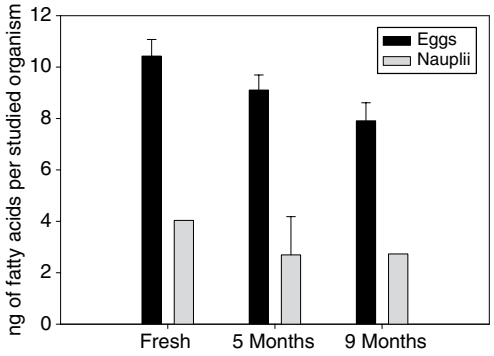

Figure 3 Acartia *tonsa* total amount of fatty acids (AVG ± SD) per studied live feed organism. For each different period of egg storage the fatty acids contents of the eggs and their hatched nauplii are shown. The number of samples for fresh, 5 and 9 months stored eggs are respectively: 4, 3 and 3. The number of samples for fresh, 5 and 9 months nauplii are respectively: 2, 3, 2.

ever, the DW was also calculated from Yùfera, Pascual, and Guinea (1993) for small (S) and large (L) strains taking into account the number of females bearing eggs (here, one egg per female). The DW were found to be 219.5 ng organism^{-1} (L strain) and 109.5 ng organism^{-1} (S strain), with an average of 164.5 ng organism^{-1}. In the medium strain used for this experiment, the calculated DW was 163.3 ng organism^{-1}, which shows that the approximations were acceptable. The DW calculated for *T. holothuriae* nauplii was found to be within the range of that for *Tigriopus californicus* nauplii, which is another small-sized Harpacticoid copepod (40–130 ng for size class 74–147 μm) (Theilacker & Kimball 1984). Further, Norsker and Støttrup (1994) reported a dry weight of newly hatched *T. holothuriae* nauplius of 71–73 ng.

Biochemical composition of the food algae

The FA contents of *R. salina* were similar to what is reported in the literature with peaks of 14:0 and 16:0 in the saturated FA fraction and peaks of the unsaturated 18:3 n-3, 18:4 n-3 and 20:5 n-3 (Norsker & Støttrup 1994; Zhukova & Aizdaicher 1995). Klein Breteler, Schogt, Baas, Schouten and Kraay (1999), described the presence of C18:1 n-9 in *Rhodomonas* sp., which seems absent in the other studies. The importance of this FA is unclear but it seems to be only present in low amounts in the subsequent trophic level in all the cited studies.

The major FAA present in our algal culture was similar to what was described by Helland *et al.* (2003a) for *R. balthica*. However, these authors presented results from two cultures (spring and autumn) and the latest was characterized by a peak of His and low amounts of Glu that was not detected in the present study. Also, in the present study, *R. salina* contained only 32% of EAA, which was lower than the 37% and 49% presented by Helland *et al.* (2003a). The peaks of FAA in the RUC algal culture were similar to those found by Klein Breteler *et al.* (1999) despite different relative quantities. It is, however, important to remember that the culture media (B1) used in the present study was different from the one used by the other authors (F/2 Guillard) and could be part of the explanation along with light intensity, salinity and other culture parameters that are known to influence biochemical composition of a given phytoplankton.

Fatty acids FA in the live feed organisms

The amount of FA found in the strains of prey organisms (8–18% FA DW^{-1}) used in this study is in accordance with other references. Norsker and Støttrup (1994) found about 5.7% FA DW^{-1} for *T. holothuriae* fed *Rhodomonas* sp. (calculated from their table). As the present study did not characterize which lipid classes were present in the samples, it is difficult to compare the proportion of FA DW^{-1} found here and the proportion of lipid reported in the literature. However, lipid contents can vary considerably in the plankton; Miliou, Moroitou-Apostolopoulou and Argyridou (1992) presented amounts of lipids in *T. holothuriae* of 9.7% DW while McKinnon, Duggan, Nichols, Rimmer, Semmens and Robino (2003) provided results on total lipid contents ranging from 11% to 25% DW in three calanoids. At the time of the experiments, *A. tonsa* nauplii were not feeding on external resources and the major part of its FA might have been used for growth rather than for storage. The fatty acid content of *T. holothuriae* (FA DW^{-1}) was higher than that for the other live prey. *T. holothuriae* nauplii and the rotifer were phagotroph at the time of the experiment and it is likely that part of the FA was originating from the phytoplankton cells from their gut. Lipids in the rotifer were recently shown to range between 9% and 28% of the dry weight (Lubzens & Zmora 2003) but has also been described to be lower by Evjemo and Olsen (1997) who found values down to 6.6% DW.

Table 5 Relative content of each studied fatty acids (FA) or group of fatty acids compared with the total amount of fatty acids in *Acartia tonsa* eggs and nauplii before and after cold storage (in percentage % ± SD)

	Non-stored eggs	5 months stored eggs	9 months stored eggs	Nauplii from nonstored eggs	Nauplii from 5 months stored eggs	Nauplii from 9 months stored eggs
12:0	0	0	0	0	0	0
14:0	2 ± 0	2 ± 0	2 ± 1	3	2 ± 2	2
15:0	0	0	0	0	1 ± 0	0
16:0	11 ± 1	9 ± 0	13 ± 1	15	16 ± 8	16
18:0	3 ± 0	3 ± 0	4 ± 0	6	8 ± 4	11
20:0	1 ± 0	1 ± 0	1 ± 0	1	1 ± 0	1
22:0	0	0	1 ± 0	1	1 ± 0	2
24:0	0	0	0	0	0	1
14:1	1 ± 0	1 ± 0	0	0	1 ± 0	0
16:1 n-7	1 ± 0	1 ± 0	1 ± 0	1	1 ± 1	1
18:1 n-9	1 ± 0	1 ± 0	1 ± 0	1	1 ± 1	2
18:1 n-7	5 ± 0	4 ± 0	5 ± 0	5	4 ± 2	5
20:1 n-9	0	0	0	0	0	1
22:1 n-11	0	0	0	0	0	1
24:1	0	0	1 ± 0	1	1 ± 1	1
18:2 n-6	2 ± 0	2 ± 0	3 ± 0	2	2 ± 1	3
18:3 n-6	0	0	0	0	0	0
18:3 n-3	19 ± 1	17 ± 1	18 ± 2	13	9 ± 5	10
18:4 n-3	13 ± 1	12 ± 1	10 ± 1	7	4 ± 2	4
20:2 n-6	1 ± 0	1 ± 0	1 ± 0	1	1 ± 0	1
20:3 n-6	0	0	0	0	0	0
20:4 n-6 (ARA)	1 ± 0	1 ± 0	1 ± 0	0	1 ± 0	1
20:3 n-3	1 ± 0	1 ± 0	1 ± 0	1	0	1
20:4 n-3	1 ± 0	1 ± 0	1 ± 0	1	0	1
20:5 n-3 (EPA)	18 ± 1	19 ± 1	19 ± 2	17	17 ± 10	16
22:5 n-6	0	0	1 ± 0	1	0	1
22:5 n-3	1 ± 0	1 ± 0	2 ± 0	1	1 ± 0	1
22:6 n-3 (DHA)	18 ± 1	22 ± 2	15 ± 2	23	25 ± 16	18
Total saturated	17	15	21	26	30	33
Total monoems	8	8	8	8	9	10
Total n-6	5	5	6	4	4	5
Total n-3	70	72	65	62	57	51
Total PUFA	75	77	71	66	61	57

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosabentaenoic acid.

The fatty acid pattern measured in *A. tonsa* was very similar to that described by Støttrup *et al.* (1999). These authors observed 16:0 as the major peak of saturated FA and 18:3 n-3, 20:5 n-3, and 22:6 n-3 as the main unsaturated peaks when *A. tonsa* was fed *R. balthica*. The present study showed a very high proportion of n-3 FA in the preys. This supports the results described by Evjemo and Olsen (1997). Furthermore, a higher DHA/EPA ratio was found for copepods than for the rotifer. This is in accordance with that described in the literature by authors working on enriched rotifers (Evjemo & Olsen 1997; Payne *et al.* 2001) but the present results did not show as high a ratio as those described by McKinnon *et al.* (2003) (*Acartia sinjiensis* = DHA/EPA ratio was 4.7) and Payne *et al.* (2001) who reported a DHA/EPA ratio ranging from 3.6 to 4.9. Sargent, McEvoy and Bell (1997), in a review, proposed an optimal DHA/EPA ratio of two for the live food organisms. In the present study, the copepods were a lot closer to the proposed optimal ratio than the rotifer. Arachidonic acid is an essential FA for fish (Sargent, McEvoy, Estevez, Bell, Bell, Henderson, & Tocher, 1999). However, these authors warned that an excess of ARA in the diet can be deleterious for teleosts. The lowest ratio of EPA/ARA reported here was found in the rotifer (4.3) and has not been shown to generate abnormal survival, growth, pigmentation or metamorphosis in fish (Sargent *et al.* 1999).

Nanton and Castell (1998) found that the content of 18:3 n-3 incorporated by *Tisbe* sp. corresponded with the relative amount in the algal diet. This was not the

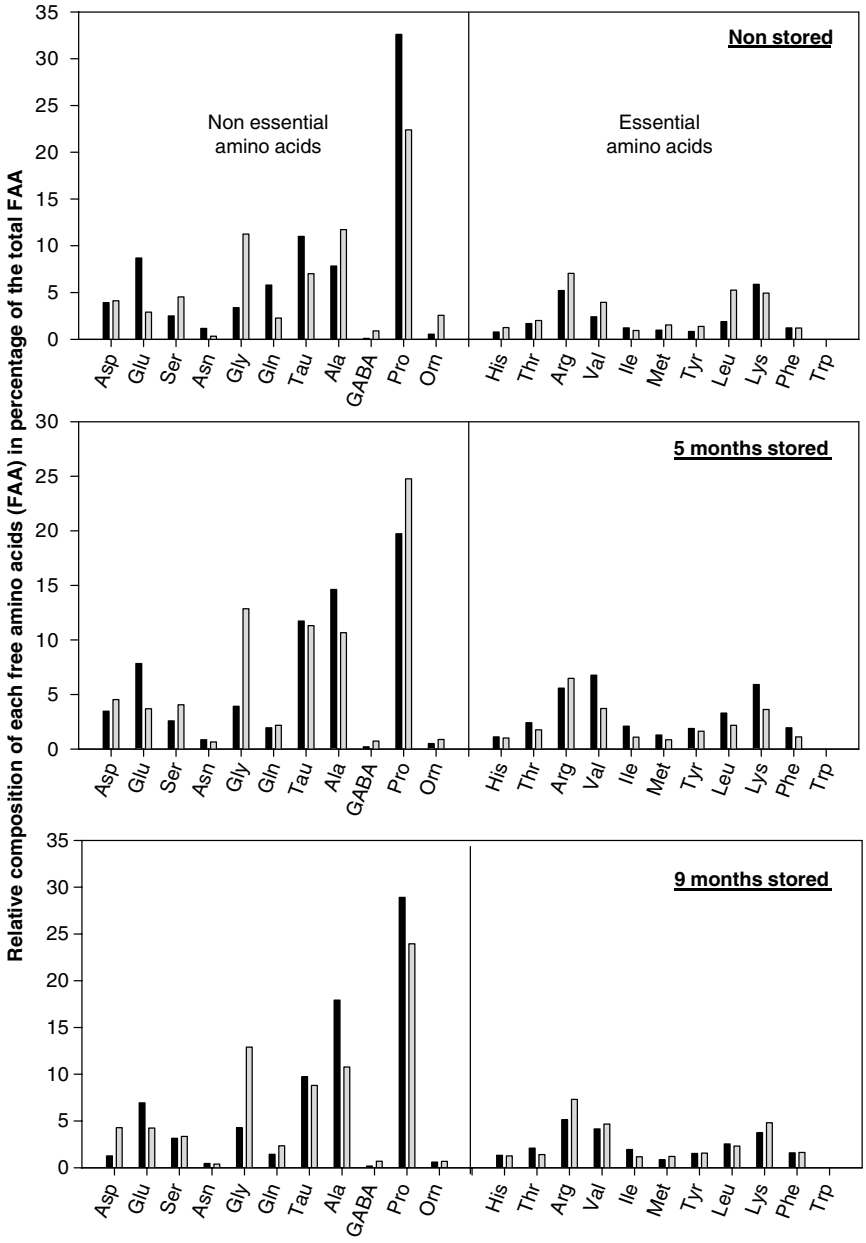

Figure 4 Relative composition of free amino acids in the eggs and the nauplii of *Acartia tonsa* before (non stored) and after 5–9 months of cold storage. Black bars show eggs, the grey bars show the nauplii. The central graph has a different scale than the two others for better reading.

case in our study where the relative 18:3 n-3 content in the algae was about three times higher than that in the copepod (17% and 5% of total FA respectively). Norsker and Støttrup (1994) and Nanton and Castell (1998) suggested that *Tisbe* sp. was able to synthesize EPA and DHA from their shorter precursors present in the algae, but did not found clear evidences of this capacity for the calanoids. Here, live preys were fed *R. salina*, which contain lower but not negligible amounts of DHA (9% of studied FA). This DHA content ($\approx$ 7%) was also found in the rotifer but surprisingly not in the copepods, where levels of this FA were

23% and 32% of the studied FA for *A. tonsa* and *T. holothuriae* respectively. This could emerge from an accumulation of this FA from the food itself or, as proposed for *Tisbe* sp. by the authors cited above, the studied copepods do contain the enzymes necessary for the biochemical conversions. The bioconversions to long chained PUFAs from shorter chain FA like 18:3 have also been proposed for *Eucyclops serrulatus* and *Paracalanus pavrus* by Moreno, De Moreno and Brenner (1979) and Desvilettes, Bourdier and Breton (1997). However, it is reported that copepods are unable to grow properly on phytoplankton lacking highly unsaturated FA (Klein Breteler *et al.* 1999). It is proposed that *A. tonsa* might also be able to synthesize DHA from shorter chain FA and that the high proportions of DHA might not originate only from food accumulation. However, as *R. salina* contained some DHA, it was not possible to achieve a genuine answer and more experiments aiming at answering this highly relevant question should be carried out.

Amino acids in the live feed organisms

Free amino acids are used by copepods to balance the osmoregulation stresses generated by salinity changes (Farmer & Reeve 1978; Goolish & Burton 1989; McAllen 2003). The pool of FAA tend to increase with the salinity and it is often accepted that mostly non essential amino acids (NEAA) are involved. There were no salinity changes involved in the present experiments (30 g L^{-1}) and so differences in FAA contents between the organisms for this reason were not expected. Also these contents are expected to be in the normality range described in the literature, i.e. between 1% and 9% of the DW (cf. review by Båmstedt 1986). Aragão, Conceição, Dinis and Fyhn (2004) reported that the FAA could vary between 2.4% and 6.6% of the DW in rotifers depending on the food, and demonstrated a very low content of FAA for *Artemia* 1.6–3.6% DW. In the present study, a distinct species-specific pattern of the FAA peaks composition when raised under the same food conditions was noticed as it has been previously suggested by Helland *et al.* (2003a) and Helland, Terjesen and Berg (2003b).

The data concerning the rotifer's relative FAA content supported partly what was described by Weltzien, Planas, Cunha, Evjen and Fyhn (1999), who showed a high proportion of EAA in their *B. plicatilis* FAA (46% in their experiment, 43% in the present study). However, in their experiment, two peaks of FAA were relatively important as compared with the others: Tyr, 13.4% and Tau, 13.6% (Weltzien *et al.* 1999). This was not the case in the present study presumably because of the different food sources used (*R. salina* in this experiment compared with *Tetraselmis suecica* and *Isochrysis galbana* in Weltzien *et al.* 1999).

The protein-bound amino acids (PAA) did not show any important variations among their proportions in the various live prey types. It has been described that when the protein contents in rotifers change, the total amino acid profiles seem unaffected by the food ration or type of food provided (Lubzens and Zmora 2003 and references therein). Also, it has been revealed that *Artemia* from different locations had differences in terms of FAA contents and composition but the AA profiles of body protein kept constant independently of the *Artemia* species (Helland *et al.* 2000). However, the diet did influence the AA from the total AA pool in *C. helgolandicus* (Laabir *et al.* 1999). Here, the results showed very compromising outcomes, *A. tonsa* and *B. plicatilis* had very similar PAA patterns and *T. holothuriae* was only slightly different because of a low content in Val in its PAA pool.

Hatching of *Acartia tonsa* eggs after cold storage to complement other live feeds strategies

The biochemical cost of hatching in live preys was studied for *Artemia* by García-Ortega, Verreth, Coutteau, Segner, Huisman and Sorgeloos (1998), but is not really well known for copepods. These authors found a reduction of the dry weight of almost 50% along the development (from cysts to instars $\approx$ 25 h) that supports the present results on copepods. Amino acid contents were studied during embryonic and naupliar development in *C. helgolandicus* by Laabir *et al.* (1999). In their study, good hatching success tended to be linked with a high amino acid content (total AA), which decreased rapidly during development, with a particular emphasis on the FAA. Helland *et al.* (2003a) found no reduction of the FAA contents from egg to nauplius, but did not measure the total amino acid content of the organisms. In the present study, the proportions of the FAA pool in eggs vs. newly hatched nauplii were followed. Nevertheless, similar conclusions were achieved supporting the results from Helland *et al.* (2003a). Glu, which is known to be implicated in the synthesis of other AAs, was not accumulated in the FAA pool during the development. The proportion of Gly, implicated in purine synthesis and present in high con-

centrations in many marine invertebrates, tended to increase during the development, which support results reported by Laabir *et al.* (1999). It is noteworthy that, for the most part, the proportions of the different protein amino acids did not change much during the storage of the eggs with the exception of two trends by Gly and Glu. These two amino acids evolved similarly in the FAA and in the PAA pools. The PAA are assumed to be used as a source of amino acids during the development of the nauplius.

The proportions of FA in the *A. tonsa* eggs and their nauplii were similar when compared with the dry weights but varied in absolute values. Working on *Artemia*, García-Ortega *et al.* (1998) observed an increase of the lipid proportion in the eggs (lipids DW^{-1}), while in the present results this proportion kept stable. Considering the fact that *Artemia* DW decreased severely with the development time, it is obvious that the absolute amount of FA also decreased during the development, which supports our results on copepods. The important loss of FA is explained by the fact that a lot of energy is required to hatch from the eggs and that some of the FA are likely to be part of the shell structure. Surprisingly, the proportion of saturated FA tended to increase during the hatching process while the proportion of PUFA tended to decrease. This could have multiple explanations that are not exclusive. The saturated FA are more used as structural support while the PUFA are a source of energy and/or there is more PUFA than saturated FA in the chorion of the eggs, and/or saturated FA are part of the reserve lipids that stay inside the nauplii when hatching, and no reserves stay in the chorion. Finally, the total amount of FA decreased in the eggs as the storage time increased as it has been described by Drillet *et al.* (2006). This could be explained by a partial metabolism of FA as energy source during the cold storage. Further, the proportion of DHA and EPA tended to increase between the eggs and their nauplii even after months of cold storage. Thus, storing *A. tonsa* eggs for later use as live feed is demonstrated to be a promising solution for fish farms.

The present study has revealed that *B. plicatilis* contain high levels of total FA per organism. However, to be used as a high-quality food source, *B. plicatilis* needs enrichment to increase its content of EPA and, mainly, DHA. Also its FA content compared with the dry weight is relatively similar to that of copepod nauplii. Because of their relative FA composition, copepods have been proposed as good live prey candidates for aquaculture by Lokman (1993) and it has previously been shown that even enriched rotifers and *Artemia* were less efficient than copepods as live feed in order to raise fish (Næss *et al.* 1995; Shields, Bell, Luizi, Gara, Bromage & Sargent 1999; Payne *et al.* 2001). Olsen, Evjemo and Olsen (1999) pointed out a high DHA catabolism in the commercially available *Artemia* sources as one of the main problems of using *Artemia* for marine cold water fish larvae with a high DHA requirement. McEvoy *et al.* (1998) and Shields *et al.* (1999) reported a better pigmentation when teleosts were fed copepods as opposed to other live feed. Presumably, copepods are better assimilated in teleosts than *Artemia* (Pedersen 1984; Luizi, Gara, Shields & Bromage 1999; Shields *et al.* 1999). The fact that copepods do not contain the same FAA pattern as commonly used live feed is likely to play an important role during the fish larval development. Because of high AA contents, copepods have been described as being of superior nutritional value for fish larvae without fully developed gastrointestinal tracts (Dabrowski & Rusiecky 1983). In the present study, both copepod species contained high levels of Tau, which have been shown to stimulate fish development and behavior (Conceição, Van Der Meeren, Verreth, Evjen, Houlihan & Fyhn 1997; Takeuchi 2001). The rotifer, on the other hand, showed low levels of Tau (2%). Rønnestad, Conceição, Aragão and Dinis (2000) demonstrated that FAA are absorbed faster and assimilated more efficiently than protein in postlarval Senegal sole (*Solea senegalensis*). Thus, copepods with high levels of FAA could be an excellent live food in this particular aspect. This is in accordance with Næss *et al.* (1995) who reported that wild zooplankton contained twice the amount of FAA as compared with enriched *Artemia*.

Reared on the same algae, different species of copepods are reported to exhibit different biochemical patterns and different vital rates like egg production; or somatic growth (Jonasdottir 1994; Kleppel, Burkart & Houchin, L. 1998). Also, the biochemical composition of the eggs and, therefore, of the first nauplii stages (before phagotrophy) is influenced by the maternal food (Guisande, Maneiro & Riveiro 1999; Laabir *et al.* 1999; Guisande, Riveiro & Maneiro 2000). Benthic copepods like *Tisbe* sp. are more adapted to grow on poor food sources. *Tisbe* seems able to elongate FA in order to synthesize the long PUFA they need (Norsker & Støttrup 1994; Nanton & Castell 1998). They have also been described as being able to grow on different synthetic diets by Guérin and Gaudy (1977). However, although fed on the same phytoplankton source, the different organisms possessed different FAA patterns in (the present

study). This is with respect to different FAA peaks, which emphasizes the importance of characterizing the FAA composition of the live food used. Each fish species has different needs to be fulfilled by administering the best prey at the right developmental stage. The present work shows that it is important to choose the right species of copepod and, probably, the right culture method in concert to achieve a perfect match between prey and predator to each specified situation. Even within the same species, copepods from different geographical origins might react differently to their diet, environment and storage conditions. To know more about this, a common garden experiment will have to be carried out by testing vital rates and biochemical compositions of the different strains under exactly the same conditions. The final solution, as it has been made for all human raised species, will be to select and mix 'strains' of the same species in order to achieve the ultimate copepod in culture that will be adjustable to various aquaculture needs. Copepods can replace or supplement rotifers and *Artemia*.

Conclusion

This study showed that the proportion of EAA was higher in a rotifer than in two copepods when raised on the same food conditions. However, the copepods had a more relevant fatty acid pattern for aquaculture purposes because of their high content in EPA and DHA. There was a species-specific pattern of the FAA in the three different live preys studied. The subclass of Copepoda is rich in species and a lot of candidates potentially relevant for aquaculture are still to be discovered. For those that can be raised easily, it is important to manipulate the culture conditions to achieve custom-made and, therefore, relevant preys. Consequently, it seems necessary to select live preys for their FAA 'type'. The right candidates should be chosen to adapt to the need of the fish before enrichment is applied. The reflection was carried on further and it is proposed that the selection of rich FAA and FA copepod strains should be carried out to build up some aquaculture 'strains'. These copepods or their eggs could be stored and sent to any fish farm industry where new cultures could be established. In small scale hatcheries developed for aquarium trade, these copepod eggs could be hatched to be used as live feed straight away.

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MANUSCRIPT 3

Improving cold storage of subitaneous eggs of the copepod *Acartia tonsa* Dana from the Gulf of Mexico (Florida – USA)

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Abstract

Developing methods to store copepod eggs is necessary to increase the availability of copepods as a live food for the aquaculture industry and aquarium trade, and also to allow the exchange of copepods between researchers. The present study, evaluated the effect of glucose and two antibiotics (kanamycin sulphate and oxytetracycline HCl) on extending the shelf life of cold-stored subitaneous *Acartia tonsa* eggs. Also, egg development effects on the survival of the eggs were tested. Glucose did not have any significant effects on the survival of the eggs. However, the addition of antibiotics to the storage vials resulted in an increase of the survival of the eggs. In the best case, the shelf life of the eggs was almost doubled. After 7 days, the kanamycin+glucose treatment led to a hatching success of 86 ± 1% of the hatchable eggs, while the untreated eggs presented a hatching success of 47 ± 6%. However, long exposure to high concentrations of antibiotics was lethal to the copepod eggs. After more than 30 days of exposure to 100 mg L^{-1} of oxytetracycline, the survival of the eggs was lower than in the untreated samples. After 45 days, oxytetracycline-treated eggs (100 mg L^{-1}) presented a hatching success of 4–5% while the non-stored eggs still had a hatching success of 9%, and the eggs treated with a lower concentration of antibiotics (10 mg L^{-1}) showed a hatching success up to 21–23%. The size of the nauplii in all trials tended to decrease as the period of cold storage at 1 °C increased. We consider that the use of antibiotics at the right dosage to be a means to increase the storage capacity of the Gulf of Mexico strain of *A. tonsa* eggs, which do not show any capacity to be stored for long periods of time, compared with some other strains. In addition eggs that were between 5 and 7 h old survived longer when stored in the cold than eggs, which were freshly spawned or closer to hatching.

Keywords: *Acartia tonsa*, copepod, glucose, antibiotics, cold storage, egg development

Introduction

The use of copepods in aquaculture is increasing because of their nutritional qualities (Støttrup 2003; Lee, O'Bryen & Marcus 2005). Copepods have proven to be an ideal food item for marine aquaculture (Evjemo & Olsen 1997; McEvoy, Næss, Bell & Lie 1998; Shields, Bell, Luizi, Gara, Bromage & Sargent 1999; Payne & Rippingale 2000; Støttrup 2000; Helland, Terjesen & Berg 2003; Drillet, Jørgensen, Sørensen, Ramløv & Hansen 2006; Wilcox, Tracy & Marcus 2006). A few industrial plants already exploit copepods as live food to raise commercially valuable fish (Engell-Sørensen, Støttrup & Holmstrup 2004). Storage methods for copepod eggs or copepod nauplii are necessary to optimize the use of copepods for extensive use in fish farming. Several articles have addressed this topic (Marcus & Murray 2001; Payne & Rippingale 2001; Drillet, Iversen, Sørensen, Ramløv, Lund & Hansen 2006; Holmstrup, Overgaard, Sørensen, Drillet, Hansen, Ramløv & Engell-Sørensen 2006). Two approaches to increase the shelf life of subitaneous eggs of *Acartia tonsa* Dana were tested. This copepod species is cosmopolitan in distribution and methodologies exist for its culture. The culture

initiated by Støttrup, Richardson, Kirkegaard and Pihl (1986) is still running at the Danish Institute for Fisheries Research (DIFRES, Denmark). For more information on the world geographical distribution of *A. tonsa* (and other planktonic copepods), see http://copepodes.obs-banyuls.fr/en/index.php.

Research has shown that dissolved organic matter (DOM) can be a source of energy for some invertebrates which absorb it through their integument (Manahan 1990). Although, it is often presumed that crustaceans are unable to use DOM because of their exoskeleton, Chapman (1981) showed by autoradiographic methods that *Neocalanus plumuchrus* Marukawa was capable of removing labelled glucose from the seawater via dermal glands and the mid gut. Gyllenberg and Lundqvist (1978) presented evidence that two other copepods, *Cyclops oithonoides* Sars and *Halectinosoma curticorne* Boeck, treated with antibiotics, ingested and assimilated dissolved glucose. Because of their shell structure, it is not known if copepod eggs are able to absorb DOM. The structure of different copepod eggs (diapause and subitaneous) has been described in the literature and the shell is known to contain different layers (Santella & Ianora 1990; Ianora & Santella 1991) which might be impermeable to DOM. The presence of glucose in storage vials could be a source of energy to increase the survival of copepod eggs, but it would also be a substrate enabling bacteria to grow, leading to anoxic conditions. Anoxia is known to negatively affect the hatching and survival of copepod subitaneous eggs, having a variable intensity depending on their developmental stage (Uye 1976; Lutz, Marcus & Chanton 1992, 1994; Marcus & Lutz 1994). Therefore, to increase the storage capacity of eggs, it seems important to maintain oxic conditions as long as possible and store the eggs at the optimum development stage. One approach to this problem would be the addition of antibiotics to a vial of eggs to eliminate bacterial growth and the onset of anoxic/hypoxic conditions.

As bacterial development is often responsible for the high mortality of developmental stages in marine organisms, the use of antibiotics during larval development of many crustaceans has proven effective (Christiansen 1971; Fisher & Nelson 1978; Pelletier & Chapman 1996) in increasing survival. Also, Chan and Lawrence (1974) observed that antibiotic treatments applied to brown shrimp larvae (*Penaeus aztecus* Ives) did not affect the respiration rates of the late larval stages, but might have decreased the respiration rates of the earliest stages. If this were the case for copepod eggs, the slowed respiration rate of the eggs could postpone the emergence of critical anoxic conditions. Another factor that affects the viability of copepod eggs exposed to hypoxic/anoxic conditions is their stage of development (Lutz *et al.* 1992, 1994; Marcus & Lutz 1994). The developmental stage of copepod eggs may have an impact on their capacity to survive cold storage, if some stages are better able to survive quiescence.

In this study, the survival of cold-stored-eggs exposed to the antibiotics (kanamycin or oxytetracycline), and with and without glucose was tested for up to 60 days. The size of nauplii that hatched from the eggs after different periods of cold storage was measured, and the effect of egg development on survival during cold storage was determined.

Material and methods

Acartia tonsa cultures

Acartia tonsa was harvested offshore, south of the FSUCML with a cod-end plankton net (3 m in length, 1 m in diameter and mesh size of 202 μm). *Acartia tonsa* individuals were isolated with pipettes and a culture was developed. This culture was kept at 17 °C in a 300 L tub and fed *Rhodomonas lens* and *R. salina* microalgae for 2 years. The eggs were harvested daily by siphoning the bottom of the tank, and letting the water pass through nested meshes (150 and 45 μm Nitex®). The eggs were subsequently rinsed through a second set of meshes (100 and 70 μm) in order to separate the harvest from faecal pellets, ciliates and nauplii. Once *A. tonsa* eggs were cleaned, they were transferred into 50 mL Falcon tubes (Fisher Scientific, Pittsburgh, PA, USA) filled to 30 mL with aerated, 1 μm filtered, UV treated, chlorinated-dechlorinated seawater adjusted to 30 g L^{-1} (further referred to as treated water). During all experiments, only treated seawater was used. For counting, a subsample (SS) was taken from the Falcon tubes to approximate the amount of eggs harvested and the tubes were stored, daily in a 1 °C incubator (Fisher Scientific) for later use as live prey for fish larvae. Before the experiments, the eggs were further cleaned manually by removing as much debris as possible with a pipette.

Antibiotics and glucose experiments

Cleaned eggs were transferred into a 3.8 L bottle filled with 2 L of treated water and mixed by shaking the bottle. Eggs were subsampled with a 2500 μL Eppen-

Table 1 Experimental setups of the two first experiments testing antibiotics and glucose in order to increase the shelf life of the cold stored eggs of the calanoid copepod *Acartia tonsa* Dana

	Experiment 1	**Experiment 2**
Treatment consisted in:	4 treatments:	16 treatments:
(final concentration in the tubes)	Control with no treatment Kanamycin sulphate 6.6 mg L^{-1} Glucose 5×10^{-4} mol L^{-1} Kanamycin sulphate 6.6 mg L^{-1} + glucose 5×10^{-4} mol L^{-1}	Control with no treatment Glucose (41×10^{-5}, 41×10^{-4} or 41×10^{-3} mol L^{-1}) Oxytetracycline HCl (1, 10 or 100 mg L^{-1}) All possible combinations with the different concentrations of glucose and oxytetracycline
Number of eggs/tube	557	338
Days of cold storage (1 °C) before hatching success experiments	7, 14 and 29	15, 30, 45 and 60
Age of the eggs when cold stored	1–5-h old eggs	3–6-h old eggs
Number of replicate during the hatching success experiment after cold storage	5	4

dorff automatic pipette from the 3.8 L bottle into 15 mL Falcon tubes and closed with a plastic lid. The antibiotics used were Kanamycin monosulphate (ICN Biochemicals, Cleveland, OH, USA) and oxytetracycline (Oxytetracycline HCl soluble powder, Terra Vet, Kansas City, MO, USA).

The tubes were filled with four SS of eggs in order to decrease the standard deviation of the average number of eggs per sample, different chemicals were added to produce the relevant treatments and the tubes were filled to 10 mL (Table 1). Once prepared, the tubes were stored at 1 °C. On the first day of the experiments an egg hatching success (HS) assessment was done. A set of four or six Petri dishes (100 mm, Fisher Scientific) with a known number of eggs, and a few drops of concentrated *R. salina* culture, was setup. The eggs were allowed to hatch 48 h at room temperature (20–25 °C), before they were fixed with Lugol's iodine solution (Lugol's). The number of nauplii that hatched was counted to calculate the HS on the first day of the experiment. Samples of each treatment were taken out of the incubator after different periods of cold storage and the eggs were hatched as for the first day HS experiments. After storage, the egg HS experiments were always done with the water from the storage vials, to which treated water was added to insure normoxic conditions.

At the end of all experiments, the relative hatching success in relation to day 0 was used to compare the sets of experiments. The HS at different times was divided by the HS at day 0. All the HS results from the antibiotics trials were compared statistically with a Tukey' multiple comparison test on ranks, with $P < 0.050$.

Size of nauplii after storage

A third experiment was conducted to check the size of the newly hatched nauplii vs. the storage time. *A. tonsa* eggs (< 24 h old) were harvested and volumetrically stocked into 30 mL Falcon tubes, so that there were approximately 100–200 eggs. An egg HS assessment was conducted the first day to check the viability of the egg pool (eight replicates). The tubes were stored at 1 °C for 1, 7, 14 and 30 days. After each of these storage periods, four replicates were used for an egg HS assessment as described above and the sizes (total length and total width) of 30 nauplii from each period of storage were measured under a dissection microscope (Motic Instruments Inc., Richmond, Canada). For statistical analysis, a Kruskal–Wallis one-way analysis of variance on ranks was made with $P < 0.05$.

Effects of egg development stage on survival during cold storage

In a last experiment, eggs produced over 1.5 h were harvested from the culture tank, cleaned and subsampled to prepare samples into 15 mL Falcon tubes containing 2589 ± 198 eggs (nine SS) in 10 mL of treated water. The samples were randomly separated into three groups called 0–2-h old eggs, 5–7-h old eggs and 10–12-h old eggs. The first age group was transferred into an incubator at 1 °C 2 h after the eggs were harvested so that the embryos were between 30 min and 2 h old when cold stored. The other groups were standing at 17 °C. Five hours later, the eggs from the second age group were transferred at 1 °C, followed 5 h later by the last age group.

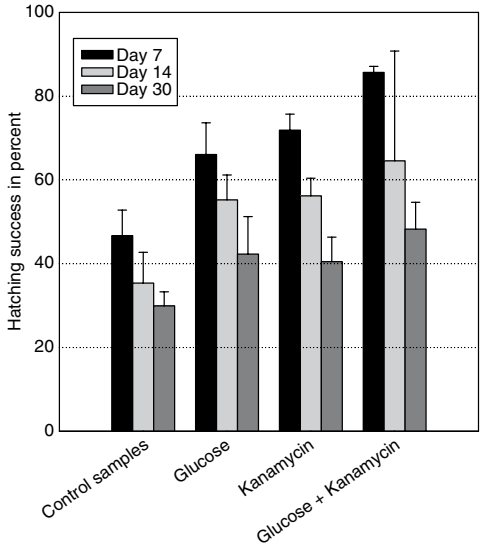

Figure 1 Rescaled hatching success of subitaneous *Acartia tonsa* eggs after 7, 14 and 29 days of cold storage at 1 °C, with or without treatment. (average ± standard deviation; five replicates for each bar). On average, over the experimental period, all HS from all the treatments were statistically different from the control ($P < 0.05$).

One set of the tubes was not cold stored and was used for an egg HS assessment as described above. Five tubes of each egg age group were used for an egg HS assessment after 5, 10, 15 and 30 days of cold storage. For statistical analysis a Tukey' multiple comparison procedure was used with $P < 0.05$.

Results

Kanamycin and glucose

The HS of the pool of eggs at day 0 was 95 ± 4%. The data computed afterwards were expressed as relative hatching success in relation to day 0. The cold storage always decreased the survival of the eggs regardless of treatment. The HS varied from 47 ± 6% (untreated eggs) to 86 ± 1% (glucose +kanamycin treated eggs) after 7 days, from 35 ± 7% (untreated eggs) to 65 ± 26% (glucose +kanamycin treated eggs) after 14 days and from 30 ± 3% (untreated eggs) to 48 ± 6% (glucose+kanamycin treated eggs) after 29 days (Fig. 1). There were statistical differences of the mean HS after 7, 14 and 29 days of cold storage (7 vs. 14 days; 7 vs. 29 days; 14 vs. 29 days; $P < 0.05$). The addition of glucose and kanamycin or their mixtures to the storage vials increased the shelf life of subitaneous *A. tonsa* eggs during cold storage. This effect tended to decrease steadily over the storage period. After 7 days, the combined treatment of glucose and kanamycin led to a HS almost double that found for the untreated eggs, while after 29 days the increasing HS effect declined to about 50% of its day 7 effect. Except for individual glucose and kanamycin treatments, over the experimental period, all HS from all the treatments were statistically different from each other ($P < 0.05$).

During this experiment, the mixed treatment (kanamycin+glucose), which gave the highest HS after cold storage, showed some filamentous growth around some of the nauplii and pieces of organic matter. This was not observed in the other treatments. All treated samples (glucose and/or kanamycin) showed better HS after 7, 14 and 29 days compared with untreated eggs.

Oxytetracycline and glucose

The eggs used for the second experiment presented a HS of 86 ± 5% at Day 0. As in the previous experiment, the relative hatching success in relation to day 0 was used. The HS, all treatments merged, showed statistical differences between the different periods of cold storage (days 15 vs. 60; days 15 vs. 45; days 15 vs. 30; days 30 vs. 45; days 30 vs. 60; days 45 vs. 60; $P < 0.05$). After 15 days of storage, the HS of all antibiotic-treated eggs were statistically higher than those of the non-antibiotic-treated eggs ($P < 0.05$), except for the oxytetracycline treatment (10 mg L^{-1}) with no glucose which was not different from non-antibiotic-treated samples (Fig. 2). On average, the highest concentration of oxytetracycline led to the highest HS. From day 30 on, this tendency changed, and the highest concentration of oxytetracycline (100 mg L^{-1}) led to the lowest HS of all antibiotic-treated samples. The HS of the eggs treated with oxytetracycline (1 and 10 mg L^{-1}) with or without glucose were statistically different from all other treatments ($P < 0.05$). There were no HS differences between the glucose-alone-treated samples and the controls. The decreased HS of the 100 mg L^{-1} oxytetracycline-treated samples was even more pronounced after 45 days of storage, where the highest concentration of oxytetracycline led to 4–5% HS, while the control samples HS averaged twofold better. However, the two lowest oxytetracycline-treated samples (1 and 10 mg L^{-1}) kept a statistically higher HS than in the other groups ($P < 0.05$), except for the

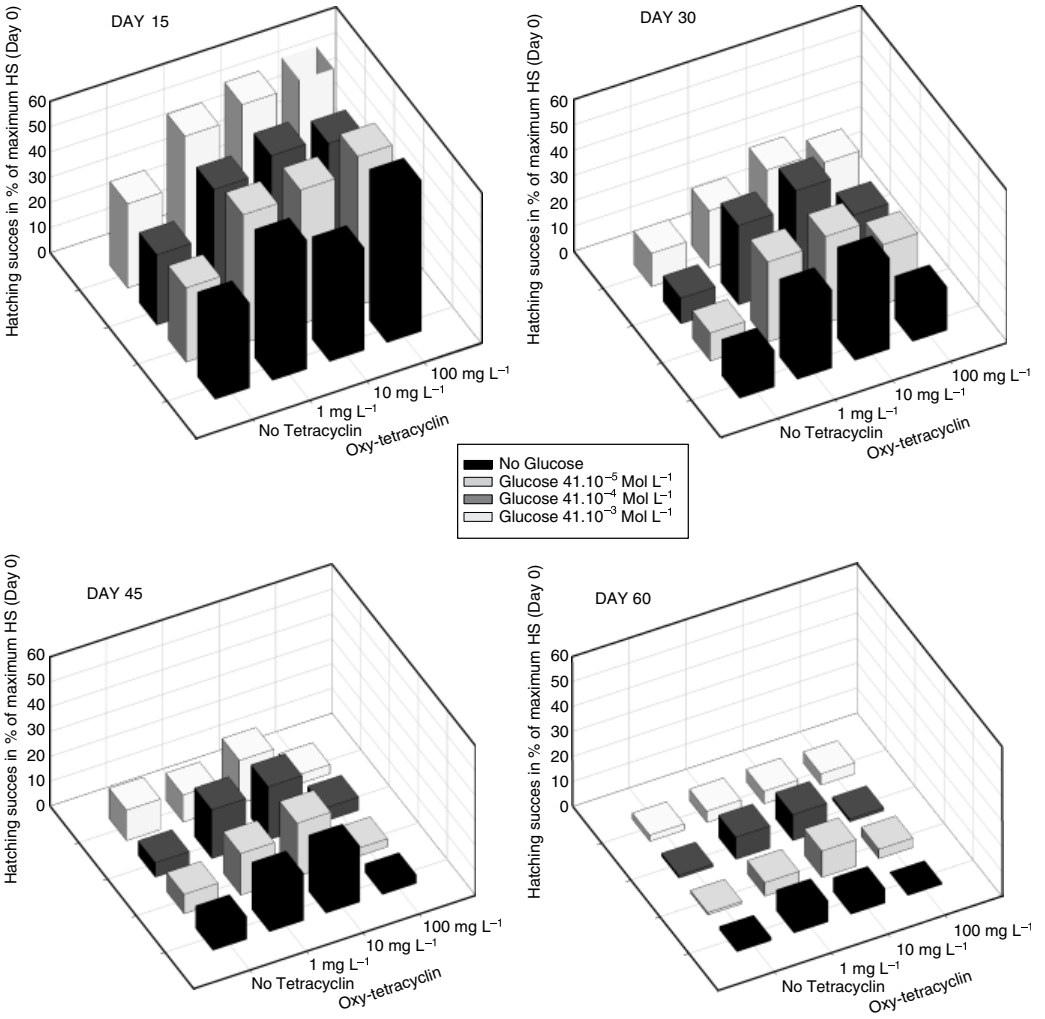

Figure 2 Rescaled hatching success of subitaneous *Acartia tonsa* eggs after 15, 30, 45 and 60 days of cold storage at 1 °C, with or without treatment. (average, four replicates for each bar).

samples treated with the highest concentration of glucose. In general, glucose alone did not increase the shelf life of the eggs, as in the previous experiment; except after 45 days, when the sample treated with the highest concentration of glucose led to a slightly higher HS than the untreated eggs. After 60 days of cold storage, the highest concentration of glucose (41.10^{-3} mol L^{-1}) tended to decrease the shelf life of the eggs in the two lowest oxytetracycline-treated samples (1 and 10 mg L^{-1}). The maximum HS were found for the samples treated with oxytetracycline at 1 and 10 mg $-L^{-1}$, without glucose or with glucose at 41.10^{-5} and 41.10^{-4} mol L^{-1}.

Size of nauplii after cold storage

This experiment was conducted to determine the size of the nauplii vs. the storage time. The HS of the eggs at the beginning of the experiment was low and with a high standard deviation (60.5 ± 18.5%). The mean length of the nauplii decreased from 122.9 ± 8.1 μm before the cold-storage, to 104 ± 11.3 μm after 30 days of storage. However, the width of the nauplii was less affected by the storage period and the trend was less obvious. The width decreased from 59.2 ± 3.5 μm for the fresh nauplii to 56.7 ± 5.1 μm after 30 days of cold storage. The width of the nauplii

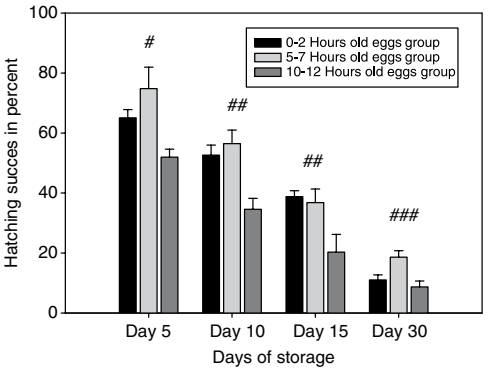

Figure 3 Hatching success of three different egg-development groups after 4 increasing periods of cold storage (mean ± standard deviation). Eggs were 0–2, 5–7 and 10–12 h old when they were first cold stored at 1 °C. #All the hatching success (HS) were statistically different from each other; ##the HS from the 0–2 h and 5–7 h groups were statistically different from the HS of the 10–12 h group; ###HS from the 5–7 h group was statistically different from the HS of the 10–12 h group ($P < 0.05$).

during the cold storage was only significantly different between day 1 and day 30 ($P < 0.05$). The length of the nauplii after cold storage was significantly reduced compared with those hatched from non-stored eggs after 14 days and 30 days of cold storage but not before ($P < 0.05$).

Effects of egg development on survival during cold storage

Another experiment was conducted to assess the impact of the developmental age (in hours) of the eggs on HS after cold storage. The HS at day 0 was 91 ± 4%. The relative hatching success in relation to day 0 decreased during the cold storage as it did for the other experiments, ranging from 75.5% for the 5–7-h old eggs group after 5 days to 8.6% for the 10–12-h old eggs group after 30 days (Fig. 3). There were statistical differences between the average HS of the different time of storage (Day 5 vs. Day 10 vs. Day 15 vs. Day 30; $P < 0.05$). There were also some differences between the groups. The more developed eggs group (10–12 h) always showed the worst HS after cold storage of all the groups, and statistical differences were found ($P < 0.05$) except when compared with the 0–2-h old eggs group after 30 days of storage. The average HS of the freshest eggs (0–2 h old) was only higher than the HS of the 5–7-h old eggs after 15 days of cold storage. No statistical differences were found between these two groups (except after 5 days of cold storage) even though there was a clear trend showing a higher survival of the 5–7-h old eggs. During the HS after storage, the non-hatching eggs from the 10–12 and 5–7-h old eggs were degrading, presenting a halo of bacterial or fungal growth around the shell while the non-hatching eggs from the 0–2-h old eggs group were not degrading, and looked opaque with no halo around the shell.

Discussion

Disinfection of copepod eggs for later use as live feed for fish larvae has been studied by Naess and Bergh (1994). Støttrup (2003) proposed to disinfect eggs from *Tisbe holothuriae* Humes by adding chlorine to the water forcing the copepods to release their egg sacs, which could be harvested, rinsed and used to hatch nauplii in axenic conditions. During the present experiments, antibiotic treatments with or without glucose were tested to determine if the shelf life of *A. tonsa* eggs stored in the cold could be further extended. Also, the impact of the stage of development of copepod eggs on their survival during cold storage was assessed.

A number of parameters, such as temperature, salinity, dissolved oxygen and food quality influence the HS of subitaneous copepod eggs (Jonasdottir 1994; Lutz *et al.* 1994; Marcus & Lutz 1994; Castro-Longoria 2003; Shin, Jang, Jang, Ju, Lee & Chang 2003; Holste & Peck 2005). The HS of the non-stored eggs during the experiments was variable and was low in the third experiment. Also the eggs used during the third experiment were between 0 and 24 h old, while the eggs were always more fresh during the other experiments. It is likely that some of the eggs produced early during the 24 h period had already hatched, increasing the number of non-viable eggs in the harvest, and decreasing consequently the HS. Generally, the eggs harvested at FSUCML hatched within 15–24 h (pers. observ.) and the HS experiment were always lasting 48 h to be sure that all viable eggs would develop to nauplii.

However, the HS of the non-stored eggs in the first two experiments and in the fourth one was high, and similar to the HS observed for the same species by Castro-Longoria (2003) in Southampton (UK) waters, who described a HS of 86 ± 4% and 97 ± 3% at 15 °C and 20 °C respectively. The HS at Day 0 of the third experiment was quite low but it still corresponded to

the HS occasionally observed in the laboratory (Jonasdottir 1994). During the present experiment, other factors may have affected the HS of the eggs. For instance, the light regime was not checked and the room temperature for hatching varied from 20 °C to 25 °C; this could have affected the HS. The survival of the eggs was almost nonexistent after 2 months of cold storage. These results were very different from those described for an *A. tonsa* culture held in Denmark, where eggs were shown to survive periods of cold storage up to 12 months; after 11 months, 70% of the eggs which still looked viable hatched (Drillet *et al.* 2006). This difference in capacity to be cold stored could be due to the 'strain' of copepods. The culture method used in Denmark (Støttrup *et al.* 1986), and the regular storing of the eggs might have selected a group of *A. tonsa* capable of surviving for a longer period (> 25 years raised in Denmark vs. 2 years in Florida). Further, the origin of the eggs might have influenced the storage capacity. The climate conditions occurring around the Baltic Sea force the copepod populations to face long periods of cold and dark conditions during the winter time, while the population from the Gulf of Mexico does not experience these conditions.

During the first experiment, the kanamycin treatment increased significantly the shelf life of Gulf of Mexico strain *A. tonsa* eggs. However, this effect tended to be less and less obvious throughout the storage period. In the sample containing kanamycin and glucose, filaments were sometimes present around the non-hatched eggs and some of the hatched nauplii. These filaments were presumed to originate from bacterial or fungal growth around the decaying material. A similar filament halo was also observed by Drillet *et al.* (2006) around decaying eggs after a long period of storage. During the present experiments, the existence of bacterial or fungal growth after the storage period was not checked unlike the study by Naess and Bergh (1994). The presence of these filaments around some of the nauplii implied that some of the nauplii were dead before the end of the HS experiment, suggesting a possible lethal effect of the treatment on nauplii, while it had no effect on the eggs. Kanamycin toxicity has been documented for high concentration (one order of magnitude higher that what was used here) by Fisher and Nelson (1978) who observed higher *Cancer magister* Dana larval mortalities with kanamycin treatment ($100\ mg\ L^{-1}$) than that in their control with no antibiotics. This obvious toxicity of some antibiotics also confirmed the results presented by Naess and Bergh (1994) for *Acartia clausi* Giesbrecht and *Eurytemora affinis* Poppe. In their experiments, some of the disinfecting treatments had no effect on the eggs, but were lethal for newly hatched nauplii. The higher egg tolerance to toxic compounds was shown for calanoid resting eggs which were proven to be 20–100 times more tolerant to rotenone treatment than later ontogenetic stages (Naess 1991a, b).

During the second experiment however, no bacterial or fungal filaments were present in the Petri dishes. The oxytetracycline treatments seemed very effective, and increased the survival of the cold stored eggs. At high concentrations however, after extended period of storage, oxytetracycline showed a toxic effect suggesting that the concentration of antibiotics used has to be adjusted depending on the period of cold storage planned. The reproductive success of *Daphnia magna* Strauss was shown to be negatively influenced by concentrations of oxytetracycline from 5 to $50\ mg\ L^{-1}$ (Wollenberger, Halling-Sorensen & Kusk 2000), while Isidori, Lavorgna, Nardelli, Pascarella, and Parrella (2005) found an acute effect of tetracycline at $22.6\ mg\ L^{-1}$ on the same target organism. Recent publication on *Daphnia* by Di Delupis, Macri, Civitareale and Migliore (1992); Wollenberger *et al.* (2000); Flaherty and Dodson (2005), showed that exposure to mixtures of antibiotics could change the sex ratio, reproductive capacities and phototactic behaviour of this crustacean. During the present experiments, the offspring growth and post storing effect of the antibiotic treatments were not followed. It would be very interesting to see how the treatments affect *A. tonsa* after cold storage. The results from other studies suggest that the offspring copepod population could be severely affected by the two highest concentrations of oxytetracycline used during the present study. It also suggests that the stored egg samples should be filtered and thoroughly rinsed before they are set out to hatch, to eliminate the disinfectant compounds, which may have a harmful effect after hatching.

Results concerning the glucose effect on the shelf life were not consistent. During the second experiment, the glucose did not engender any beneficial effect on cold storage survival like it appeared to during the first experiment. It is possible that glucose, which is a quite large molecule, will not pass through the shell because of the tegument structure, but its presence in the media containing the eggs might have changed the osmotic equilibrium between the eggs and their environment. More investigations should be launched to understand which molecules are able to pass through the tegument of the calanoid eggs.

The third experiment was conducted to assess the size of the nauplii vs. the storage time under cold conditions. However, statistical differences between the nauplii length of non-stored and cold stored eggs were found after 14 and 30 days of storage only. This might have been due to a decrease in the availability of biochemical reserves. It was previously shown for another 'strain' of *A. tonsa* that the biochemical contents of eggs changed during cold storage (Drillet *et al.* 2006) without affecting the capacities of the future adults to produce viable offspring.

Finally, the development stage of the embryo was shown to affect the capacity of the eggs to survive cold storage. This supports the results of Lutz *et al.* (1992, 1994), and Marcus and Lutz (1994) who observed that the development of eggs affected their HS and viability when exposed to hypoxia/anoxia. During the present study, the 10–12-h old eggs group presented the worst hatching success at all sampling times, suggesting that developed eggs are more sensitive to cold storage than newly laid eggs. No groups over 10–12 h old were tested as some eggs are known to hatch after 15 h and this would have affected the results. Until 7 h of development, the eggs showed a better survival to cold storage conditions. The 0–2-h old eggs group tended to present a lower hatching success than the 5–7-h old eggs group even though no statistical differences were found. This result was interesting since the non-hatching eggs present in the 10–12-h old samples were degrading very fast and bacterial or fungal growth was observable indicating that the non-hatching eggs were dead, while the non-hatching eggs in the 0–2-h old eggs group did not seem to be decaying, indicating that they were still alive. We propose two speculative hypotheses concerning this observation. The eggs may have been unable to develop further because the temperature shock blocked the development during one of the division processes, and the eggs were surviving without developing, until their reserves were gone. These effects of cold treatment upon embryonic development have been well described for different fish species, showing that tolerance to low temperature was relatively low during the earliest developmental stages (particularly cleavage stages) and at some other periods of the development (Sasaki, Kurokura & Kasahara 1988). Another possible explanation for the non-degrading eggs (0–2-h old samples) is that the eggs switched into a deeper resting stage because the temperature shock happened early enough in their development, and needed a stronger signal or delay, than just a quick increase of the temperature before they hatch. None of the non-hatching eggs from the 0–2-h old eggs group were left to hatch for longer than 48 h. Also, no trials were launched to test the viability of these non-hatching eggs after long periods of cold storage, so no clear answers are evident.

Conclusion

The results presented here allowed us to develop a storage protocol for subitaneous *A. tonsa* copepod eggs. Firstly, cold storage forced eggs to switch to a quiescent state. The short shelf life of *A. tonsa* eggs from Florida was increased by the addition of antibiotics in the storage vials during cold storage. However, the antibiotics had a lethal effect on eggs and nauplii when their concentration was too high or when the exposure period was too long. The addition of the proper dose of oxytetracycline or kanamycin to storage vials appears to be a useful procedure to increase the shelf life of *A. tonsa* eggs stored in the cold. The amount of antibiotic used needs to be adjusted depending on the period of cold storage planned. The effect of glucose was not consistent, and no significant results were achieved when using it. Long storage periods are likely to decrease the quality of the eggs and subsequent nauplii, which tend to become smaller after extended periods of cold storage. Finally, the egg development stage was shown to be important to keep a good HS after cold storage of the eggs. The best stage of development for storage was 5–7 h old, very young and very old eggs had the poorest hatching success after cold storage.

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Strain-specific vital rates in four *Acartia tonsa* cultures, I: Strain origin, genetic differentiation and egg survivorship ☆

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ABSTRACT

Successful development of planktonic copepods for use as live feed in marine aquaculture relies on optimization of environmental conditions for population growth and egg storage. This study examined strain-specific differences in egg survivorship during cold-storage-induced quiescence in four cultures of the widespread marine calanoid *Acartia tonsa* Dana, 1849. Experimental cultures were obtained from Øresund, Denmark (DIFRES), Kiel, Germany (KIEL), Turkey Point, Florida, USA (FL), and Mobile Bay, Alabama, USA (AL), and were shown to derive from three highly distinct mitochondrial clades. Eggs from Gulf of Mexico strains had low tolerance for cold storage, and showed very low hatching success after 10 days. In contrast, Baltic Sea strains produced eggs able to tolerate up to 150 days of cold storage, with the DIFRES strain showing the highest egg survivorship during the experiment. Eggs from the Kiel strain showed an increase in hatching over time, indicating the presence of dormant eggs. Parental rearing temperature was also found to affect egg survivorship during quiescence in the DIFRES strain, with lower hatching success observed among eggs produced at 25 °C than at 17 °C. The DIFRES strain is recommended as the optimal strain for use in aquaculture, and conditions for cold storage of eggs are discussed.

1. Introduction

Dormancy is an important strategy used by marine planktonic copepods to survive poor environmental conditions. Copepods have been described to enter dormancy at various stages of development, including over-wintering copepodites and resting eggs that settle into sediment layers (Mauchline, 1998). Resting eggs can be important in the population dynamics of coastal species due to their role as benthic seed banks for pelagic populations (e.g., Katajisto et al., 1998). Recovery of resting eggs from the sediment is thought to vary depending on physical and biological conditions in the sediment, but hatching success can be in the range of 21.4%, with seed banks containing on the order of 10^6–10^7 viable eggs m^{-2} (Marcus, 1984; Marcus et al., 1994; Katajisto et al., 1998) (see reviews on dormancy by Grice and Marcus, 1981; Dahms, 1995; Marcus, 1996; and Alekseev et al., 2007).

Based on the work of Mansingh (1971), resting eggs have been classified into two distinct categories (Grice and Marcus, 1981): diapause eggs, which are obliged to arrest development for extended periods of time due to maternal hormonal control over egg development, and quiescent eggs, which are subitaneous eggs (produced to hatch rapidly) that arrest development due to the sudden appearance of poor environmental conditions (Grice and Marcus, 1981). Diapause eggs are produced in response to cues that precede poor environmental conditions (Grice and Marcus, 1981). In the case of diapause eggs, the eggs will not resume development even if conditions are optimal before the refractory period is over, while in quiescent eggs, the eggs resume development as soon as environmental conditions are again optimal. A third type of dormant egg, called "delayed-hatching", was later observed in *Labidocera scotti* and *Pontella meadi* by Chen and Marcus (1997) as another resting strategy, although no physiological data has distinguished them from the two other egg types. The duration over which copepod resting eggs remain viable in marine sediments has been rarely studied, but may have important ecological consequences. Resting eggs extracted from sediment cores have been shown to hatch after up to 40 years in the natural environment (10–13 years, Baltic sea; Katajisto, 1996; 40 years, Rhode Island, USA; Marcus et al., 1994).

Copepods have been shown to be of greater value than traditional live feeds for fish, such as *Artemia* and rotifers, when used either as a dietary complement or alone (see review by Støttrup, 2003, Table 5.2 and references therein; Rajkumar and Vasagam, 2006; Wilcox et al., 2006). Copepods have higher nutritional value (see review by Støttrup, 2003; Drillet et al., 2006b), and extensive outdoor cultures have been successfully used in fish enclosures for use with first-feeding larvae (Engell-Sørensen et al., 2004; Sørensen et al., 2007). There are two main difficulties in increasing the use of copepods in

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aquaculture: (1) the lack of a well-developed intensive copepod culturing technology (*but see* Sun and Fleeger, 1995; Schipp et al., 1999; Payne and Rippingale, 2001a) and (2) the long-term storage of copepod products (eggs and nauplii; Støttrup et al., 1999; Marcus and Murray, 2001; Payne and Rippingale, 2001b; Drillet et al., 2006a,b, 2007). Much of the biological data required for development of large-scale copepod cultures, for example optimal food conditions, temperature, salinity, and the impact of cannibalism, can be obtained from ecological studies, with a number of recent papers also addressing the effect of stocking density on egg production and survival (Medina and Barata, 2004; Peck and Holste, 2006; Jepsen et al., 2007). Studies on survival of *Acartia* spp. have shown that eggs from this genus can survive low temperature and refrigeration (Toledo et al., 2005; Holmstrup et al., 2006). However, recent studies on survivorship and hatching success of *Acartia tonsa* copepod eggs stored at cold temperatures have shown conflicting results. *A. tonsa* eggs from the Baltic Sea area survived for up to a year in storage at 4–5 °C, whereas eggs from the Gulf of Mexico area did not survive longer than a month under the same conditions (Drillet et al., 2006a,b, 2007). Differences in stage development between two allopatric populations of *A. clausi* also have been observed in previous studies, highlighting the potential importance of geographic origin of strains (Leandro et al., 2006). These results, among others, suggest that there may be strain-specific differences in life history traits that impact growth and storage in culture.

A. tonsa is a cosmopolitan planktonic copepod species that is abundant in neritic and coastal waters of the global ocean (Razouls et al., 2005–2007). As dominant members of coastal planktonic communities, *Acartia* species serve as important food sources for fish populations in the wild (Sheridan, 1978 in Putland and Iverson, 2007; Fox et al., 1999; Möllmann et al., 2004; Maes et al., 2005). *A. tonsa* also is known to produce dormant eggs, in either true diapause or quiescent stages, in order to survive periods of poor environmental conditions (Zillioux and Gonzales, 1972; Castro-Longoria, 2001). For these reasons, *A. tonsa* may be particularly valuable for use in marine aquaculture. However, the taxonomy of the genus *Acartia* is known to be incomplete (Bradford-Grieve, 1999), and genetically distinct clades have been reported within the nominal species *A. tonsa* (Caudill and Bucklin, 2004; Hill, 2004; Chen and Hare, 2008). These clades have been shown to be ecologically differentiated in some cases (Hill, 2004; Chen and Hare, 2008). Many broadly-distributed neritic and estuarine species, including other *Acartia* species, also have genetically distinct populations that exhibit levels of differentiation comparable to species-level differentiation in other copepod genera (e.g., McKinnon et al., 1992; Trujillo-Ortiz et al., 1995; Lee, 2000; Kelly et al., 2006; and Burton and Trujillo-Ortiz, unpub. data). Given these observations, it appears likely that the nominal species *A. tonsa* includes a number of genetically divergent lineages or cryptic species that may have specific habitat or culturing requirements.

In an effort to understand how the geographic origin and genetic lineage of *A. tonsa* strains impact optimal culture conditions and egg storage capacity, we conducted two common garden experiments on four *A. tonsa* strains isolated from the Baltic Sea and eastern US coast (Fig. 1). The objectives of our experiments were to describe the genetic differentiation between strains, test for strain-specific differences in life history traits and egg survivorship during cold storage, and identify the lineage/population with the most optimal suite of life history traits for sustainable mass cultivation and storage of eggs for use in aquaculture. Four experimental strains were examined for differences in developmental rates, mortality, sex ratio, egg production and hatching success, egg size, resting egg survivorship following cold storage for up to 150 days, biochemical composition of eggs and adults (polyunsaturated fatty acids and free amino acids), and genetic differentiation at two mitochondrial gene loci. Results on genetic differentiation between strains, egg size, and egg survivorship following cold storage are presented in the present paper, with a related paper describing results on life history traits and biochemical composition of strains (Drillet et al., 2008). We find that the European (DIFRES, Kiel) genetic lineage exhibits the highest egg survivorship over storage times up to 150 days, and is therefore recommended as optimal for cold-storage procedures for aquaculture applications.

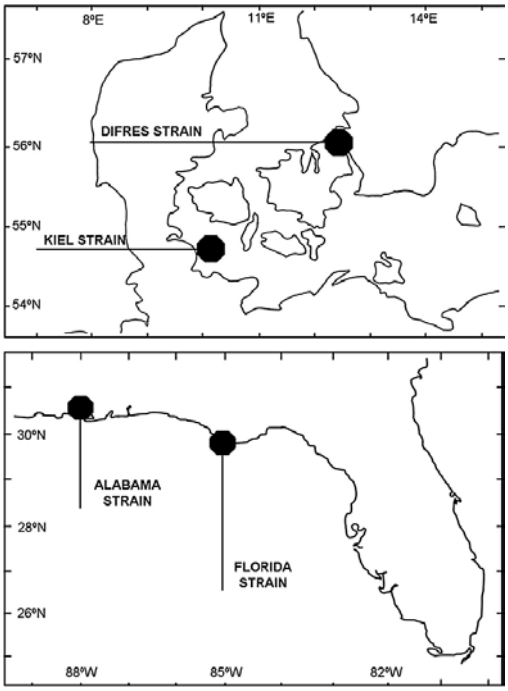

Fig. 1. Geographic origin of the four investigated strains.

2. Materials and methods

Experimental cultures of *A. tonsa* were established from four geographically distinct populations in the Baltic Sea and Gulf of Mexico: (1) Øresund, Denmark (DIFRES), (2) Kiel, Germany (KIEL), (3) Turkey Point, Florida, USA (FL), and (4) Mobile Bay, Alabama, USA (AL). Eggs were obtained from local cultures and stored in test tubes, cooled down and sent by express transport (<2 days) to the Danish Institute for Fisheries Research (DIFRES), where cultures were launched immediately. Cultures were maintained under constant laboratory conditions for more than 8 weeks (~2 generations) to eliminate maternal effects. Temperature and salinity were kept at 17.6±0.3 °C and 34 ppt, respectively. Cultures were held in semi-opaque tanks providing dim light that followed the natural diel cycle (April to August). All strains were fed in excess with the chrypto-phyte *Rhodomonas salina* (8 μm equivalent spherical diameter). *Rhodomonas* was grown at 17 °C on B1 media (Hansen, 1989) under 14L/10D light conditions (66 μmol m^{-2} s^{-1}). Sea water used during the experiment was filtered at 0.22 μm. Eggs were harvested from the culture tanks by siphon and cleaned by successive filtrations through 100 μm and 63 μm Nitex meshes. Cleaned eggs were used to initiate the egg storage experiment. Adults from each strain were sent for morphological identification to I. Vaglio and G. Belmonte (Lecce, Italy), and slight morphological differences were observed between strains (pers comm).

2.1. Origin of the four strains (Fig. 1)

2.1.1. DIFRES

The DIFRES strain originated from individuals isolated in 1981 from the Øresund (N 56°/E 12°; Denmark) and were assigned the identification code DFH-ATI (Støttrup et al., 1986). The strain has been maintained under constant temperature and light conditions for over 25 years (34 ppt, 17 °C, dim light), and has been fed a monoalgal diet of *R. salina*. Eggs were routinely harvested and cold stored for later use.

2.1.2. Kiel

The Kiel strain originated from adults isolated in August 2003 in the Kiel Bight (Germany), south-western Baltic Sea (N 54°/E 10°; Holste and Peck, 2005). In the laboratory, cultures were maintained at 18 ppt, 18 °C and 13L/11D and fed *Rhodomonas* sp. daily. Eggs were collected daily and cold stored for later use. This strain is currently in use as live prey for cod (*Gadus morhua*) in some Danish hatcheries (J.G. Støttrup, pers comm).

2.1.3. Florida

The Florida strain originated from Turkey Point, Florida, USA. The copepods were collected from local waters outside the Florida State University Coastal and Marine Laboratory (FSUCML; 29°55′N/84°30′ W) between 2002 and 2004, and the culture was established from adult females. The culture was maintained in the laboratory for multiple generations at 30 ppt, 17 °C, and 14L/10D, while being fed a mixed algal diet (*R. lens*, *R. salina*, *Akashiwo sanguinea*). Eggs were harvested on a regular basis and stored in a dark incubator at 1 °C (±0.5) until later use. This strain has been successfully used for first feeding of Southern Flounder, *Paralicthys lethostigma* (Wilcox et al., 2006) and Floridian Pompano, *Trachinotus carolinus* (Marcus N.H. and Wilcox J., pers comm).

2.1.4. Alabama

The Alabama strain originated from Mobile Bay, Alabama, USA. Copepods were collected on June 2005 along a transect in the ship channel in the lower bay (N 30°/W 87°). Animals were cultured at 30 ppt, 19 °C, 20L/4D on a mixed dinoflagellate diet including *Prorocentrum micans*, *Scrippsiella trochoidea*, and *A. sanguinea* (*see* Oppert, 2006).

2.2. Genetic analysis of strains

Individuals from the four cultures were isolated alive, preserved in 95% ethanol, and stored at −80 °C until further analysis. DNA was also extracted directly from a few live individuals of the Kiel strain. DNA was extracted from individual adult females or copepodites using a standard lysis buffer protocol (Lee and Frost, 2002; Goetze, 2003), and 2–5 μL of extract was used in each polymerase chain reaction. DNA sequence data were obtained for two mitochondrial genes, cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA), from all four culture populations. These gene loci are linked, and although they do not provide independent assessments of the true tree, they are useful for identifying pseudogenes and for comparisons of genetic divergence to other calanoid copepod genera. A 702-bp region of the 5′ end of mitochondrial COI was amplified using the primers COI_VH and L1384 (Folmer et al., 1994; Machida et al., 2004). A 400-bp region of mitochondrial 16S rRNA was amplified using primers 16S CB and 16SAR (Palumbi et al., 1991; Braga et al., 1999). Annealing temperatures were 42 and 45 °C, for COI and 16S rRNA, respectively. Although the experimental strains were not isofemale lines, they were, in many cases, established from a small number of females and mitochondrial diversity within the culture is expected to be absent or very low. Sequence data were obtained from 5–17 individuals of each strain at each gene locus, for a total of 31 and 24 individuals for COI and 16S rRNA, respectively. New sequences are available in GenBank under accession numbers EU196678–EU196732.

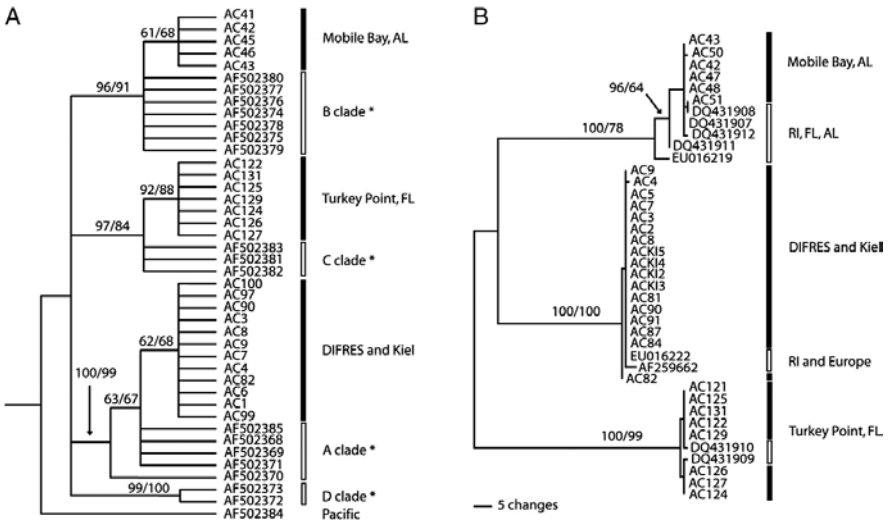

Fig. 2. (A) Maximum parsimony consensus tree of *A. tonsa* lineages and culture strains, based on the mitochondrial gene 16S rRNA. Numbers beside each node indicate bootstrap support for the node (MP/ML), and the topology of the 50% majority-rule consensus tree is based on 10 equal-length trees. Strains used in the present study are indicated in black bars, open bars are clades sampled by Caudill and Bucklin (2004). The tree is rooted by the Pacific lineage of *A. tonsa* (GenBank No. AF502384). (B) Maximum parsimony phylogram of *A. tonsa* strains and lineages, based on the mitochondrial gene COI. This is one of 150 equal-length MP trees, which differ only in the position of haplotypes within the clade containing Mobile Bay, AL and RI, FL, AL. Numbers beside each node are bootstrap support for the node (MP/ML), and black bars indicate strains used in the present study. The tree is unrooted, with GenBank accession numbers as indicated (Bucklin et al., unpub; Durbin et al., 2008).

Both sequenced strands were aligned and checked for accuracy using VectorNTI software (Invitrogen), and multiple sequence alignments were generated using ClustalW (Thompson et al., 1994). The final COI alignment contained 658 bp. Because the primary goal was to place the culture populations within the larger phylogeographic structure of the species, the 16S rRNA alignment included all *A. tonsa* data reported in Caudill and Bucklin (2004) (GenBank Nos. AF502368–AF502385), and was therefore restricted in length to an unfortunately short fragment of 129 bp. Parsimony and likelihood analyses of each gene were conducted in PAUP* 4.0b 10 (Swofford, 2002), using heuristic tree searches with TBR branch swapping and random stepwise addition. For the 16S rRNA tree, node stability in the parsimony analysis was estimated by 1000 bootstrap replicates, with 1000 random stepwise additions per replicate. Maximum likelihood analyses included the K81 (with unequal base frequencies) substitution model, which was selected as the best-fit model by AIC (Modeltest; Posada and Crandall, 1998), and ran for 100 bootstrap replicates, with 10 stepwise additions per replicate. Analysis of COI data included 750 bootstrap replicates, with 10 random stepwise additions per replicate in the parsimony analysis, and 100 bootstrap replicates with 10 random stepwise additions in the likelihood analysis. The best-fit model as selected by AIC for the COI data was the HKY (+G) model. All nucleotide sites were included in the analysis. Clades with ≥70% bootstrap support were considered well-supported. The average number of nucleotide differences between experimental strains was calculated in DnaSP (Rozas et al., 2003).

2.3. Egg storage and morphology

Eggs from the four strains were observed under an Olympus BX50F microscope. Images were captured with a Colorview camera, and the image-processing program AnalySIS was used to measure egg diameter (10–15 eggs). Newly spawned eggs, less than 12 h old, were collected from the culture tanks and cleaned as described above. Between 514 and 826 eggs were stored in 14 mL plastic tubes and stored in GF/F filtered sea water (34 ppt) at 5.4±0.7 °C (DIFRES17=647; DIFRES25=826; Kiel=704; Florida=514; Alabama=715). Hatching success (HS) was measured from all four strains for eggs incubated at 17 °C. In addition, eggs from the DIFRES culture were incubated at 25 °C (8 weeks acclimation) in order to observe any effects of parental rearing environmental temperature on the tolerance of eggs to cold storage. Hatching success was measured over a period of 2 days starting on 0, 10, 20, 30, 60, 100 and 150 days of storage. Five replicate tubes from each strain were transferred in their entirety to large Petri dishes (150 mm) and left for 48 h to hatch in a 17 °C constant temperature room. Samples were fixed with Lugol's solution and hatched nauplii were enumerated. Hatching success was calculated by dividing the number of hatched nauplii by the initial number of eggs.

3. Results

3.1. Genetic differentiation between experimental strains

The mitochondrial COI alignment was 658 bp in length, and contained 140 variable sites, 136 of which were parsimony informative. Across all strains, and including data from 31 individuals, a total of 8 haplotypes were observed. The average number of nucleotide differences between haplotypes within each strain did not exceed 0.16%, and within strain nucleotide diversity (π) ranged from 0.00036–0.00163. The 16S rRNA alignment was 129 bp in length, following truncation of the alignment to include only those nucleotide sites shared with *A. tonsa* sequences from Caudill and Bucklin (2004). The alignment included 39 variable sites, 29 of which were parsimony informative, and no indels were present in the alignment. A total of four 16S rRNA haplotypes were observed among all strains. A maximum of 0.03% in the average number of nucleotide differences between haplotypes within each strain was observed at this gene locus, and within strain nucleotide diversity ranged from 0–0.00129.

The four experimental strains derived from three highly distinct mitochondrial clades within the nominal species *A. tonsa* (Fig. 2A, B). The DIFRES and Kiel strains were genetically indistinguishable at both COI and 16S rRNA gene loci, indicating that they originate from the same lineage of *A. tonsa* within the Baltic Sea. The Turkey Point, FL and Mobile Bay, AL strains were highly divergent from one another, as well as from the DIFRES/Kiel European lineage (Table 1, Fig. 2). As estimated by Kimura 2-parameter distance measures, these three lineages were divergent by 14–17.45% at COI and 10.95–16.49% at 16S rRNA (Table 1). The average number of nucleotide differences between lineages ranged from 12.51–14.90% at COI and 10.08–14.90% at 16S rRNA (Table 1).

Phylogenetic results from mt COI and mt 16S rRNA analyses were in agreement regarding the position of the experimental strains within the phylogeny of *A. tonsa*, as expected given linkage of these gene loci. Caudill and Bucklin (2004, Fig. 1) described four divergent 16S rRNA mitochondrial clades, which they identified as containing 'A', 'B', 'C', and 'D' haplotypes. We will continue to refer to these clades by their letter designations. Analysis of 16S rRNA sequences found four reciprocally-monophyletic clades, which had bootstrap support values of 84–100% in both parsimony and likelihood analyses (Fig. 2A). The DIFRES/Kiel strain was found to be closely related to the A clade, while the Mobile Bay, AL and Turkey Point, FL strains were members of the B and C clades, respectively (Fig. 2A). Our experimental strains differed from haplotypes of these previously identified clades by 0–2.4% (Kimura 2-parameter distance estimates). However, given the very short length of this gene fragment (129 bp), there was insufficient information present in the data to resolve relationships among the four dominant clades. They are therefore presented as a basal polytomy in Fig. 2A, as observed in the topology of the 50% majority-rule consensus tree of 10 equal-length MP trees.

Analysis of mt COI data identified three reciprocally-monophyletic clades, each of which had between 78 and 100% bootstrap support in parsimony and likelihood analyses (Fig. 2B). Data from our experimental strains was analyzed in conjunction with *A. tonsa* COI sequences available in GenBank (Bucklin et al., unpub; Durbin et al., 2008). Current sampling at COI has recovered only three of the four known mitochondrial clades, with the Mobile Bay, AL and RI, FL, AL clade corresponding to clade B, DIFRES/Kiel and RI/Europe corresponding to clade A, and Turkey Pt, FL representing clade C (Fig. 2B). Although parsimony analysis recovered 150 equal-length trees, these trees differed only in the relative positions of haplotypes within the Mobile Bay, RI, FL, AL clade (B) and were stable with regard to placement of haplotypes across clades and the positions of haplotypes within clades A and C. One observation of note is that one of the DIFRES/Kiel strain haplotypes was 100% identical to a haplotype sampled in Rhode Island, USA.

Table 1
Genetic differentiation between experimental strains of *A. tonsa*, based on (A) 658 bp of mt COI and (B) 129 bp of mt 16S rRNA

	Alabama	Florida	DIFRES and Kiel
A. COI			
Alabama	****	14.79%	12.51%
Florida	17.24–17.45%	****	14.90%
DIFRES and Kiel	14.00–14.44%	16.93–17.13%	****
B. 16S rRNA			
Alabama	****	10.08%	11.69%
Florida	10.95%	****	14.02%
DIFRES and Kiel	12.64–13.58%	15.50–16.49%	****

Kimura 2-parameter distance estimates between strains are listed below the diagonal, and average numbers of nucleotide differences are listed above the diagonal.

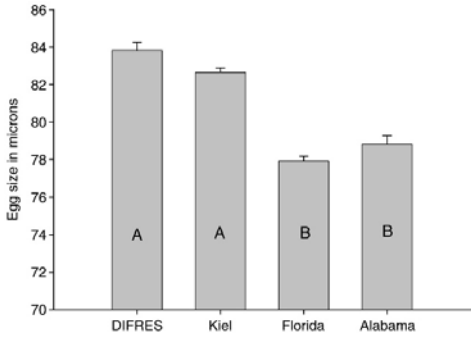

Fig. 3. Egg size of *A. tonsa* cultures from four geographic areas, raised in a common garden experiment at 17 °C. Bars labeled A and B have significantly different mean values (Kruskal–Wallis on Ranks, Dunn's method, $P<0.001$).

3.2. Egg morphology

Egg diameter was 83.8±0.4 μm, 82.7±0.2 μm, 77.9±0.3 μm, and 78.8±0.5 μm for DIFRES, Kiel, Florida, and Alabama eggs, respectively (mean±S.E.; Fig. 3). There were significant differences in egg size between the Gulf of Mexico and Baltic strains (Kruskal–Wallis on Ranks, Dunn's method, $P<0.001$), but not within these two groups of strains.

3.3. Egg storage

Egg survivorship during cold storage varied between strains (Figs. 4 and 5). Eggs from Florida and Alabama rapidly lost their viability over time, and showed increased mortality after 30 days of cold storage (Fig. 4). After 60 days of cold storage, no survival was observed in eggs from either strain. Florida and Alabama hatching successes were significantly lower than the DIFRES strain from day 20 onwards (all pairwise multiple comparisons, Student Newman–Keuls methods, $P<0.05$; Fig. 4). Eggs from DIFRES were more resistant to cold storage, and 5.0±1.2% hatched after 150 days. Hatching success of DIFRES eggs was significantly different from the other three strains on all days except Alabama on day 10 (all pairwise multiple comparisons,

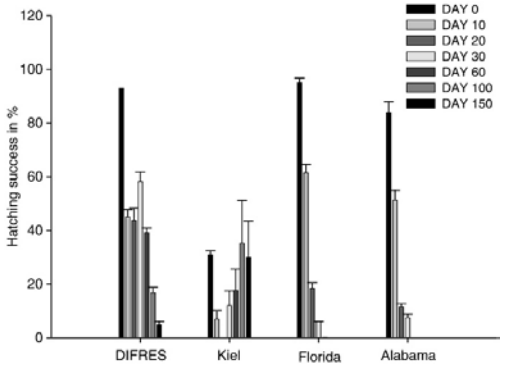

Fig. 4. Hatching success at 17 °C of eggs from the four *A. tonsa* strains produced at 17 °C and stored subsequently at 5.4±0.7 °C for a range of storage durations. For each strain, bar color indicates increasing periods of storage (inset legend). Figures represent the mean±S.E.

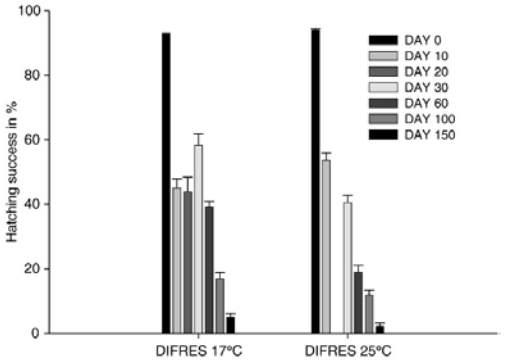

Fig. 5. Hatching success at 17 °C and 25 °C of eggs from the DIFRES strains of *A. tonsa* produced at 17 °C or 25 °C and stored subsequently at 5.4±0.7 °C for a range of storage durations. For each strain, bar color indicates increasing periods of storage (inset legend). Figures represent the mean HS± S.E.

Student Newman–Keuls methods, $P<0.05$). Eggs from the Kiel population showed the most surprising trend in hatching pattern during the cold-storage period. At day 10, the lowest hatching success was observed for this strain, but subsequently increased with the length of the cold-storage period from 7.0±0.7% hatching after 10 days to 35.4±2.0% hatching after 100 days and 30.1±3.7% hatching after 150 days of cold storage. The hatching success of Kiel eggs was significantly different from all strains on all days with the exception of Alabama and Florida on day 30 (all pairwise multiple comparisons, Student Newman–Keuls methods, $P<0.05$).

Parental rearing temperature also was observed to be an important factor influencing egg survivorship during cold storage (Fig. 5). Eggs produced by DIFRES females kept at 25 °C showed a constant decrease in hatching success over the storage period while eggs produced at 17 °C seemed to have a more stable hatching success, around 40%, between day 10 and day 60. After 100 days of cold storage, the HS of eggs produced at 17 °C and 25 °C had declined to 16.8±2.0% and 11.8±1.5%, respectively. After 150 days, the hatching capacities of eggs produced at both temperatures were low, but some survival was still observed. From day 30 onwards, eggs produced at 25 °C showed lower survival than eggs raised at 17 °C (all pairwise multiple comparisons, Student Newman–Keuls methods, $P<0.05$).

4. Discussion

Egg survivorship during cold storage was low for both strains originating from the Gulf of Mexico in comparison to the Baltic Sea strains. However, the two Baltic Sea strains most likely produced two distinct egg types (delayed hatching and subitaneous), which did not exhibit the same pattern of survival over the storage period, despite the fact that they originated from the same mitochondrial clade. The Florida and Alabama strains were also found to derive from two additional, genetically distinct mitochondrial clades. The Kiel and DIFRES strain produced eggs that were best for aquaculture purposes (in terms of storage), but the unpredictable presence of dormant eggs is non-optimal for short-term storage. Hence, further studies need to be conducted on the environmental cues that induce production of delayed-hatching eggs.

4.1. Genetic differentiation of strains and phylogeography of A. tonsa

Genetic results from two mitochondrial gene loci indicate that the four experimental strains originate from three highly divergent mitochondrial clades within *A. tonsa*. These three experimental strain lineages are closely related to previously described mitochondrial

clades A, B, and C (Caudill and Bucklin, 2004), but are highly divergent from one another. Genetic differentiation between strains on the order of 10–17% at 16S rRNA and 14–18% at COI is comparable to the level of divergence observed between species and, in some cases, genera of other planktonic calanoid copepods (Bucklin et al., 1995, 2003; Goetze, 2003). These divergent strain lineages may represent distinct, currently undescribed species that have unique environmental tolerances and preferred habitats (e.g., Chen and Hare, 2008). Observed strain-specific differences in life history traits and egg survivorship during cold storage in our common garden experiments may result from the fact that these strains originate from distinct taxa (this paper, Drillet et al., 2008).

The broad-scale phylogeographic structure of *A. tonsa* is currently unclear, due to insufficient sampling in prior studies. However, this nominal species contains at least four deep mitochondrial clades, of which, clade A appears to have colonized European brackish waters. The DIFRES/Kiel experimental strain, isolated from waters in the Øresund and southern Baltic, was found to contain a COI haplotype genetically identical to a haplotype present in Rhode Island, USA, which may indicate an East coast US source for the invasive populations in Europe. *A. tonsa* was first reported in European waters in the Caen canal (France) in 1927 (Rémy, 1927), followed by subsequent reports extending the range of the species to estuaries throughout Europe, including the Baltic, Mediterranean and Black Seas (e.g., Redeke, 1934; Brylinski, 1981, and references therein; Belmonte et al., 1994; David et al., 2007). European populations of *A. tonsa* may result from either single or multiple invasions, and substantial field sampling would be required to test hypotheses regarding colonization pathways into and among European brackish waters.

4.2. Strain-specific differences in egg survivorship

Our results demonstrate that Baltic Sea strains of *A. tonsa* show higher egg survivorship during long-term cold storage than Gulf of Mexico strains. For example, survival of cold-stored eggs from the Florida strain decreased from 75.5 to 8.6% within 30 days, as observed in earlier studies (Drillet et al., 2007; Sedlacek C. and Marcus N.H., unpublished data). The egg survival of the Alabama strains at day 30 was also low (7.5%). In both cases, survivorship was reduced to zero after 60 days of storage. Given the inability of these strains to tolerate cold conditions, it appears unlikely that resting egg banks in the sediment are an important aspect of the population dynamics of these populations in their natural environment. In contrast, Baltic Sea strains were able to tolerate up to 150 days of storage at ~5 °C (Fig. 4), although hatching success declined with age in the DIFRES strain. Previous studies have also shown that the DIFRES strain produces some eggs that can withstand cold storage for up to 1 year (Drillet et al., 2006a), and Peck and Holste (2006) approximated egg mortality to 4% every 20 days.

The Baltic Sea strains appear to have produced eggs with two different resting strategies, quiescence and delayed hatching, under these common garden conditions, despite a lack of genetic differentiation at two mitochondrial loci between these laboratory strains. The DIFRES strain produced eggs that had maximal hatching success at initiation of the experiment and declined with age, as would be expected for subitaneous eggs. The Kiel strain, however, produced eggs with relatively low hatching success at the beginning of the experiment (31%), with a minimum in hatching at 10 days (7%), followed by increased hatching success later in the experiment (up to 35% after 100 days). Eggs cold stored for 150 days had high levels of viability, and showed a 30% final hatching success. Freshly spawned eggs from the Kiel strain also showed a significantly prolonged hatching period in a related stage-development experiment (Drillet et al., 2008), which they considered unusual for *A. tonsa* subitaneous eggs at this temperature. Based on these results (and ongoing studies), we conclude that a fraction of the Kiel eggs were entering a delayed-hatching state, rather than simply being in a state of quiescence. It is, however, currently unclear which environmental conditions acted as cue(s) for induction of this type of dormancy in eggs of this strain, as eggs from previous generations were hatching in a pattern similar to that presently observed in the DIFRES strain (Pers. Obs.). The light regime was intentionally chosen to follow the spring/early summer rhythm, because *A. tonsa* is known to exhibit winter diapause in the egg stage (Castro-Longoria, 2001). Hence, it seems unlikely that light conditions were acting as cues for the partial dormancy switch. One possibility is that *A. tonsa* resting egg production may be affected by the presence of copepod metabolites in the relatively dense culture, as has been demonstrated for *Eurytemora affinis* (Ban and Minoda, 1994). Stocking densities were not recorded systematically in our main cultures, and it is therefore impossible at present to relate the suggested occurrence of delayed-hatching eggs to the accumulation of metabolites in the water. Further research needs to be done on this subject, since this particular aspect could easily be controlled in future intensive copepod cultures (Zadereev, 2007). Given the genetic similarity between the two Baltic strains, it may also be possible to deliberately induce dormant eggs in the DIFRES strain under the correct environmental conditions. However, the DIFRES strain may be less sensitive to environmental factors that induce delayed-hatching egg production, as this culture is known to have partially lost its diel cycle over 15 years in culture (Tiselius et al., 1995).

Egg survivorship in quiescent eggs of the DIFRES strain may be impacted by oxygen concentrations during cold storage. Egg survivorship in the DIFRES strain, despite being the highest reported among the four strains studied here, was significantly lower than observed in previous studies using the same culture population under similar conditions (Drillet et al., 2006a). This difference in survivorship could simply be due to inherent batch to batch variability, but it could also be due to the fact that in previous studies, the eggs were stored in quantities up to ~40,000 to 100,000 eggs per storage tube, providing anoxia within a relatively short period of time, while in the present study egg quantity did not exceed 826 eggs per storage tube and tubes were initially filled with fully oxygenated water. Katajisto (2004) and Holmstrup et al. (2006) have shown high survivorship of *Acartia* spp. eggs under low oxygen concentrations, and consider hypoxia and anoxia to be good conditions for high egg survival. Hence, anoxic conditions during storage may be crucial for successful storage of eggs for aquaculture applications.

Parental rearing temperature also was observed to be an important factor influencing egg survivorship during cold storage (Fig. 5). Eggs produced by DIFRES females kept at 25 °C showed higher mortality than eggs produced at 17 °C. This result could either be due to increased embryonic development at 25 °C, as development time has been shown to affect survival under cold conditions (Drillet et al., 2007), or to a maternal effect of temperature on the egg tolerance to cold storage.

4.3. Egg storage and applications in aquaculture

Diapause eggs have been proposed as a potential source of copepod nauplii for fish aquaculture, because eggs have been shown to survive up to 17 months in laboratory cold storage (Marcus and Murray, 2001), and up to >40 year old eggs have hatched successfully from marine sediments (Marcus et al., 1994). Furthermore, dormant copepod eggs in freshwater systems have been reported to hatch after a maximum of 332 years in the sediment (Hairston et al., 1995). This suggests that successful long-term storage of copepod eggs should be achievable under the correct laboratory conditions. This long storage capacity can be observed in the present experiment, where the highest hatching success after 150 days was found for the delayed-hatching eggs. However, these eggs showed low hatching success when stored for shorter periods of time. Little is known about both the mechanism by which dormancy is induced in copepods and about

termination of the refractory period. In order to circumvent this problem, subitaneous eggs, which hatch as soon as environmental conditions are optimal, seem to be the solution. The technique of cold storing eggs is currently in routine use at DIFRES for keeping inocula for restarting cultures, and cold storage has been shown not to affect the reproductive capacities of the generation arising from the stored eggs (Drillet et al., 2006a). One important aspect not studied here is the effect of long-term egg storage (months) on the biochemical composition of eggs, which could also affect naupliar quality (Støttrup et al., 1999; Drillet et al., 2006a,b). Such an effect was not observed during short-term storage of subitaneous eggs from the Florida strain (Sedlacek C. and Marcus N.H., unpublished data). Changes in biochemical composition of eggs and nauplii may influence mortality rate, impact their palatability and nutritional value to fish. These aspects should be investigated in future studies. Obtaining full control of quiescence and dormancy strategies could be useful for both short and long-term production of copepod nauplii and should be developed for aquaculture and aquarium trade purposes. However, for both purposes we recommend use of copepod lineages that can survive long periods of cold temperatures, such as those from the Baltic clade.

4.4. Long-term culturing and selection effects

The DIFRES strain has been in culture for over 25 years and has been restarted routinely from cold-stored eggs. This laboratory treatment may create selection pressure for an enhanced ability to enter quiescence and to survive longer periods of cold storage in this strain. The Florida and Kiel populations have also undergone this treatment, but have been in culture for a much shorter period of time (3–5 years). Strong directional selection pressure might be usefully employed in aquaculture applications to increase desirable phenotypic traits, such as rapid development times, high egg production, and high egg tolerance to cold storage.

5. Conclusion

Successful use of copepods as live feed in marine aquaculture hinges on optimization of environmental conditions for population growth in addition to storage of copepod eggs. One key aspect for optimizing both properties is to identify strain differences in life history traits and also tolerance of eggs to cold storage in order to select the most optimal strain for aquaculture applications. This study demonstrated significant variability in resting egg tolerance to cold storage between genetically and geographically distinct strains of the nominal species *A. tonsa*. A related study also identified significant differences between strains in mortality, egg production, hatching success, and biochemical composition of eggs and adults (Drillet et al., 2008). Given that early naupliar stages are the primary developmental stage used as food for fish larvae, and little differentiation was observed in egg biochemical composition between strains (Drillet et al., 2008), the most important criteria for selection of strains will be life history traits that maximize ease in large-scale culturing. Desirable traits include high egg production, fast development times, low mortality, and high egg survivorship during cold storage. Following these criteria, the DIFRES strain appears to be optimal for large-scale culturing in marine aquaculture. Although the Florida strain has been successfully used for first-feeding purposes (Wilcox et al., 2006), both American strains are sub-optimal for aquaculture applications under the conditions studied here due to poor egg survivorship during cold storage.

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Strain-specific vital rates in four *Acartia tonsa* cultures II: Life history traits and biochemical contents of eggs and adults

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ABSTRACT

The need of copepods as live feed is increasing in aquaculture because of the limitations of traditionally used preys, and this increases the demand for an easy and sustainable large-scale production of copepods. In this study, 4 strains of the copepod *Acartia tonsa* Dana, 1849 were compared in a common garden experiment to identify a strain with life history traits essential for sustainable mass cultivation of copepods and to identify a strain which produces preys with a highly valuable chemical composition. The strains originated from 4 coastal sites in Øresund, Denmark (DIFRES), Kiel, Germany (KIEL), Turkey Point, Florida, USA, and Mobile Bay, Alabama, USA. Stage development and mortality were analysed at 17 °C, 34 ppt. When the copepods reached adulthood, individual females were isolated to determine the egg production and hatching success. The biochemical contents of both eggs and adults were evaluated for all strains. The DIFRES and Alabama strains had both shorter generation time (13.7 and 14.6 d^{-1}, respectively) and lower mortality (4.0 and 5.7% d^{-1}, respectively) than other strains, making them attractive for mass cultivation. Strain-specific patterns were observed in egg production, with highest productivity observed in the DIFRES strain. Hatching success of the eggs ranged from 47.3 to 83.6% in the DIFRES, Kiel and Alabama strains, but showed reduced hatching in the Florida strain (1.6 to 7.2%). The strains had similar total free amino acids (FAA) content in the eggs in comparison to their dry weight (6–8%). In general, the FAA pattern of the eggs was dominated by Proline, while the females had a more diffuse FAA pattern. The fatty acid ratio between DHA and EPA in the eggs was lowest for the Florida strain (0.82) and highest in the Alabama strain (1.48), and adults had even higher ratios (2.1 to 2.45). Apart from the low DHA/EPA ratio in the Florida eggs, we find that all the strains met nutritional values needed for the first feeding of marine finfish larvae. However, essential criteria for mass cultivation such as fast development, low mortality and high egg production led us to recommend the use of the DIFRES strain of *A. tonsa* under the present growth conditions. A study to optimize the copepod cultivation to meet industry's criteria should include deliberate selection pressure on copepod to promote the needed traits for first feeding of finfish larvae.

1. Introduction

Aquaculture is one of the fastest growing animal food-producing sectors. Worldwide, the sector has grown at an average rate of 8.8% per year since 1970 (FAO, 2006), and development and implementation of new methods for successful fish farming are required. In marine fish hatcheries, the use of live feeds is essential for successful larval rearing. Traditionally-used live feeds, such as *Artemia salina* and rotifers (*Brachionus* spp.), after enrichment, meet the nutritional requirements of many domesticated fish species but are still a limiting factor for success in fish hatcheries because of their rather poor nutritional value. This was revealed by previous studies demonstrating that planktonic copepods can be used, either for substitution or as supplement to traditional live feeds, to diminish the nutritional deficiencies that result from exclusive use of rotifers and *Artemia*, and increase survival, development and pigmentation of fish larvae (Shield et al., 1999; Støttrup and McEvoy, 2003; Lee et al., 2005; Rajkumar and Vasagam, 2006; Wilcox et al., 2006). Semi-intensively grown copepods have already been implemented in some fish farms (Toledo et al., 1999; Støttrup, 2000; Engell-Sørensen et al., 2004; Sørensen et al., 2007) and methods for intensification of culture are developing (Schipp et al., 1999; Payne and Rippingale, 2001a). However, all copepod species are not suitable for mass cultivation because of their different life history traits. Calanoids are interesting because of their pelagic life cycle and because some species produce resting eggs which are also valuable for aquaculture industries (Marcus, 2005). The calanoids of the *Acartia*

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genus have been identified as particularly interesting because of their small size (0.1–1.5 mm), short generation time and ease to grow (O'Bryen and Lee, 2005). Also, the ability of *A. tonsa* eggs to enter quiescence, such as during cold storage, was shown to be crucial to aquaculture (Drillet et al., 2006a,b; Drillet et al., 2007). The uses of local populations have been advised because of the risk of exotic species introductions (O'Bryen and Lee, 2005).

Nonetheless, differences in stage development within one nominal copepod species have been observed in previous studies, pointing out possible effects of geographical origin on life history traits (Leandro et al., 2006). Such differences in local strains may also affect their value as live feed for use in aquaculture. As an example, laboratory raised *A. tonsa* has been reported to exhibit strain-specific density-dependent egg production and egg survivorship under cold storage (Medina and Barata, 2004; Drillet et al., 2006a,b; Peck and Holste, 2006; Drillet et al., 2007; Jepsen et al., 2007; Sedlacek C and Marcus N.H., unpub. data). The reason for these differences between strains could be due to culture conditions or to inherent biological differences between strains.

In this study, four strains of *A. tonsa* from distinct geographic areas in the Baltic Sea and Gulf of Mexico regions were reared in a common garden experiment at 17 °C and 34 ppt. Our specific objectives were to identify a strain with a particularly interesting set of life history traits, necessary for sustainable mass cultivation of copepods, and a strain with a highly valuable chemical composition. In Drillet et al. (in press), origin and genetic differentiation of four of *A. tonsa* strains and their egg survivorship under cold storage were characterized. Here, these strains were evaluated for stage development time, mortality, egg production and hatching success, as well as for fatty acid and free amino acid content in adults and eggs.

2. Materials and methods

Four *Acartia tonsa* cultures were compared in a common garden experiment. Cultures originated from populations from (1) Øresund, Denmark, DIFRES, (2) Kiel, Germany, (3) Turkey Point, Florida, USA, and (4) Mobile Bay, Alabama, USA (for more information on the strains and the culture methods, *see* Drillet et al. in press, this issue).

2.1. Stage development

Batches of eggs from all cultures were harvested when the eggs were less than 12 h old. Eggs were cleaned according to Drillet et al. (in press), and transferred to 2 L Nalgene ® bottles. Eggs were then subsampled from the bottle using a 10 or 25 mL kip automat NS 29.2/32 (Witeg, Germany) into 10×20 mL Petri dishes for enumeration of the eggs. The eggs were incubated at 17 °C for 48 h for hatching, fixed in 1% final concentration of acid Lugol's solution, and enumerated under a dissection microscope (Olympus SZ 40). The ratio of nauplii to eggs was used to evaluate hatching success (HS) of the eggs on day 0 (D0). Following harvesting, the remaining eggs in the bottle were used to inoculate new 2 L acid-washed bottles, for the next step of the common garden experiment. Four replicate bottles were inoculated with 4000–15,000 eggs for each strain. Under these conditions and at these densities, *A. tonsa* has been demonstrated to develop and survive without any apparent negative density dependence (Drillet et al., 2006a). Average algal concentrations (*Rhodomonas salina*) were kept at ≥20,000 cells mL^{-1} (corresponding to ≥950 μg C L^{-1}) during the experiment and were measured daily with a Coulter Counter Z3 (Beckman Coulter Corporation). This concentration is considered optimal (Kiørboe et al., 1985a; Berggreen et al., 1988). The bottles were completely filled with seawater, plastic film was placed over the bottle opening, and the bottles were randomly fixed on a plankton wheel (0.3 turn min^{-1}), above which a light was placed to maintain photosynthesis and thereby avoid hypoxia in the bottles. The light was maintained throughout the experiment. Subsamples of between 20 and 75 mL were taken daily from the bottles using a 10 or 25 mL kip automat to estimate the density, mortality and stage development of each strain. In order to compare the stage development data from all incubation bottles, fractions between the major development stages (eggs, nauplii, copepodites, adults) were used. Fractions were calculated from the individuals present in the subsamples over the course of the experiment (average of 4 replicate bottles) except for the last day of the incubation when the entire content of the bottles was counted for more accuracy. To prevent pooling from multiple generations, egg counts were terminated when the first adults were observed in any given bottle and naupliar counts were stopped the following day. For all replicates, the time at which 50% of the culture had metamorphosed to the next major development stage was estimated from graphs using interpolation between the two data points framing the "50% switch point".

Oxygen and pH were measured daily throughout the experiment with a LDO™ HQ20 Portable Dissolved Oxygen/pH meter. Four-fifths of the seawater in each bottle was then removed by reverse filtration (52 μm) using a siphon, and the removed seawater was used to monitor algal concentrations during the experiment. The bottles were refilled with clean fully-oxygenated water containing the appropriate concentration of algae, closed, and fixed randomly on the plankton wheel. This daily protocol continued for 10 days following the emergence of adult copepods in the incubation bottles, and the experiment ran for approximately three weeks in total.

2.2. Mortality

Instantaneous rates of mortality (Z d^{-1}) were calculated according to Breteler et al. (2004) using the equation $N_t = N_0 e^{-zt}$, where N_0 is the number of individuals L^{-1} at time 0 and N_t is the number of individuals L^{-1} at time t. N_t was corrected for sampling mortality (due to removal of individuals) by multiplying the observed number of individuals by $V(n^{-1})/[(V-v1)\ (V-v2)\ \ldots\ (V-v(n^{-1}))]$, where V is the volume of the experimental bottle, v is the volume of the sample and n is the rank number of the sample.

The instantaneous rates of mortality were calculated for each bottle as an average over the entire experiment for the total population. The hatched number of nauplii in the bottles was calculated from the number of eggs incubated and multiplied by the hatching success of that batch of eggs.

2.3. Sex ratio

Sex ratios were determined from the first day adults appeared in the bottles. Sex ratios were obtained by counting the number of males and females within each subsample or in the entire incubation bottle on the last day of the experiment. The number of females was divided by the total number of adults to obtain the sex ratio per day and this was averaged over the last days of incubation.

2.4. Egg production

In order to monitor egg production, two days after the first adults appeared in a bottle of each strain, a set of 10–15 females (0–2 days old) were isolated and incubated individually in 600 mL acid-washed polycarbonate bottles with phytoplankton (*R. salina* at a density of 20,000 cells $mlmL^{-1}$) as food. Over four days, the entire contents of each incubation bottle were daily filtered gently through two mesh sizes (180 and 53 μm) to separate females from spawned eggs. The females were re-incubated in fresh food suspensions and the spawned eggs were counted and incubated in small Petri dishes for 48 h before being fixed with Lugol's and hatching success was calculated. Following the incubation, the female's cephalothorax length was measured and carbon content calculated according to Berggreen et al. (1988), using the equation $W = 1.11 \times 10^{-5} \times L^{2.92}$ where W is the female

body weight (ng Carbon) and *L* prosome length in micrometers. Egg carbon content was calculated according to Kiørboe et al. (1985a; 1985b and ref therein) using the equation $W=0.14\times10^{-6}$ µg C µm^{-3}, where *W* is the egg volume content of carbon (µg C) (measurement of the eggs are described in Drillet et al., in press). The specific egg production (SEP) per day was calculated. This represents the quantity of carbon used per day for egg production divided by the carbon weight of the female. Results from the first day were different from the 3 following days (Newman–Keul, $P<0.05$), reflecting the adaptation of the copepods to the incubation environment and were thus eliminated from the calculations.

2.5. Amino acids and fatty acids

For analysis of fatty acids and amino acids in egg and adults, samples were prepared by concentrating between 3000 and 4775 eggs or between 15 and 25 adults on a 45 µm mesh, from which they were gently transferred to 1.5 ml mL glass vials. The glass vials had previously been filled with a chloroform:methanol mixture (2:1 vol:vol) for the fatty acid analysis or MilliQ water for the amino acid analysis. After preparation, all samples were immediately stored at −80 °C and kept at this temperature until analysis. Fatty- and amino acid analyses were conducted as thoroughly described in Drillet et al. (2006b). Lipids of 5 to 10 replicates were extracted in the chloroform: methanol mixture followed by trans-esterification of the lipids by acetyl chloride in methanol. The fatty acid methyl esters were analysed by gas chromatography–mass spectrometry (GC–MS). The only difference was that the internal standard used for fatty acid analysis was C23:0 and that the fatty acid standard was a pre-prepared mix from Selco (INVE, Belgium). The analysis of fatty acids focussed on docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) because of their importance to marine fish larval development. For amino acids, between 3 and 10 replicates were used and the analysis focussed on content and composition of free amino acids (FAA). FAA are easily metabolized and involved in a variety of metabolic processes in marine invertebrates, including especially maintenance of osmotic pressure (Kube et al., 2007). Potentially, FAA may have a higher nutritional value than protein-bound amino acids. The FAA in eggs and adults were extracted by heating the samples to 95 °C for 5 min after which the samples were centrifuged at 4000 *g* for 5 min. The supernatants were filtered through 13 mm 0.2 µm pore size Acrodisc® GHP polypropylene membrane filters (VWR International, Denmark) and were assumed to include the intracellular pool of FAA. The FAA were quantified by HPLC. To compare the FAA content of the eggs, the absolute amount of FAA in the eggs was normalized to their dry weight (DW), rather than egg number, because the eggs varied in size across strains (see Drillet et al., in press). Egg DW was estimated for each strain by the relation between egg carbon weight-DW as proposed by Kiørboe et al. (1985a).

2.6. Statistical analysis

The software, SigmaStat 3.10 (Systat Software Inc.) was utilized to do the statistical analysis. Differences between strains were detected

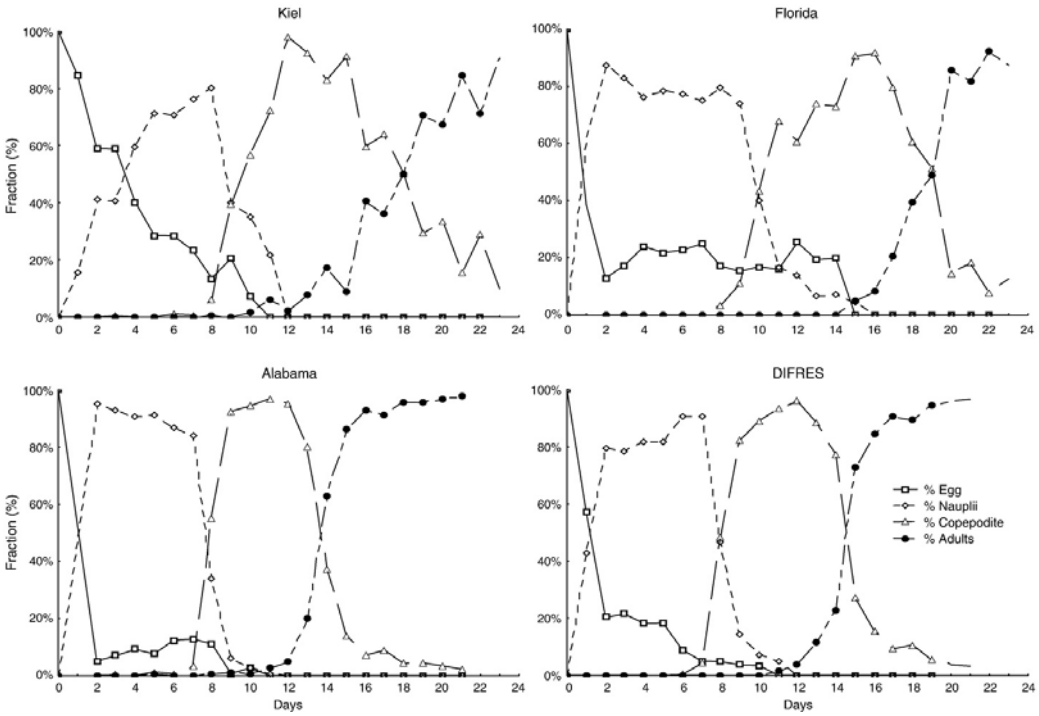

Fig. 1. Fractional developmental stages of four *A. tonsa* strains (DIFRES, Kiel, Florida and Alabama) from egg to adult. Data points represent the means of four replicates (incubation bottles).

Table 1
Mean duration (days — d^{-1})±S.E. (n=4 replicates) of the different development stages

	DIFRES	Kiel	Florida	Alabama
Nauplii	1.3±0.1a	2.8±0.5b	1.2±0.2a	1.1±0.0a
Copepodites	8.4±0.3a	9.9±0.6b	10.3±0.2b	7.9±0.2a
Adults	14.6±0.1a	17.5±0.6b	18.6±0.2c	13.6±0.1a

Within each row, there are no statistical differences between means annotated with the same letter (SNK, P<0.05).

Table 2
Mean sex ratios calculated for all the strains

	Sex ratio (female/total)
	%±SEM
DIFRES	57.1±3.1a
Kiel	53.9±3.7a
Florida	69.3±5.8b
Alabama	59.9±2.4a,b

There are no statistical differences between means annotated with the same letter (SNK, P<0.05).

using analysis of variance (ANOVA) followed by identification of significant differences in means (All Pairwise Multiple Comparison Procedures; P<0.05) using the Student Newman–Keuls test (SNK), or an ANOVA on ranks, (Kruskal–Wallis) followed by a comparison of groups using the Dunn's method when data were not normally distributed.

3. Results

3.1. Water quality

Dissolved oxygen and pH values in the incubation bottles remained relatively constant during the experiment, ranging from 4.5 to 9.4 mg O_2 L^{-1} and 8.0 to 8.3 pH units, respectively. The salinity and the temperature were constant at 34 ppt and 17 °C, respectively.

3.2. Stage development

The four strains showed distinct patterns of development. For all strains except Kiel the main peak in nauplii was observed within 2 days of incubation (though a few eggs from DIFRES hatched a bit later), while eggs of the Kiel strain developed into nauplii later and peaked at 8 days (Fig. 1). Copepodite stages appeared after 7 to 8 days for all strains. Adult stages were observed after 11, 10, 15 and 10 days in the DIFRES, Kiel, Florida and Alabama strain, respectively. Average time required for hatching (time to switch from egg to naupliar stage) was higher for the Kiel eggs than for the three other strains (SNK, P<0.05) and ranged from 1.05±0.02 days to 2.78±0.45 days for Alabama and Kiel, respectively (Table 1). The time required to switch to the copepodite stage was shortest in the Alabama strain (7.9 days), followed by DIFRES (8.4 days). These two strains differed significantly from both Kiel and Florida (SNK, P<0.05). Copepodites of the Alabama strain were the first to reach adulthood (after 13.7 days), followed by DIFRES (after 14.6 days), while copepodites from Kiel and Florida strains reached adulthood after 17.5 days and 18.6 days, respectively. The DIFRES and Alabama strains required a similar time period for development from egg to adulthood.

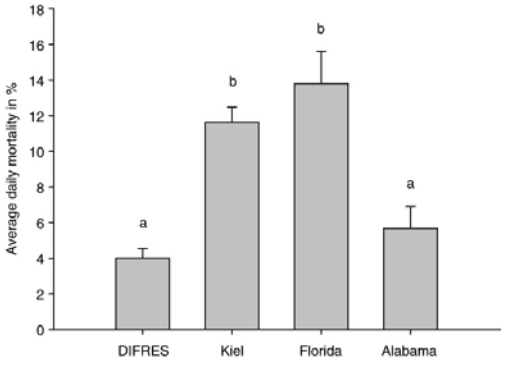

Fig. 2. Mortality rate for *A. tonsa* DIFRES, Kiel, Florida and Alabama strains (mortality d^{-1}). Bars and whiskers are for the mean±SE. There are no statistical differences between means annotated with the same letter (SNK, P<0.05).

3.3. Mortality

The average, daily mortality of the strains varied from 4.01 to 5.67% (DIFRES and Alabama strains), and from 11.6 to 13.8% (Kiel and Florida strains) (Fig. 2). The mortality rates of the DIFRES and Alabama strains were statistically different from both the Kiel and the Florida strains but not from one another (SNK, P<0.05).

3.4. Sex ratio

In all strains, the sex ratio was in favour of females, making up 53.9 to 69.3% (Table 2). In comparisons between strains, the sex ratio for the Florida strain was found to be significantly different from DIFRES and Kiel sex ratios, but not from Alabama (SNK, P<0.05, n=27). Within strains, the differences between genders were significant for DIFRES, Florida and Alabama strains (P<0.01 n=9, 6 and 10) but not for the Kiel population (P<0.01 n=8).

3.5. Egg production

Significant differences in egg production were observed among the four strains. Individual females produced between 0 and 78 eggs day^{-1}. The daily mean egg production over three days for each strain varied from 37.2 to 50.5 eggs. The DIFRES strain had higher average egg production than the Kiel and the Florida strains (SNK, P<0.05), and the Alabama strain had higher egg production than the Kiel strain (SNK, P<0.05) (Table 3).

The average specific egg production (SEP), calculated as production of carbon (C) relative to C content for each female over 3 days, ranged from 17.2 to 55.8% C F^{-1} d^{-1} (Fig. 3). However, on particular days some females had a SEP as high as 60.9% of their carbon body weight day^{-1}. Adult cephalothorax length ranged from 850 to 900 μm, and mean egg

Table 3
Daily mean egg production and accumulation of daily egg production

	DIFRES		Kiel		Florida		Alabama	
Days	Egg F^{-1} d^{-1}	*A*	Egg F^{-1} d^{-1}	*A*	Egg F^{-1} d^{-1}	*A*	Egg F^{-1} d^{-1}	*A*
1	– No data –							
2	47.5±2.8	47.5	38.6±2.8	38.6	45.7±4.2	45.7	44.6±4.7	44.6
3	55.0±2.1	102.5	37.2±4.0	75.8	41.3±2.4	87.0	46.3±3.5	90.9
4	49.1±4.7	151.6	35.8±6.4	111.6	37.8±1.5	124.8	42.8±4.0	133.7
Av.	50.5±1.9	a	37.2±2.6	c	41.6±1.7	b, c	44.6±2.3	a, b

The accumulated egg production (*A*) was obtained by adding the mean egg production by each female during three days. (n=8 to 13). There are no statistical differences between means annotated with the same letter (SNK, P<0.05).

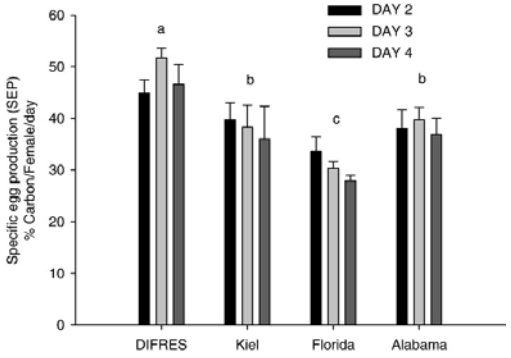

Fig. 3. Specific egg production (SEP) of *A. tonsa* DIFRES, Kiel, Florida and Alabama strains. Means±SE (n=8 to 13) shown. There are no statistical differences between means annotated with the same letter (Dunn's, P<0.05).

diameter was 83.8±0.4 μm, and 82.7±0.2 μm, 77.9±0.3 μm, and 78.8±0.5 μm for DIFRES, Kiel, Florida, and Alabama eggs, respectively (Drillet et al., in press). The average SEP over three days ranged from 30.3 to 51.8%. All strains showed significantly different SEP values, with the exception of Alabama vs. Kiel (Dunn's, P<0.05).

Hatching success, defined as mean percentage of hatched eggs within 48 h, ranged from 47.3 to 83.6% (Fig. 4) for eggs of the DIFRES, Kiel and Alabama strains, but were significantly lower in the Florida strain (Dunn's, P<0.05), ranging from 4.1 to 7.2%.

3.6. Free amino acids and fatty acid ratio in eggs and females

Eggs of all strains were characterised characterized by a high fraction of Pro (34–43%, Table 4), though, the Floridian eggs showed a lower proportion of Pro than the two Baltic strains. The Floridian eggs also had a higher content of Asn than the other three strains and higher content of Tau (taurine, a non-protein amino acid) than the Baltic strains (7.7 and 11.4%, respectively) (P<0.05, Table 4). The DIFRES strain had a lower content of His than all the other strains (4.8% vs. 7.9% or more) (P<0.05). Finally, the DIFRES eggs had a higher proportion of Ile than all the other strains (1.9% vs. 1.2 or less) (P<0.05). No significant differences were observed between strains in egg content of FAA relative to dry weight. All strains contained approximately 6–8% FAA relative to DW.

In the females, there was no single dominant FAA. Instead, a few FAA made up between 8% and 15% of all FAA (Table 5). Common FAA were His, Tau, Arg, Ala and Pro, and to a lesser extent, Leu and Lys. Kiel and Alabama females had a significantly higher proportion of Tau than the other groups (P<0.05). Kiel females also presented a lower proportion of Tyr and Val than the other strains (P<0.05).

Significant differences in the fatty acid ratio DHA/EPA were observed between eggs but not between adults of different strains (Fig. 5A, B). Mean ratios of DHA to EPA in the eggs were 0.82, 1.37, 1.34 and 1.48 for Florida, DIFRES, Kiel and Alabama strains. The differences were significant between eggs of all strains, but not between DIFRES and Kiel eggs (SNK, P<0.05). In the adults, the DHA/EPA ratio was

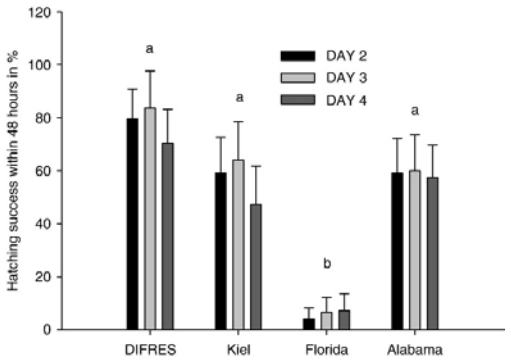

Fig. 4. Hatching success within 48 h of eggs. Means±SE (n=8 to 13) shown. There are no statistical differences between means annotated with the same letter (Dunn's, P<0.05).

Table 4
Relative quantities of free amino acid (FAA) in eggs (% of total FAA±SE) and number of replicates (n) used for analysis

	(D) DIFRES (n=5)	(K) Kiel (n=6)	(F) Florida (n=10)	(A) Alabama (n=5)
Asp	1.9±0.7	1.4±0.1	2.0±0.1a	0.7±0.1f
Glu	7.0±0.8f	8.2±0.4	8.6±0.2ad	6.7±0.4f
Ser	4.1±2.6	1.3±0.1a	1.6±0.1	6.1±1.5k
Asn	1.9±0.7f	2.7±1.0f	7.7±0.5dka	4.5±2.0f
Gly	3.2±0.3	2.4±0.1f	4.3±0.3ka	2.6±0.2f
Gln	1.0±0.1ak	0.7±0.0d	0.7±0.0	0.6±0.0d
His	4.8±0.7kaf	11.4±0.7df	7.9±0.8kd	9.5±0.5d
Tau	7.3±0.9f	8.0±0.2f	11.4±0.4kd	8.6±0.4
Thr	2.8±0.8	1.9±0.1	2.2±0.1a	1.6±0.1f
Arg	2.9±0.4fk	3.7±0.1ad	3.8±0.1ad	2.5±0.1fk
Ala	8.5±0.4f	6.2±0.3	5.2±0.1da	7.5±0.2f
Pro	39.1±4.1f	42.5±1.4f	34.2±0.3kd	39.2±0.4
Tyr	1.2±0.3k	0.5±0.1ad	0.8±0.1	1.0±0.0k
Val	2.4±0.4	1.5±0.3f	2.2±0.1k	2.0±0.1
Met	1.3±0.2af	0.8±0.2	0.8±0.0d	0.7±0.0d
Ile	1.9±0.3akf	1.1±0.2d	1.2±0.1d	1.0±0.1d
Orn	1.1±1.0	0.1±0.0	0.1±0.1	0.3±0.1
Leu	2.6±0.3	1.7±0.4	1.9±0.1	1.6±0.1
Lys	3.3±0.2	2.9±0.6	2.1±0.3	2.3±0.2
Phe	1.6±0.2af	1.1±0.2	0.9±0.0d	0.9±0.0d

For any given amino acid, the means annotated with a letter are different from the ones in the column the letter is referring to (P<0.05).

Table 5
Relative quantities of free amino acid in females (% of FAA±SE) and number of replicates (n) used for analysis

	(D) DIFRES (n=4)	(K) Kiel (n=5)	(F) Florida (n=5)	(A) Alabama (n=12)
Asp	2.8±0.2	3.1±0.4	3.0±0.1	3.1±0.3
Glu	2.7±0.4	3.3±0.3	3.4±0.4a	2.1±0.3f
Ser	2.5±0.1	2.6±0.2	2.2±0.1a	6.2±1.2f
Asn	3.1±0.1	2.5±0.4	2.6±0.2	3.0±0.5
Gly	4.3±0.2	3.1±0.4	3.4±0.1	2.9±0.3
Gln	1.8±0.0a	1.6±0.1	1.7±0.1a	1.4±0.1df
His	8.3±0.8	12.5±2.2	6.4±0.6	11.3±1.3
Tau	6.8±0.6ka	14.6±1.2df	9.0±0.6ka	12.9±0.9df
Thr	3.6±0.1	3.3±0.4	3.6±0.1	3.4±0.2
Arg	8.7±0.1kf	12.0±0.8d	11.4±0.9d	9.5±0.4
Ala	9.5±0.2	9.2±0.9	9.4±0.4	9.1±0.2
Gaba	0.3±0.0	0.5±0.1	0.3±0.0	0.4±0.1
Pro	10.5±0.5	10.3±1.1	9.9±0.6	7.4±1.1
Tyr	3.0±0.1k	1.5±0.4dfa	2.8±0.1k	2.7±0.1k
Val	4.5±0.2k	2.6±0.7fda	4.8±0.3k	4.2±0.2k
Met	2.9±0.1k	1.7±0.4fd	2.9±0.0ka	2.3±0.1f
Ile	4.4±0.2k	2.5±0.7d	3.9±0.2	3.6±0.3
Orn	0.0±0.0	0.1±0.1	0.0±0.0	0.7±0.3
Leu	7.4±0.3k	4.6±1.2df	7.3±0.1k	6.3±0.2
Lys	9.3±0.1a	4.8±1.2	8.1±0.2a	4.4±1.0df
Phe	3.5±0.0	3.6±0.9a	3.4±0.1	2.9±0.1k

For any given amino acid, the means annotated with a letter are different from the ones in the column the letter is referring to (P<0.05).

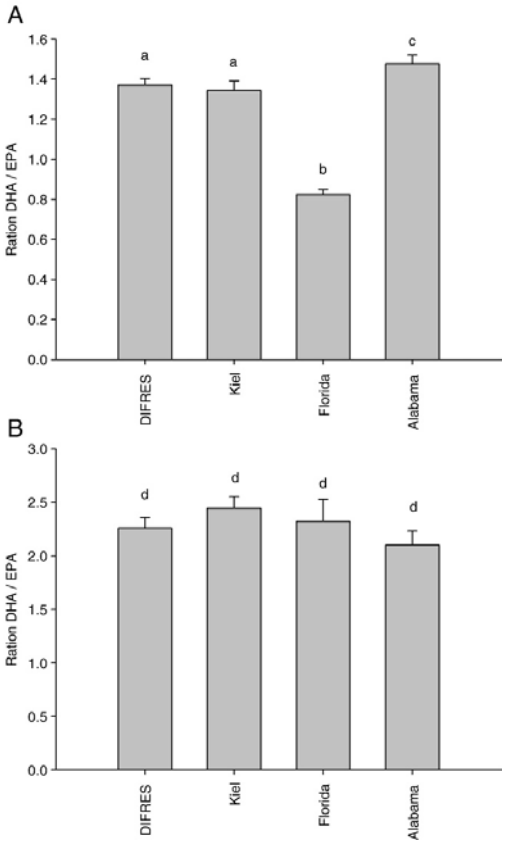

Fig. 5. A, B. Ratios between docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contents in all four strains. Panel (A) eggs, panel (B) females. Mean ratio ± SE (n = 4 to 12) shown. There are no statistical differences between means annotated with the same letter (SNK, P<0.05).

higher and ranged from 2.1 to 2.45, but no significant differences were observed (SNK, P<0.05).

4. Discussion

Analysis of life history traits and chemical content of 4 strains of the calanoid copepod *A. tonsa* showed that the DIFRES and the Alabama strains had the highest aptitude for large large-scale culture under the present culture conditions because of their fast development and low mortality. The use of the Kiel and Florida strains as candidates for mass cultivation was questioned because of some non non-attractive life history traits (slow developmental rate, high mortality) and chemical composition (low DHA/EPA ratio in the Florida strain).

4.1. Mortality, sex ratios and generation times

The oxic conditions in our bottles were well above the lower limits for optimal growth and survival of *A. tonsa*, i.e. 1.5 mL L^{-1} (~2.14 mg L^{-1}) (Marcus et al., 2004). The daily mortality rates of adult *A. tonsa* in the present study (4 to 14%) agree with daily mortality rates of 5 to 10% reported by Berggreen et al. (1988) and ref. therein and Medina and Barata (2004) for *A. tonsa*. An elevated mortality as observed for the Kiel and Florida strains has previously been found for *A. tonsa* but this was likely due to intense manipulation of the copepods (Jepsen et al., 2007). In our study, the increased mortality might have been caused by sub-optimal conditions for some of the strains with respect to salinity and temperature. Thus, the Kiel strain was raised in the laboratory at 18 ppt for multiple generations before our experiments (see history of the strain in Drillet et al., in press) and the Florida strain was raised at 30 ppt under laboratory condition. In Turkey Point, Florida, the natural salinity for *A. tonsa* ranges from 15 to 32 ppt (Hayes and Menzel, 1981) and may mean that this strain potentially perform better in lower salinity. Temperature may have been another important factor influencing the mortality. The experiments were run at 17 °C. The Florida strain is experiencing from 10 to 30 °C in its natural habitat (Gulf of Mexico), while the Baltic strains more likely face temperature ranging from 5 to 15 °C.

The observed sex ratios of 53.9 to 69.3% females correspond to previous findings of 56 to 67% (Medina and Barata, 2004). The statistical differences observed in this study were caused by a lower fraction of males in the Florida strain compared to the three other strains. Manipulation of sex ratios in favour of females in copepod cultures would be an interesting tool to promote a higher egg production per fed individual as long as enough males are present to fecundate the females. However, optimal sex ratio vs. egg production remains unknown and deserves attention in future studies (O'Bryen and Lee, 2005). Holste and Peck (2005) applied an 80% female fraction and still obtained highly viable egg productions. Here, the strains had a rather similar sex ratio, except for the Florida strain, and none of the strains performed "better" than the other on this particular aspect.

The observed generation times of the *A. tonsa* strains of 13.7 to 18.6 days and being shortest for the DIFRES and Alabama strains, corresponded to the range in generation times of 7–25 days reported in the literature for *A. tonsa* (Mauchline, 1998 and ref. therein).

4.2. Hatching success and egg production

During the stage development experiment, DIFRES, Florida and Alabama eggs hatched within 1.25 days, while Kiel's showed a longer hatching time of 2.78 days. Applying the relationship between temperature and *A. tonsa* egg development (Narragansett Bay population) as proposed by McLaren et al. (1969), a calculated egg development time of 1.85 days was found, supporting the rapid egg development of the 3 fastest strains but not the Kiel strain. In another study on the Kiel strain, Holste and Peck (2005) showed that at 18 °C, a hatching success of 80% was reached within 40 h. This is a much faster development than the present findings for the same strain (up to 10 days). However, for unknown reasons, the Kiel strain sometimes appears to have slow and variable egg development time (Drillet G., unpublished data). This reflects the presence of delayed hatching eggs as it was previously described only 3 times for *Labidocera scotti* and *Pontella meadi* (Chen and Marcus, 1997) and *A. tonsa* in the Baltic Sea (Katajisto, 2006). As hypothesized in Drillet et al. (in press), the copepods' own metabolites might have induced the delayed hatching of the eggs as has been suggested by Ban and Minoda (1994) for another copepod, *Eurytemora affinis* and other aquatic invertebrates (Zadereev, 2007, and ref. therein).

The first naupliar stages of copepods are the most desirable for many fish larvae (Kleppel et al., 2005). This means that a constant and fast supply of newly hatched nauplii would be an optimum source of live feed in the aquaculture industry. A fast hatching (within 48 h) was observed in all the present strains, except the Kiel strain. Unpredictable slow hatching could lead to catastrophic first feeding management and therefore strains producing dormant eggs under optimal food, light and temperature conditions should be avoided as proposed

by Zadereev (2007). One solution to circumvent the lack of live feed at a given day is to cold-store nauplii from one day to another (Payne and Rippingale, 2001b).

The egg production in this study of 19 to 55 eggs F^{-1} d^{-1} agrees with rates of 18 to 50 eggs F^{-1} d^{-1} found for this species in other studies (as cited in Mauchline, 1998, and ref. therein). Our SEP rates also compared well to the rates of 14.5 to 71.8% C F^{-1} d^{-1} reported by Sullivan and McManus (1986). The present SEP rates at the applied conditions appear to favour the use of the DIFRES and the Alabama strains in aquaculture industries, but SEP rates of other strains cannot be considered abnormal or out of range and might differ at other environmental conditions. It has been shown that changes of diet, temperature and salinity can affect both the total egg production and the hatching success (Støttrup and Jensen, 1990; Jonasdottir, 1994; Kleppel and Burkart, 1995; Holste and Peck, 2005). Here, the hatching success was found acceptable with no strain-specific differences, apart from the low hatching success in the Florida strain for which no obvious explanation was found. For the same strain, Oppert (2006) presented a hatching success of more than 90% which were close to the present observations for the other strains.

4.3. Content of free amino acids and fatty acid ratio

Content of free amino acids (FAA) in natural zooplankton populations has been found fairly constant (see review by Båmstedt, 1986; Helland et al., 2003) and this was also observed in the present study. The quantity of FAA relative to DW was almost identical between the four different strains (~6–8% DW), and this aligns results as observed in other species (Jeffries and Alzara, 1970, in Båmstedt, 1986; Drillet et al., 2006b). All the strain's eggs presented a peak of Pro, but had otherwise a rather similar proportion of many FAA, except for a few differences between Asn, His, Arg, Ala, Tau and Pro among the strains. No dominant FAA were found in the adults and the composition of FAA was more variable. In a comparison between two different copepod species (*A. tonsa* and *Tisbe holothuriae*) raised under similar conditions, Drillet et al. (2006b) noted a possible species-specific pattern of FAA. Composition of FAA may be an important parameter in the aquaculture industry as palatability of prey is likely to be affected by the FAA composition. For fish larvae, essential amino acids (EAA) have been found to be: Thr, Leu, Met, Lys, Arg, Val Ile, Trp, His and Phe (Tacon, 1987). In the present study, the proportion of essential FAA appeared similar between the strains in eggs and adults. Although eggs are not used directly as prey, biochemical composition of the eggs can be partly extrapolated to the nauplii (Drillet et al., 2006b). Interestingly, the Florida strain showed a high level of Tau which is known to stimulate fish development and behaviour (Conceição et al., 1997; Takeuchi, 2001), and this could partly explain the success of first feeding of Southern Flounder, *Paralicthys lethostigma* when fed this strain (Wilcox et al., 2006). Based on the results from our FAA study, no strains would be considered more efficient for fish farming, but the slight differences could be used to fit particular demands of some fish larvae.

Fatty acids are important for fish larvae (Sargent et al., 1997). Among fatty acids, the ratio between docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has been found to indicate the nutritional value of fatty acids, and should have an optimum value of about 2 (Sargent et al., 1997). Evjemo and Olsen (1997) found DHA/EPA ratios from 1.83–1.97 in adult *T. logicornis* and *Eurytermora* sp. while Drillet et al. (2006b) found a ratio of 1.35 in *A. tonsa* nauplii from the DIFRES strain reared under the same conditions as here. The low DHA/EPA ratio of Florida eggs suggests that this strain is the least suited for first feeding in aquacultures. Under the present environmental conditions, the adults from all strains presented the optimal proposed DHA/EPA ratio (2.2 to 2.5), suggesting that all four strains at the adult stage are suitable as live feed for this aspect. Manipulation of copepod DHA/EPA levels and FAA pool, if required for aquaculture feeding, can be done through feeding different phytoplankton (Guisande et al., 1999; Laabir et al., 1999; Støttrup et al., 1999).

4.4. Strains, cultivation and aquaculture

The Kiel and DIFRES strains had different stage development times and mortality rates under constant experimental conditions, but may originate from the same mitochondrial clade (Drillet et al., in press). These differences could originate from the extended period of indoor culturing of the DIFRES strain (25 years) as compared to the Kiel strain which was cultured only 2–3 years (see Drillet et al., in press). Long-term cultivation time has previously been shown to transform some life history traits (Tiselius et al., 1995). The selective pressures might modify or even eliminate some of the life history traits and could be used to increase attractive phenotypic traits as rapid developmental time and high egg production that are valuable for copepod mass cultivation. Other differences in life history traits between the 4 studied strains may partially be explained by genetic differentiation as indicated by differences at two mitochondrial gene loci between three of the four strains (Drillet et al., in press). This differentiation may represent distinct species with different optimal conditions and preferred habitats.

Among the four strains studied here, the Florida population seems to be the least suited for aquaculture purposes because of its slow development rates and relatively poor biochemistry (though nutritional quality must be confirmed by feeding-trials), while the DIFRES and Alabama strains exhibited both shorter generation times and lower mortality. These attributes are attractive for mass production of copepods, enhancing the overall fraction of copepods in culture that are entering the reproductive stages and decreasing the risk of sudden failure in the tanks. However, additional investigations have to be completed in order to find the optimal conditions of each of the strains. As an overall conclusion, we advice aquaculture breeders aiming at using calanoid copepods for first feeding to avoid extrapolating all the available data on a certain species from the literature when using a different strain from those they are isolating. Hence, we recommend testing of specific strains to characterize particular life traits.

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MANUSCRIPT 6

Status and opinion on marine copepod cultivation for use as live feed

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ABSTRACT

Copepods are important crustaceans studied because of their key role in ecology, trophic biology, fisheries management, in modeling the flow of energy and matter, ecotoxicology, aquaculture and aquarium trade. This paper discusses various aspects of the state of knowledge of copepod culture at large scales and provides the scientific community with ideas and concepts that could improve and quicken the development of copepod mass cultures. As a framework for discussion, we use a conceptual scheme from Teece (1988) and adapted it to our goal: 'How to capture value from a copepod product'. The suggestions include: 1) optimize cultures by automation and implement recirculation technology for improving water quality; 2) use harpacticoid and cyclopoid copepods in industries that can produce large amounts of these prey on site at any given time; but use calanoid copepods for industries limited in production time and those that export copepod products (e.g. eggs); 3) select preferentially local copepod species and if possible species with lipid conversion capabilities; 4) optimize sex ratio and selection/cross-breeding to develop suitable copepod strains for aquaculture; 5) explore the use of probiotics for improving the fitness of copepod cultures; 6) encourage copepod producers/retailers to use/develop an efficient sales and marketing strategy.

Keywords: Copepod culture, live feed, live prey, cannibalism, density, water quality, population selection, recirculation, probiotic, storage and shipping, economics and commercialization

INTRODUCTION

Free living copepods have been intensively studied because of their impact as key players in the marine pelagic environment. In terms of biomass, copepods can represent up to 80% of the zooplankton (Mauchline et al. 1998). They are an important food source for planktivorous fish and fish larvae in general (e.g. Fox et al. 1999; Möllmann et al. 2004). Their life cycles, vital- and physiological rates are intensively studied to understand the effects of various environmental conditions on the pelagic ecosystem. Experimental zooplankton studies have benefited from the small-scale cultivation of copepods. Also, when compared to field sampled specimens, the advantage is that the history and condition of cultivated copepods are known and can even be manipulated. Information derived from the experiences and practices of copepod culturing is important for a variety of research areas such as copepod genetics, feeding behavior, population dynamics, parameterization of standing stocks, production rates for ecosystem models etc.

Since the 1960's culturing copepods has become increasingly more reliable and approximately 60 copepod species have been successfully raised (Mauchline et al. 1998). To our knowledge, the oldest copepod culture is from the Danish Technical University of Denmark (*Acartia tonsa*) which was isolated from Øresund (between Denmark and Sweden) in 1981 (Støttrup et al. 1986). The World Copepod Culture Database was initiated in 2006 at Roskilde University (http://copepod.ruc.dk/main.htm) in an attempt to supply and share knowledge between copepod scientists, aquaculturists, and the public at large. The database contains details on various cultures and up-to-date recent knowledge on cultivation procedures. This far, approximately 30 copepod cultures have been referenced in the database (Table 1).

Genus/Species	Geographical origin	Cultivation conditions, temperature, salinity, light regime, food
Acartia grani	Barcelona Harbor (Spain)	19°C / 38ppt / 12L:12D / *Rhodomonas salina*
Acartia sinjiensis	Townsville Chanel, (Australia)	27-30°C/ 30-35ppt/ 18L:6D/ *Tetraselmis chuii* and T-iso
Acartia southwelli	Pingtung (Taiwan)	25-30 °C / 15-20ppt / 12L:12D / *Isochrysis galbana*
Acartia tonsa	Øresund (Denmark)	17°C / 30ppt / dim light / *Rhodomonas salina*
Apocyclops royi	Pingtung (Taiwan)	25-30 °C / 15-20ppt / 12L:12D / *Isochrysis galbana*
Centropages typicus	Gulf of Napoli (Italy W Mediterranean Sea)	19-21°C / 38ppt / 12L:12D / *Prorocentrum minimum / Isochrysis galbana / Tetraselmis suecica*
Eurytemora affinis	River Seine Estuary (France)	10-15°C / 15ppt / 12L:12D / *Rhodomonas marina*
Eurytemora affinis	Gironde Estuary (France)	10-15°C / 15ppt / 12L:12D / *Rhodomonas marina*
Eurytemora affinis	Loire Estuary (France)	10-15°C / 15ppt / 12L:12D / *Rhodomonas marina*
Eurytemora affinis	Baie de l'Isle Verte / St Laurent Estuary (Canada)	10-15°C / 15ppt / 12L:12D / *Rhodomonas marina*
Euterpina acutifrons	Mediteranea	19°C / 38ppt / 12L:12D / *Rhodomonas salina*
Gladioferens imparipes	Swan River (Perth Australia)	23-27°C / 18ppt/ continuous dark / T-Iso and *Chaetoceros muelleri*
Mesocyclops longisetus	Florida (USA)	http://edis.ifas.ufl.edu/IN490
Microcyclops albidus	Florida (USA)	http://edis.ifas.ufl.edu/IN490
Oithona davisae	Barcelona harbour (Spain NW Mediterranean)	20°C / 30ppt / natural light / *Oxhyrris*
Pseudodiaptomus annandalei	Pingtung (Taiwan)	25-30 °C / 15-20ppt / 12L:12D / *Isochrysis galbana*
Temora longicornis	North Sea	15°C / 30ppt / continuous dark / *Thalassioiera weissflogii, Rhodomonas salina, Heterocapsa, Prorocentrum minimum*
Temora longicornis	Plymouth (Devon, UK)	Temp according to current sea temperatures, 30-36ppt / 12L:12D / mixture of *Isochrysis galbana, Rhodomonas* and *Oxyrrhis*
Temora stylifera	Gulf of Napoli (Italy W Mediterranean Sea)	19-21°C / 38ppt / 12L:12D / *Prorocentrum minimum / Isochrysis galbana / Rhodomonas baltica*
Tisbe battagliai	Brixham (Devon, UK)	?

Table 1: Extract of the copepod culture list from the World Copepod Culture database (data are from January 2009).

When copepod cultures are required in fundamental research fields such as phylogeny, physiology and ecology, there is a need for reliable continuous cultures. This includes the potential availability of copepods for toxicity testing in bio-assays as was demonstrated by Karlsson et al. (2006) and Palacios-Caceres and Pereira (1997) with more references of eco-toxicity studies found at http://cfpub.epa.gov/ecotox/. Furthermore, aquaculture and the aquarium trade are in desperate need of copepods to supply the rapid development of their respective industries. Støttrup (2000) and Payne et al. (2001) put forward the hypothesis that the inclusion of copepods in aquaculture will enhance the number of successfully reared fish species. The same hypothesis can be applied to increase the production of various species of ornamental fish for the aquarium trade which would lead to a decrease in fishing pressure on natural stocks. The benefits include a reliable delivery of fish for the end-users and a unique chance for protecting delicate environments like coral reefs. One of the keys to successful larval finfish rearing is the high nutritional quality of copepods compared to other traditionally used live prey (Drillet et al. 2006b). Extensive cultures of copepods have already been achieved in order to supply aquaculture industries and aquarium trade needs with high quality live feed (Engell-Sørensen et al. 2004; Evjemo et al. 2003; Olivotto et al. 2008; Rajkumar and Vasagam, 2006; Sørensen et al. 2007; Toledo et al. 1999; Van der Meeren et al. 2008; Wilcox et al. 2006) and systems including the use of copepods have been patented (patents numbers: CN1288967 and JP2007044042, as examples).

The present contribution evaluates different limitations that slow down the development and realization of new cultivation methods, while at the same time, proposes possible novel and necessary actions based on a multi-disciplinary approach. The group of authors represents university researchers with backgrounds in zooplankton, aquaculture, business and management, and professionals from the private sector with expertise in microbiology and aquaculture consulting.

We present our evaluations and suggestions in three major parts dealing with: 1: Competitive manufacturing - Biological challenges and perspectives; discusses the challenges of copepod culture at the biological level, 2: Complementary technologies – Technical challenges and perspectives; provides technical information and innovations about copepod cultures, 3: Sales and marketing – Commercialization status and perspectives; proposes a suite of advices and suggestions which are crucial for further investigations to develop profitable intensive copepod cultivation.

PART 1: COMPETITIVE MANUFACTURING - BIOLOGICAL CHALLENGES AND PERSPECTIVES

1.1. Which copepod to raise?

Some copepods have more potential for live feed cultures because of particular life history traits. For instance, a fast reproductive cycle and low mortality are two factors that matter in terms of viability of commercial copepod cultures, but one could also argue for biochemical composition or/and swimming behavior. The relevant candidates for commercial culturing belong to three orders of copepods for which advantages/disadvantages have been reviewed (Støttrup, 2006). In short, harpacticoid copepods are epi-benthic copepods with in many cases pelagic nauplii and have been proven to have superior nutritional qualities compared to *Artemia* and rotifers (Cutts, 2003; Drillet et al. 2006b). Species of flatfish, gobies, salmonids, ciaenids, and blennies are sometimes considered to be obligatory harpacticoid feeders, at least for a period in their life cycle (Coull, 1990). Most harpacticoids can be grown at high densities (e.g. from 10,000 to 400,000 ind.L^{-1}, Støttrup, 2003) in high area/volume systems provided by incorporating 3D structures in the rearing tanks (Fig 1). However, most harpacticoids are egg carriers (Williams and Jones, 1994) and harvest of nauplii can become difficult in large systems. This has been recently solved in a semi-automatic rearing system producing 1-2 million nauplii daily in average in a 600L system (Steenfeldt, 2008) and production systems that are able to generate up to 8.2 millions of *Nitokra lacustris* nauplii per day in 266L systems exist (Rhodes, 2003). Cyclopoid copepods with obligatory pelagic nauplii are pelagic and are used occasionally in aquaculture and densities of ~5000 ind.L^{-1} are possible to achieve in cultures (Phelps et al. 2005; Su et al. 2005). Cyclopoids definitely offer a great potential for aquaculture and aquarium trades but they have not been intensively studied maybe because of the difficulties in harvesting nauplii from culture (Støttrup, 2006) and in the lack of storage possibilities for eggs. Calanoid copepods are pelagic and present in the entire water column. They have been particularly studied in aquaculture because they are natural prey for fish larvae. Nauplii of calanoid copepods have successfully been fed to a wide range of fish species including cod, halibut, flounder and barramundi. Several calanoid copepod species have been proposed as candidates for aquaculture production; and the commonly studied species are from the following genera *Acartia, Eurytemora, Gladioferens, Parvocalanus* and *Centropages* (O'Bryen and

Lee, 2005). Advantages from some of the calanoids include the capacity of storing eggs (Drillet et al. 2007; Marcus and Murray, 2001) but limitation resides in the difficulty of attaining high density cultures without negative side effects, like a decrease in hatching success or high mortalities (Jepsen et al. 2007; Peck and Holste, 2006). Maximum densities of the cultivated calanoid species proposed for aquaculture purposes are within the range of 100-1000 ind.L^{-1} (Støttrup and McEvoy, 2003), but recent studies have shown small-scale cultures attaining densities of 4000 *Acartia tonsa* ind.L^{-1} while keeping a low and density-independent mortality (Drillet et al. 2006a).

1.2. Food

The amount and quality of food are essential parameters that enhance the production of copepod cultures. Effects of food quantity and quality have been assessed in natural populations because of their importance at the ecological level. Most of the information present in the literature can be extrapolated and used for culture purposes. Quantity-wise, all copepods do not have the same need to reach their maximum growth and production potentials even when closely related (Jonasdottir, 1989). Too little food tends to slow down stage development, increase the competition for food between the individuals, and ultimately increases mortality (Berggreen et al. 1988). Most of the studies reported focus on the effects of optimal food concentration at the individual level. However, the improvement of copepod cultures increases the competition for food resources and investigations on optimal food concentration in high density cultures should be performed (also because phytoplankton production is costly).

Food quality should be adjusted for the target number of copepods, and the size of the prey should be consistently adapted not only to the species but also to the development stages that need to be fed (Berggreen et al. 1988, Hansen et al. 1994). Previous research has shown that a mixture of different diets generally enhanced the somatic growth and egg production of copepods in the laboratory (Harris, 1977; Jonasdottir, 1994; Klein Breteler, 1980), but also the right food item at the right development stage increases the overall success (Koski et al. 2006; Murray and Marcus, 2002). The food items should be adapted to the copepod feeding habits for example whether the copepods raised are raptorial feeders or suspension feeders. In the case of a raptorial feeder, phytoplankton might not be the only food item that copepod cultures could require. In cultures of the carnivorous copepod *Rhincalanus nasutus*, *Artemia* nauplii mixed with diatoms were used with success (Mullin and Brooks, 1967). In the

case of feeding on a non-algal diet (Takano, 1971a, b; Guérin and Gaudy, 1977), it is relevant that the cultivated copepods also use the microbial- and ciliates prey associated with the degradation of the added algal diet as a food source. The capacity to survive on purely bacterial food was described for *Tisbe holothuriae* by Rieper (1978), and Zillioux (1969) described microphageous ciliates as a source of food in his *Acartia* culture system. Ciliates are used as the sole food source for raising other copepod species (Suárez et al. 1992). The studies by Klein-Breteler et al (1980) give good ideas of the potentials given by hetertrophic dinoflagelates in copepod cultures showing they may also be interesting food items. Nassogne (1970) tested different food organisms on the development and culture of *Euterpina acutifrons*, as did Støttrup and Jensen (1990) for *Acartia tonsa* and Abu Rezq et al. (1997) for *Tisbe furcata*. The list of food items of relevance for various target species could be exhaustive and data concerning potential algae as food and their effect on growth and reproduction of copepods could be a review all by itself. Hence, out of the scope of this contribution. In all copepod cultures nuisance organisms like microphageous and histophageous ciliates, nematodes, and even rotifers regularly appears. In case they bloom they may exhibit a negative effect directly by predating on copepod eggs or indirectly as food competitors leading to waist of algal food. Recently a method to eradicate rotifers was proposed by Steensfeldt and Nielsen (2010), and abrupt salinity drop is by personal experience effective against ciliates, but nematode fauna is still difficult to eradicate. However, some microbial components are beneficial as food items for the copepods. New possibilities are to establish a balance between the intended copepods and their algal food and relevant protozoans is recommendable. If successful it will provide the copepods with food supplements to its algal food with a different biochemical composition and prey size. This strategy is most likely a lot more realistic than constantly fighting nuisance organisms, but it requires research to achieve this delicate balance in the cultures.

Biochemically, some copepod species seemingly have the capacity to bio-convert from shorter chains of fatty acids (e.g. 18:3) to long chain poly-unsaturated fatty acids (PUFAs). This has been proposed for *Eucyclops serrulatus* and *Paracalanus parvus* by Desvilettes et al. (1997) and Moreno et al. (1979). Nevertheless, it has also been reported that some species of copepods cannot grow well on phytoplankton lacking highly unsaturated fatty acids (Støttrup and Jensen 1990; Klein Breteler et al. 1999). If some species of copepods can bio-convert fatty acids, this would permit one to feed them diets based on cheap and easily accessible low quality food items like flour or yeast as it was shown by Kahan and Azoury (1981), Kahan et al. (1982) and Takano (1971a; b) for *Nitocra spinipes,*

Gladioferens imparites and *Tigriopus japonicus*, respectively. These cheap food sources would be of great interest for lowering the cost of copepod productions; although one of the problems generated by the use of flour or yeast as food for copepods in the same manner as used for rotifers and *Artemia*, is that the resulting water quality is often poor. Two reports (Chapman, 1981; Gyllenberg and Lundqvist, 1978) proposed that copepods *Neocalanus plumchrus* and *Cyclops oithonoides* could use dissolved glucose as a food source. However, for water quality reasons, this would not be an optimal solution for feeding copepods in cultures due to expected problems with uncontrolled growth of heterotrophic bacteria and the potential risk for developing pathogenic microorganisms in the system.

1.3. Density

Density of individuals is crucially important to copepod cultures. Some copepods are much more sensitive to high density (hundreds per liter; Ban and Minoda, 1994) than other live preys like rotifers (millions ind.L^{-1}; Moretti et al. 1999). Thus, they have been used only occasionally in fish larval feeding. The density question is of central importance because production costs are directly related to possible densities of copepod cultures. Calanoids are considered poor candidates for mass cultivation simply because they have difficulties in surviving at high densities, but harpacticoids and cyclopoids can commonly be raised at densities reaching a few thousand ind.L^{-1}or more (see Lee et al. 2005; Støttrup and McEvoy, 2003; Steenfeldt, 2008). The trade off is most often that increased density is negatively affecting survival, developmental time, growth, reproduction and egg hatching success (Zhang and Uhlig, 1991; Kahan et al. 1988; Ban and Minoda, 1994; VanderLugt and Lenz, 2008); suggesting that a balance must be achieved. However, it is interesting that for various species of the same genus and even populations of the same species, density-dependent egg production and hatching success showed highly divergent results (Camus and Zeng, 2009; Jepsen et al. 2007; Medina and Barata, 2004; Peck and Holste, 2006) but these differences may be due to inherent differences between populations or to growing conditions as proposed by Drillet et al. (2008b) and therefore fast conclusions should be avoided. Nevertheless, high culture densities increase the intensity of resting egg production of *Eurytemora affinis* and *Acartia sinjiensis* (diapause and delayed hatching eggs) (Ban and Minoda, 1994; Camus and Zeng, 2009). To our knowledge, this has never been shown for any other copepod species but is well known in other crustaceans (e.g. *Daphnia* spp.; Zadereev, 2007). This

density-dependent diapause egg production seriously raises the question of the viability of intensifying batch cultures and possibly that chemical communication between copepods could be a limit to enhancement. On the other hand, this effect could also be investigated in order to deliberately aim at producing these particular types of eggs for use in aquaculture. This strategy might allow long-term storage of resting eggs, which opens up new perspectives for storage and shipping (see section 2.4.). However, if accumulation of chemical cues/metabolites in the water can lead to detrimental effects on the culture, then changing the water regularly or even applying water treatment technologies like circulation (see later) allows batch cultures to reach high densities as in the case of calanoids (e.g. >4000 ind.$L^{-1}$with 5% mortality day^{-1}; Drillet et al. 2006a).

1.4. Egg and nauplii cannibalism

A problem facing dense copepod cultures is cannibalism due to the increased encounter rates of individuals in the rearing tanks. Cannibalism has been reported for many copepod species (e.g. *Sinocalanus tenellus, Centropages abdominalis, Tigriopus fulvus, Acartia sinjiensis*). Often, smaller individuals (younger stages) are more likely to be the victims to the more developed stages (Daan et al. 1988; Ohman and Hirche, 2001; Ohno and Okamura, 1988; Uye and Liang, 1998). Hada (1991) and Hada and Uye (1991) showed for *Sinocalanus tenellus* that the ingestion rate of their own nauplii increased with increasing nauplii density to an asymptotic value and that cannibalism took place even when alternative food was available. When adult females occurred at a density of 10 ind.L^{-1}, the mortality due to cannibalism was 99.2% during the naupliar stages. Nauplii stages of *Rhincalanus nasutus* developed escape behaviors to avoid predation from adults (Mullin and Brooks, 1967). Lazzaretto and Salvato (1992) reported the existence of cannibalistic behavior in *Tigriopus fulvus* females towards first-stage non-related nauplii, which demonstrates that mothers can spare their own offspring. Yet, if this mother/offspring recognition raises interesting questions about chemically mediated communication between copepods, then it would not matter to intense cultures where young stages face cannibalism from non-related individuals. The safe way to counteract this effect is to separate nauplii from the adults (Brandl, 1972). More developed systems as presented by Payne and Rippingale (2001a) and Zillioux (1969) are of great importance. Kang and Poulet (2000) and Liang et al. (1994) showed that egg cannibalism was also important, suggesting that separation of the swimming

stages and the eggs is crucial. Sedimentation and regular harvest of the eggs from a culture is necessary to increase the harvest of egg production per unit. This regular harvest also keeps tanks clean by removing fecal pellets, exuvia, detritus particles, sedimented algae, etc. (pers. obs.).

1.5. Optimization of sex-ratio

Another way to improve the productivity of copepod cultures is to control the sex-ratio to optimize the number of females producing offspring. This is based on the hypothesis that males feed nearly as much as females and their unique role is to fertilize the females. Hence, decreasing the density of males will be cost efficient in terms of feed expenditure and culture volume.

Based on Fisher's (1930) fundamental law of natural selection, the sex-ratio should be 1:1. If one sex is in short supply in a population, then an allele that leads to the production of the rarer sex will be favored (Fisher, 1930). Only when the sex-ratio is exactly 1:1 will the expected success of a male and a female be equal and the population stable. Though the sex-ratio in the natural environment is often observed as different from what was expected by Fisher's law, this has been explained by a higher mortality of males compared to females because of their risky motility pattern and speed when seeking mates (higher risk of predation) (Hirst and Kiørboe, 2002; Kiørboe, 2006, 2007). Sex changes which are believed to be under strong environmental control (food limitation being the strongest factor) may be an important mechanism determining the adult sex ratio (Gusmão and Mckinnon, 2009). The fertility of intersex is unknown, but there are some indications that they can have a reproductive outcome (Miller et al., 2005; Sillett and Stemberger, 1998; Dharani and Altaff, 2002). Species with intersex may have an advantage if they are able to reproduce successfully, because they are able to secure the population in a highly patchy planktonic environment and thereby give a higher reproductive outcome. As long as we do not know if the intersex species have a high successful reproductive outcome, it is of interest to keep good food quantity to prevent sex changes from occurring. However, if natural conditions can affect the sex-ratio of populations, this could be a highly useful tool to optimize copepod production. In several species, one spermatophore is probably enough to fertilize eggs for ~ 2-2.5 month (e.g. *Acartia clausi,* Uye, 1981, *Calanus* spp. Marshall and Orr, 1955, *Eurytemora affinis,* Katona, 1975). In other species (e.g. *Centropages* spp., *Temora stylifera, Acartia tonsa*), females lack seminal receptacles and thus the ability to store sperm except in the

spermatophores and need to replenish their sperm supply continuously (Ohtsuka and Huys, 2001). Several studies have shown that during experimental time females tend to outlive males (e.g. Rodrígues-Granã et al. 2010), although the fecundity of the females does not seem to be affected due to their ability to save sperm. Behavioral and field observations together with population models suggest that in some species (e.g. *Oithona davisae)* population growth is severely limited by fertilization rate and by the shortage of males, even when population densities are high. There are many factors to understand in order to optimize the sex ratio of copepod cultures and increase the productivity and research in this direction will be necessary in the future.

1.6. Selection and cross-breeding to obtain optimized populations

In all food production sectors, activity increases by selecting populations that have particular traits needed for rendering the production economically valuable. In the marine environment, this selection procedure started late as compared to land-based food sectors but improvement of reared marine organisms is done intensively (Duarte et al. 2007). The selective reproduction technique is only applied to a small percentage of the hundreds of organisms raised in aquaculture, and therefore there is large room for improvement of reared species which have not yet been selected in any way (FAO, http://www.fao.org/biodiversity).

Selection of groups of individuals has to be combined with the development of techniques for storage and selection of sperm, embryos and other products from desirable parents (see *Conservation and shipping* section). Within the subphylum of Crustacea, preliminary studies can be realized by using *Artemia* as a model because many of the factors thought to be responsible for genetic differentiation and speciation in other organisms are observable in these organisms (Barigozzi, 1982; in Eimanifar et al. 2006). For instance, studies to produce fast hatching *Artemia* have already been performed (Briski et al. 2008) and similar studies could be achieved by using relevant copepods.

Indeed, little work on selection has been conducted for copepods, but the selection of heritable traits that are valuable for aquaculture is an interesting suggestion. Most likely, the relative stability of culture conditions (salinity, temperature, feed type) is likely to result in a genetic selection in the live prey population. An on-going study using an *A. tonsa* population validates this hypothesis showing that

phenotypic variability decreased significantly over the first five generations while under a constant laboratory environment and staying almost constant thereafter. After isolation from the field, the average body size of *A. tonsa* remained almost stable over 15 generations under controlled laboratory conditions (C. Halsband-Lenk, Unpublished). However, this relative stability is probably not developed as far as it would be necessary for aquaculture purposes at this point in time.

Recent common garden experiments revealed that different copepod populations of the same species could actually exhibit different life history traits which could be selected to develop a particular population for aquaculture purposes (Drillet et al. 2008a,b; Leandro et al. 2006); although none of these studies tried further to breed the different populations for numerous generations. However, cross-breeding allopatric populations can both result in an increased fitness of the following generations (heterosis or hybrid vigor) or decreased fitness of the following generations (outbreeding depression or hybrid breakdown) and this is due to the recombination processes over the multiple generations (Edmands et al. 2005, and references therein).

1.7. Enrichment and use of probiotics

Enrichment of live prey in aquaculture is a well-known practice and innovation in this field is necessary. However, copepods are often used as sole feed when compared to enriched prey because work performed in this area are mainly focused on comparisons of biochemical composition and effects upon the predator using various live prey organisms reflecting common husbandry in fish rearing. Among alternative methods developed to increase the production or survival of live prey for aquaculture, beneficial bacteria and microbial preparations gathered under the generic term "probiotics" are now currently used. These probiotics can contribute to limiting the establishment of pathogenic bacteria (Rengpipat et al. 1998; Rengpipat et al. 2003). They can also enhance performances of crustaceans by stimulating immunity of the host species like it was observed with *Penaeus monodon* and *Peneaus vannamei* (Alavandi et al. 2004; Gullian et al. 2004), or improving the digestive physiology and the stimulation of digestive enzymes of *Litopenaus stylirostris* (Frouël, 2007). Probiotic microorganisms might improve the host physiology by contributing to the host nutrition supply of vitamins (vitamin C, vitamin B12, retinol and others), proteins, amino acids, and highly unsaturated fatty acids (Gapasin et al. 1998; Gorospe et al. 1996; Yu et al. 1988), and delivering

enzymes which stay active in the host intestine. Microorganisms could facilitate diet assimilation and decomposition of refractory compounds (Hood et al. 1971) and convert all dissolved nutrients, normally unavailable because of non appropriate molecular size (Verschuere et al. 1999). All these aspects may improve the development and the survival rate of the crustacean by enhancing the diet assimilation.

However, research on the effects of probiotics on copepods is, to our knowledge, limited to a few text lines referring to the use of a "commercial probiotic A" in a book chapter (Su et al. 2005) where the effect of a mixture containing probiotics increased by two fold the production of a copepod culture. Recently, a study where heat inactivated *Lactobacillus* was added to an A. *tonsa* culture leading to significantly larger sized females with a higher egg production (Drillet et al. submitted). Considering the potential of probiotics for copepods, it seems obvious that more research on the subject is needed.

PART 2: COMPLEMENTARY TECHNOLOGIES - TECHNICAL CHALLENGES AND PERSPECTIVES

2.1. Copepod cultures

Vijverberg (1989), in a paper aimed at evaluating vital rates of copepods and cladocerans, noted that marine copepod culture techniques were more advanced than fresh water ones. Zillioux (1969) presented one of the very first culture systems for copepods, followed by research groups who improved and intensified the system (Payne and Rippingale, 2001a; Schipp et al. 1999; Sun and Fleeger, 1995). Their technical descriptions are excellent for the aquarium trade but more adaptations and improvements are needed to reach the huge demand of mass cultivation systems required by the aquaculture industry.

Wild or semi-wild copepods are used successfully to raise fish larvae (Conceição et al. 1997; Engell-Sørensen et al. 2004; Evjemo et al. 2003; McEvoy et al. 1998; Næss et al. 1995; Toledo et al. 1999). In these semi-extensive inland tank systems, isolated populations from the natural environment are either caught and used directly or grown in 1000's m^3 tanks; in which occasional phytoplankton blooms are

stimulated by the addition of inorganic nutrients to the water. Semi-extensive systems require low maintenance and allow production of large amounts of copepods, eggs and nauplii. However, they are weakened by their large volume and are susceptible to contamination by other copepod species, other zooplankton and parasites that use copepods as intermediate hosts (Markowski, 1933).

Whatever the risks of contamination, recent studies on production and biochemical composition of copepod production all year round have shown that outdoor culture and wild copepods were highly valuable for fish production (Sørensen et al. 2007; van der Meeren et al. 2008). Thus, improvement and sustainable enhancing procedures are achievable and should be further optimized by a thorough research effort.

2.2. Temperature-salinity-oxygen-light

Temperature, salinity, dissolved oxygen and light are factors that greatly influence the success of a copepod culture. Most of these factors have been considered in environmental studies and therefore plenty of literature information is accessible to culturists (Mauchline et al. 1998). The primary need for information with regards to large-scale and dense copepod cultures resides in the changing effects of these parameters during intensive cultivation. Temperature and salinity have to be adapted to each population of copepods and are often analogous to the conditions the population is facing in the natural environment because populations have adapted to these particular environmental conditions. Because to our knowledge the optimal temperature and salinity do not change during intensification processes, preventing extreme conditions is, most of the time, sufficient to avoid a restraining effect on copepod cultures.

Low oxygen tension in culture tanks is most likely to increase the mortality and decrease the fecundity of the copepods. Marcus et al. (2004) reported that 1.4 $ml.l^{-1}$ O_2 (2 $mg.l^{-1}$ O_2) do not affect the mortality but decreases the fertility among *A. tonsa*. At 0.7 $ml.l^{-1}$ O_2 (1 $mg.l^{-1}O_2$), both fertility and mortality are adversely influenced. However, within a species, some populations experiencing routinely natural hypoxia could evolve into a more resistant population as suggested by the work of Oppert (2006).

Light rhythm is an important factor controlling the physiological performances of copepods, activating sometimes the production of diapause stages (Marcus, 1982; Alekseev et al. 2007). This factor affects

egg production and hatching success of *Acartia* sp. cultures; longer light exposure increased the 48 hour hatching success of the eggs (Camus and Zeng, 2008; Peck et al. 2008). However, cultivation procedures kept constant over time under particular conditions are likely to select for specific traits. For example, cultures of *A. tonsa* from the Danish Technical University (DTU-Aqua) have lost their diel feeding and egg production rhythm due to excess food availability and absence of predators through 150 generations (Tiselius et al. 1995).

Light spectra is also a parameter that should be studied because ultraviolet (UV) radiations usually generate harmful effects by lowering egg hatching success and increasing mortality, though some supra-littoral species are more resistant than open water species (Chalker-Scott, 1995; Kane and Pomory, 2001; Karanas et al. 1979; Saito and Taguchi, 2003).

2.3. Water quality, recirculation and automation of cultures

Antibiotic treatments to increase survival, growth, and lifespan of crustaceans including copepods have occasionally been used (Corkett and Urry, 1968; Fisher and Nelson, 1978; Pelletier and Chapman, 1996). However, the use of antibiotics at the industrial scale must be avoided because of potential contamination of the natural environment by resistant bacteria. Also, exposures to antibiotics could generate toxic effects to crustaceans including copepods (Di Delupis et al. 1992; Drillet et al. 2007; Flaherty and Dodson, 2005; Isidori et al. 2005; Wollenberger et al. 2000).

Since the introduction of governmental regulations on waste water quality from production plants and because of the need for high water quality in intensive systems, the industry has developed re-circulated fish farming systems. This is because low water quality leads to poor growth and fertility, increased disease pressure, and mortality (Boyd and Tucker, 1998; Schreck et al. 2001). The recirculation of water through a proper system helps at controlling the abiotic factors such as NHx compounds, phosphorus, pH and CO_2, which greatly affect fish/crustaceans welfare. Indoor copepod cultures are often kept in small volumes where water change is easy to perform but up-scaling the volume of culture renders this impossible. A solution resides in the recirculation technologies which should be adapted since copepods may require different rearing conditions compared to fish (see typical recirculation components in Losordo et al. (1998) and Fig.1).

With biofilters, organic and inorganic substances are removed using a biologically active biofilm, which is created when bacteria grow on the surfaces of the biofilter element (Lazarova et al. 1994; Juretschko et al. 1998). The great variety of bacteria in the biofilm makes this technology adaptable to a wide range of environments although its success to enhance the water quality depends on the ability to control the processes in the biofilter (Tal et al. 2003; Tseng and Wu, 2004). An efficient control of suspended solids as food residuals, exoskeletons and feces, is essential for the stability of biofilters (Okabe, 1996; Piedrahita, 2003). The particle content affects the oxygen level, as less oxygen can be dissolved in water with impurities than in clean water and because the efficiency of UV disinfection is decreased by the presence of particles in the water (Summerfelt and Sharrer, 2004).

UV radiation is the most common water disinfection method in aquaculture industries and is used to reduce the presence of harmful bacteria, parasites and viruses in the water. Chlorination-dechlorination using chlorine and thiosulfate is another effective method to disinfect water before batch cultivation (see method in Støttrup and McEvoy, 2003). The use of ozonation is another way of decreasing the bacterial development in cultures and was shown to be effective at low levels (400-500mV oxidation-reduction potential), increasing the growth and survival of the southern rock lobster (*Jasus edwardsii,* Ritar et al. 2006). One of the risks related to copepod cultures in intensive fish farms is the pollution of copepod cultures by rotifers which may take over the systems. This was partly solved in a recent study where the use of flubendazole as a therapeutic agent was used to remove *Brachionus plicatilis* from copepod cultures (Steenfeldt and Nielsen, 2010).

Another issue with copepod cultures is that they demand many man hours for daily routines, such as algal culture maintenance, copepod culture maintenance, sorting copepods, egg harvest, etc. Automation of these procedures with an automatic control of feeding by dosing pumps, sorting of copepods and egg harvest by size fraction mechanical filters and the use of an efficient electronic control system is a key to success with large-scale copepod cultures. Automatic control systems ensure stable water quality by using probes to measure O_2, pH, salinity and CO_2 and are already introduced to counteract water quality fluctuations by calculating from earlier known scenario, and decreasing costs for O_2, NaCl, NaOH, etc. Those control systems are expected to function as a forecast and help to develop copepod mass cultivation systems.

2.4. Conservation and shipping

An additional challenge for the enlargement of copepod use in fish farming and in the aquarium trade is the development of storage processes for various development stages. These techniques would allow the shipping of copepod products between producers and end-users and help producers to ensure back-up of cultures for re-inoculations and re-stocking of their systems. Payne and Rippingale (2001b) stored fresh nauplii of *Gladioferens imparites* for up to 12 days at 8°C (survival 99%) but mortality was almost 100% after 36 days. One alternative resides in the storage of eggs. Marcus and Murray (2001) stored diapause eggs of *Centropages hamatus* for 17 months under anoxia keeping a hatching success >80%. Around 50 copepod species belonging to Acartiatiidae, Centropagidae, Pontellidae and Temoridae have been described to produce resting eggs as part of their life cycle (Mauchline et al. 1998). However, there are plenty of complexities regarding the induction of diapause and the cues breaking diapause are scarcely understood. Some authors propose that peroxide production due to the oxidation of fatty acids to prostaglandins by light inside the embryo may trigger hatching in *Brachionus* sp. (Hagiwara et al. 1995), while other authors working on *Artemia* spp. manipulated intra-cellular pH to force hatching during the refractory period (Drinkwater and Crowe, 1987).

Alternatively, Drillet et al. (2006a) stored subitaneous eggs of *A. tonsa* for up to a year at low temperatures (2-3°C), forcing eggs to switch to a quiescent state. The authors showed that cold storage does not affect the fitness of the next generation of copepods, although they observed an increased mortality during the ontogenetic development (likely due to the lack of reserves transferred from the stored eggs into the first nauplii). However, the biochemical profile of eggs and nauplii is negatively affected by cold storage (Drillet et al. 2006b; Støttrup et al. 1999) and egg ontogeny affects their capacities to be cold stored (Drillet et al. 2007). Additionally, under similar storage conditions, different populations of *A. tonsa* showed totally different capacities of cold storage, showing the importance of the population origin (Drillet et al. 2008a).

Previous studies have shown that optimal cold storage conditions of wild harvested eggs from a Danish estuary were: low temperature (<5ºC), medium salinity (10-20) and anoxia (Holmstrup et al. 2006). Eggs from a culture of *Acartia tonsa* even survived storage at room temperature in distilled water for weeks keeping a viability of 15-20% (Højgaard et al. 2008). Thus, in the marine environment eggs can survive in the sediment as long as 40-69 years (Marcus et al. 1994; Sichlau et al. In revision). This

gives researchers a lot of room for improvement of storage techniques for copepod eggs. Recently, first steps towards cryo-conservation of eggs were conducted by Ohs et al. (2009) but, as stated by the authors, much more work has to be achieved in this field.

Limitations to the shipping of copepod eggs are the transfer of parasites, histophageous ciliates and bacteria from one system to another. Ciliates can be removed from egg samples by simple fresh water rinsing (personal observation) and chemicals can be used to disinfect properly the eggs as they are known to be much more resistant to various chemicals than nauplii (Næss, 1991a,b: Næss and Bergh, 1994). For egg carriers, Støttrup and McEvoy (2003) proposed to disinfect eggs (*Tisbe holothuriae* Humes) by adding chlorine to the water, whereby the egg sacs detach from the dead adults, can be harvested, rinsed and used to hatch nauplii under axenic conditions.

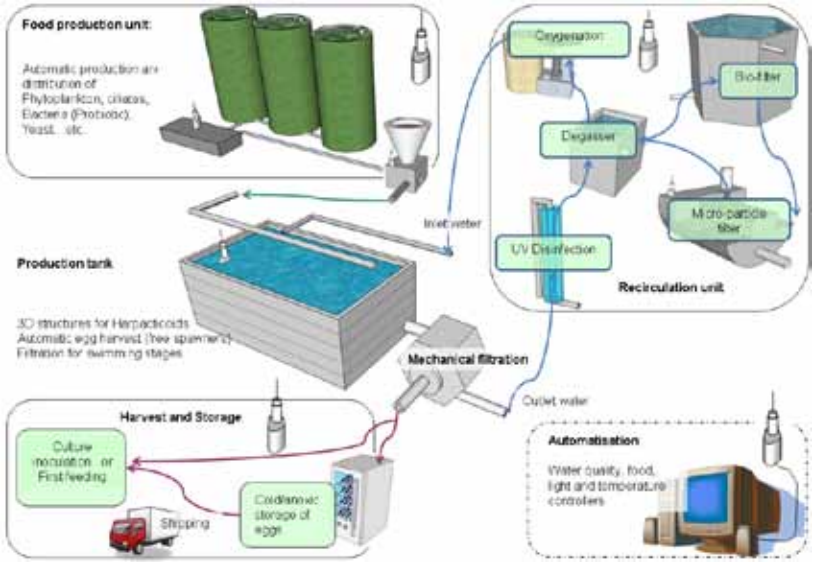

Figure 1: Schematic ideal large-scale copepod production. Copepod culture for free spawning and egg carrying species are incorporated. Benthic copepods are produced in systems containing 3D structures. Harvest of nauplii/eggs for further utilization or storage decreases the risk of cannibalism. Recirculation procedures provide high quality water to which automatic feeding is added. A surveillance system equipped with multi-sensors keeps track of variations in vital parameters in cultures, allowing a minimum of labor force during production (figure made using Google SketchUp 7™ and MS office™).

PART 3: SALES AND MARKETING - COMMERCIALIZATION STRATEGY FOR COPEPOD PRODUCTS

It has been argued over the years that constructing a successful commercialization strategy is important to capture the value from research and development activities (in our case *Copepod products,* Figure 2). However, the economic value and commercialization of copepods has, to our knowledge, never been assessed even though patent for fish breeding including the use of copepods do exist (JP2007044042, CN1288967). Here, we adapted Teece's conceptual framework (1988) with the objective to identify the major gaps in our knowledge. The framework lists the main parameters influencing the success of the product in focus. Some of these have been discussed earlier and the ones related to management are discussed here. We consider that it is essential to differentiate between centralized vs. decentralized productions when analyzing the economical and commercialization aspects. One may like to keep in mind two levels of considerations:

1: The copepod production is held on site at the end-user production plant (decentralized production), where the copepods are needed for first feeding and in this case, the price of the copepod products is bound to the capital investment for the production system (culture system and eventually expertise from consultancy) and the cost of production (labor and variable costs = maintenance, electricity, consumables etc.).

2: The copepod production is completed by a firm that sells copepod products to end-users (centralized production). The copepod producer needs to incorporate in his selling price the price due to the capital investment, cost of production, marketing, and potential shipping prices. The end-users benefit from a decrease in labor cost, capital investment cost and need for expertise, but they are also restricted to the price set by the competition/availability of copepod products on the market.

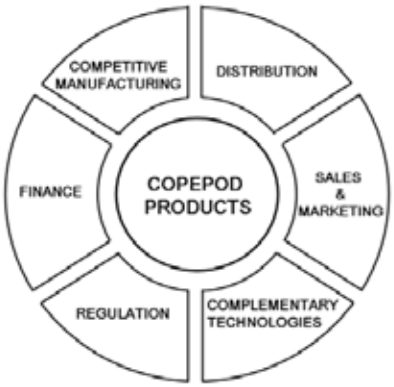

Figure 2: 'How to capture value from a copepod product'. Conceptual scheme modified from Teece (1988) showing the most important parameters influencing commercialization of copepod products.

We reviewed and discussed *Competitive manufacturing and complementary Technologies* earlier in this paper (Part 1 and 2) and ask here two simple questions: "How much does a copepod cost?" and "How to commercialize a copepod?" The aquaculture sector occasionally uses copepods from semi-extensive *in situ* systems but no live copepod products are available for large aquaculture businesses. Only a few published studies deal with the economic aspects of copepod production. Molejon and Alvarez-Lajonchere (2003) proposed to hold productions in countries with low "qualified labor" costs because in hatcheries from industrialized countries, for example, salaries and benefits represent the largest cost-component of production at 37% (Juarez and Martinez-Cordero, 2004). Alvarez-Lajonchere and Taylor (2003) took copepod cultures into account in their calculation of cost efficiency in the production of juvenile common snook, but no details for price of production are presented (price per amount of produced copepod) as can be found for *Artemia* production (Lim et al. 2001). In the aquarium trade sector, however, it is possible to find distributors, retailers and customers of live copepod products through internet searches, but access to these products is limited and prices vary drastically from one retailer to another (Table 2). One of the reasons for this could be that firms producing copepods sell their products only in their own geographical areas, using only the local languages and networks, making national or international research for copepod products relatively difficult. In our compilation, only one retailer proposed its products in more than one of the major languages (Table 2).

Firm	Product	price	Language
AlgaGen	*Pseudodiaptomus pelagicus* (*1000)	50 US$	English
AlgaGen	*Acartia tonsa* (*1000)	50 US$	English
AlgaGen	*Parvocalanus* sp. (*1000)	100 US$	English
AlgaGen	*Tisbe* sp. (*1000)	35 US$	English
Guernesey Sea farms	*Acartia tonsa and Tisbe* sp. females (*300 up to *1500)	160-255 £ 225-355 €	English
SeaHorseMania	mix of Calanoid and Cylopoid copepods 1000ml	29.80 €	English German French Spanish Italian
SeaHorseMania	*Acartia tonsa* adults (*1000)	65 €	English German French Spanish Italian
SeaHorseMania	*Nitokra lacustris* 200ml	18 €	English German French Spanish Italian
SeaHorseMania	*Acartia tonsa eggs* *10.000 / *50.000 / *100.000 / *1.000.000	11 € / 18 € / 27.5 €/ 150 €	English German French Spanish Italian
Seahorse Sanctuary	*Gladioferens imparites* nauplii (*5.000-10.000)	AUS $110	English
SeaHorseMania	*Tisbe* sp. 200ml / live ReefPods *Tisbe* sp. 200 ml	15 € / 18 €	English German French Spanish Italian

Table 2: List of COPEPOD PRODUCTS available from retailers advertising and selling on the internet (January 2009, with authorization from companies). (*1000= 1000 individuals)

Another important limiting factor could be the difficulty of import/export of live products (*Distribution*) in rather small amounts across borders due to custom *Regulations* which differ from country to country and small aquarium businesses may not be well prepared for international export (Adelaide Rhodes, personal communication). In our opinion, economic and market studies are lacking because the effects of an improved availability of copepod products could increase the number of fish species raised through their entire life cycle not only affecting the aquarium trade. An important gain

would be, for example, to preserve natural habitats by decreasing the demand/availability ratio of some declining/over-caught fish populations and therefore keeping sustainable natural resources. This is even more relevant when taking into consideration that many reef fish are captured using destructive fishing practices such as the use of cyanides generating long lasting or even devastating ecosystem and social impacts (see Barber and Pratt (1997) and Johannes and Riepen (1995) in Sadovy and Lau (2002)). In addition, it seems that most of the research and development on copepod cultivation and copepod products is performed using public *Finance*. A systematic study of the price of copepod production is necessary to raise the interests of business angels and venture capitalists. Also, in association with commercialization, the intellectual property rights (IPRs; i.e. patents, trademarks etc.) must be considered because they play a central role behind capturing value from knowledge assets (Teece, 1998). Many authors argue that strong IPRs encourage investment because it gives a fair degree of certainty on return of investment (but surely it doesn't give an absolute guarantee of profit maximization) though, as stated by Teece (1986): "the protection offered by patents is fairly easily enforced".

The last important gap in knowledge we identified, though it is a rather personal opinion, resides in the fact that apart from a few professionals, too few people in the aquaculture and aquarium industry actually know about copepods and their potentials as live feed. Screening the internet for copepod businesses, it was obvious that many aquaculture consultants/aquarists do not know enough about copepods. It is a paradox that knowledge about the most numerous multicellular organisms on the planet and the optimal live feed for larval fish exists for researchers but not yet for the obvious end-users. Hence and to our knowledge, the concept is only utilized in rare cases.

CONCLUSIONS AND RECOMMENDATIONS

There are several aspects of copepod cultivation techniques and use that have been studied and reviewed (see Lee et al., 2005 and Støttrup, 2003). However many important and novel parameters that could help the improvement of copepod cultivation techniques should be carefully studied. The scientific community for instance should launch research to understand catabolic lipid pathways in various copepod species as this could help developing inexpensive food items for copepods able to bio-

convert fatty acids. Also, the need for understanding mating processes, sex changes, sex-ratio and chemical communication between copepods seems obvious and the amount of effort put in these areas should be increased before transferring this knowledge to applied aquaculture. There are other factors that are interesting to study and these could include for example the selection of highly productive populations (with or without cross-breeding) and this also suggest the need for developing storage solutions of embryos. We also encourage the scientific community to produce detailed review papers on the effect of food sources on copepod development in order to condense the vast amount of data available in the literature to the culturists. Finally, the development of probiotic products as it is done with other live prey seems an interesting way to counteract some of the issues that are specific to copepods.

In the present contribution, we tried to condense a very large amount of discoveries and reviews that have helped directly or not the development of copepod cultures. We give here a few recommendations for culturist, some of them being personal opinions based on our knowledge at this time, and most of it based on related scientific work described earlier in the article.

- We recommend using harpacticoids and/or cyclopoids in industries that can produce a large amount of these prey on site at any given time, and using calanoid cultures for industries limited by time for production and industries which need to export copepod products (e.g. eggs). Copepod cultures of non-native species should be reared in closed systems to avoid invasions of surrounding environments.
- We recommend feeding dense copepod cultures continuously using automatic procedures, rather than feeding once or twice daily to ensure constant optimal food concentrations in the water.
- We suggest that water recirculation in copepod cultures is essential for further development because water quality is essential in cultures. The recirculation system could be linked to an automatic system for harvest of nauplii, eggs and debris to decrease the cannibalistic effects and to facilitate the storage of copepod eggs.
- We recommend launching research with the purpose of selecting strains or populations of copepods that can tolerate high densities, exhibit a fast development and growth with moderate mortality, exhibit a relatively high resistance to stress, and present a highly qualitative nutritional value for fish larvae
- We recommend using light regimes that are not known to increase the production of resting eggs (e.g. 12D/12L, UV free) for producing copepods, although this should be adapted for individual

species. Also, we recommend hatching eggs with increased light exposure because this seems to enhance the hatching success of some copepod species.

- We recommend using an automation of copepod cultures to decrease labor work and cost of production. We believe that it should be trivial to transfer some technologies from mass fish farming to copepod mass culturing.
- We recommend copepod scientists/culturists/businesses to use/develop an efficient and effective Sales and Marketing strategy to reach and educate consumers and develop a sustainable business. This has to be tightly coupled with a thorough study of economical feasibility.

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MANUSCRIPT 7

Do microbial preparations with probiotic effect improve life history traits of the copepod *Acartia tonsa*? Zootechnical observations from cultures

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Abstract:

We have tested a microbial preparation with probiotic effects (PSI: Sorbial A/S Danisco) on the calanoid copepod *Acartia tonsa* (Dana) development time and reproduction effectiveness in culture. The hypotheses were that PSI increases the productivity and quality of copepods in culture (increased egg production and hatching success, HS). This was carried out because the use of copepods as live prey in aquaculture could increase the number of fish successfully raised through their entire life cycle. However, the availability of copepods is limited by their difficulty to be effectively raised. Our results show that the addition of PSI increased the individual size of the adult females and their egg production. The PSI also increased the HS of the eggs produced by PSI treated females. These effects were observed despite that the biochemical analysis of the PSI revealed that it is a nutritionally poor food lacking essential fatty acids and hence, it cannot be used alone to raise copepods but instead as an food additive. This is the first demonstration that the effectiveness of copepod culturing can be improved using microbial preparations as a food additive.

Key words: Copepod, Acartia, Probiotic, Live prey, Culture, Microbial preparation

Running title: Effects of microbial preparations on copepods

Introduction

Aquaculture is the fastest growing food production sector in the world (FAO 2006). The difficulty in recruitment of a large number of fish larvae is the bottleneck in commercial aquaculture of many marine fish species. This can be partially attributed to the large input of essential nutrients required at the small fish larval stage, particularly several essential fatty acids and amino-acids which fish larvae are unable to synthesize *de novo*. In some cases fish meal can supplement these needs but the use of traditional organisms as live prey like *Artemia* sp. and rotifer is sometimes required. Nevertheless, the enrichment procedures of these preys have shown limitations and rotifers and *Artemia* sp. may be sometimes inadequate food. In many cases copepods have been shown to be much better food for fish larvae because of their high nutritional value (e.g. high contents of EPA and DHA fatty acids) and long passage time through the fish gut (Drillet *et al.* 2006b, Pedersen 1984). Herbivorous or omnivorous

copepods have been shown to be valuable in this regard by nutritionally supplementing traditional live prey or by replacing them altogether (Wilcox *et al.* 2006).

The euryhaline and eurytherm calanoid species *Acartia tonsa* is an interesting candidate for use as live prey in aquaculture due to its previously described nutritional benefits and its capacity to produce eggs that can be stored for relatively long time (Drillet *et al.* 2006ab, 2007).

The difficulty in using copepods for live feed lies in the lack of refined cultivation knowledge although advances in indoor culturing of copepods have allowed for the implementation of some semi-intensive copepod fed fish hatcheries (Schipp *et al.* 1999, Payne and Rippingale 2001). One solution to increase the yield of production and the resistance to infections of copepods in culture could be the use of microbial preparations with probiotic effects which have been used successfully in aquaculture, including on live prey (Verschuere *et al.* 1999, Patra and Mohamed 2003, Balcazar *et al.* 2006). We aimed at evaluating the use of a fermented milk product based on two heat inactivated *Lactobacillus* spp. strains developed by Sorbial A/S (Allonnes, France, DANISCO) as a food additive with probiotic effect in *A. tonsa* cultivation, specifically the effects on developmental rate, fecundity, and HS of a well described *A. tonsa* laboratory population.

Material and Method

A. tonsa eggs were harvested from our cultures at Roskilde University (Denmark) and are originally isolated from the Øresund sound in 1981 (Støttrup 1986). The culture was maintained in 50L tanks kept in dark/ 17°C/salinity 30 and fed *ad libitum* with *Rhodomonas salina* that was maintained in log phase in B1 media (Hansen 1989) at 17°C /24h light. The eggs were harvested <24h after production, cleaned by vigorous washing through a 56 µm filter, and stored in GF/F filtered seawater (30 g mL^{-1}) in containers completely filled and sealed with plastic wrap in 4°C/dark.

Lactobacillus farciminis CNCM MA27/6R and *Lactobacillus rhamnosus* CNCM MA27/6B were cultured on a dairy-like medium. The live *lactobacilli* cells were enumerated on MRS agar (Mann, Rogosa and Sharpe, Merck laboratory) to adjust the dose before heat inactivation process (Process protected by a "Pli Soleau") and the additive was prepared and named PSI. The PSI C:N ratio and potential nutritive qualities (fatty acids contents) were determined using the methods described by Drillet *et al* (2006b). The bacterial preparation was always sonicated before use as a food additive and

its particle size distribution was evaluated over time using an electronic particle counter (Z3 multisizer Beckman Coulter Corporation).

Cold stored eggs (less than 3 weeks) were used to inoculate bottles for the stage development study following the method described by Drillet *et al* (2006a, see description later). Some of these eggs were used to determine a baseline HS (HS) of our cold stored eggs and incubated at 17ºC for 48 hours before being fixed with 1% acid Lugol's solution (hereafter Lugol) and stored for counting.

Five treatments were prepared in three replicates in acid washed 2L Nalgene ® bottles filled with seawater (30 g mL^{-1}, 2 experiments). One treatment contained *R. salina* at a concentration of 20,000 cells mL^{-1} (36.7pgC.cell-1; Kiørboe et al. 1985) the actual concentration added was adjusted daily by determining cell density with the particle counter. This concentration of *R. salina* has been shown to saturate *A. tonsa* grazing rate (Berggreen *et al.* 1988) (Control treatment). One treatment, a negative control to test the nutritive value of PSI, was a suspension of PSI alone with an adjusted carbon content to match the control treatment (5,961 $mg.l^{-1}$ PSI, 948 µg $C.l^{-1}$) (Treatment PSI). The other treatments contained *R. salina* 20,000 cells ml^{-1} with PSI at 2.5, 5, and 10 mg l^{-1} and are referred as P2.5, P5, P10 treatments. All bottles were sealed with plastic wrap and attached to a plankton wheel (0.3 rpm) in 17°C/24h light to prevent anoxia by ensuring algal photosynthesis. Oxygen content and temperature were measured daily with a LDO™ HQ20 Portable Dissolved Oxygen/pH meter and 30 or 50 ml subsamples were taken with a 10ml kip-automat, fixed in Lugol, and stored until counting. Approximately 4/5 of the remaining water was removed by reverse filtration siphoning using a 52 µm filter and new seawater with the initial concentrations of *R. salina*, PSI, or both were added to the bottles until full. Samples of the incubation suspension were retained for particle size distribution and concentration analysis with the particle counter. When during the ontogenetic development of the copepods, the algal concentration in the bottles was observed to be decreasing below the point where it would no longer be saturating to the copepods; the concentration of *R. salina* was increased up to 120,000 $cell.ml^{-1}$ in order to maintain saturating levels (Drillet *et al.* 2006a). The entire procedure was repeated daily for 14 days after which 15 fertilized adult females from each treatment were isolated for egg production experiments (see later).

In each samples, the counted numbers of the four major groups of copepod stages: egg, nauplius, copepodite, adult were compared as fractions of the entire number of individuals present. To avoid bias from the experimental copepods progeny, eggs were not counted after mature copepods appeared

in the samples (day 10) and nauplii were not counted from one day after that (Drillet *et al.* 2006a). The time at which 50% of the individuals in a given replicate had molted to the next group of major stages was determined using linear regression, incorporating the most dominant point of the outgoing group to the most dominant point of the incoming group. The day at which adults first appeared in the sample was determined by linear regression for individual replicates and averaged.

Fifteen fertilized adult females from each treatment were selected at day 14 of the stage development experiment and placed individually in acid washed 600ml Nalgene ® bottles filled with GFF seawater (30 g mL^{-1}) and their corresponding treatment (*R. salina* or *R.salina* ± PSI) and were incubated on the plankton wheel. The entire content of each bottle was filtered daily through two filters sequentially (180µm (female capture) and 60µm (eggs capture)) and the female was transferred to GFF seawater with the corresponding treatment. The eggs were cleaned and transferred to Petri dishes and counted, then allowed to hatch for 48h at 17ºC before being fixed with Lugol and counted again. This daily procedure continued for 4 days after which the female prosome length was measured under an Olympus binocular in order to calculate the specific egg production (SEP) which represent the proportion of carbon a female invest into egg production compared to her own weight. We assumed an egg carbon weight of 45.7pg based on (Kiørboe *et al.* 1985a). For the egg production data, we ignored the egg production results from the first day of incubation to take into account the adaptation time from the females. After the stage development experiment, females prosome length and width were also measured using a camera and Image J picture analysis program, and their bio-volume was calculated using the formula of an ellipsoid ($=4/3*314*(L/2)*(W2/2)^2$) in order to observe if there were size differences in body volume between females from the different treatments.

ANOVA or ANOVA on ranks (when non parametrical) with an all pairwise multiple comparison procedure (Holm-Sidak method/Dunn's Method) where used to test the different parameters studied using SigmaStat 3.5. The graphs were done using SigmaPlot 10 (Sigma©).

Results

The C:N ratio of PSI was found to be of 11.8 (%C of dry weight = 49.13; %N of dry weight = 4.18) with a dry weight/wet weight ratio of 0.346± 0.024. The size distribution of PSI particle is shown in figure 1 and was stable in salt water over 24 hours incubation in rotating bottles. The PSI was

composed of very small particles of <4µm while the phytoplankton cells were larger (~7µm in spherical equivalent diameter, See Figure 1).

Fatty acids	***R. salina*** **% DW**	**PSI** **% DW**
14:0	1.03	0.13
15:0	0.03	0.00
16:0	1.25	3.29
18:0	0.09	2.00
20:0	0.01	0.04
22:0	0.00	0.02
24:0	0.01	0.02
14:1	0.02	0.00
16:1 n-7	0.26	0.09
18:1 n-9c	0.33	4.02
18:1 n-9t	-	0.16
18:1 n-7	0.77	-
20:1 n-9	0.01	0.06
22:1 n-11	0.01	0.04
24:1	0.00	0.01
18:2 n-6c	1.16	1.45
18:2 n-6t	-	0.00
18:3 n-6	0.31	-
18:3 n-3	2.73	0.09
18:4 n-3	3.54	-
20:2 n-6	0.00	0.03
20:3 n-6	0.02	0.01
20:4 n-6 (ARA)	0.25	0.02
20:3 n-3	0.00	0.01
20:5 n-3 (EPA)	2.29	0.01
22:6 n-3 (DHA)	1.44	0.01
Total Fatty acids	16.17 % DW	11.65 % DW
C:N ratio	4.54	11.8
DW/cell (pg)	86.50	-
DW/WW	-	0.346

Table 1: Fatty acid (FA) composition in % DW of *R. salina* and PSI. The FA values are recalculated from FA proportions from (Drillet et al. 2006b), DW of *R. salina* was averaged from Brown et al. (1998) and Kiørboe et al. (1985a). C:N ratio of *R. salina* was averaged from Dutz et al. (2008), Berggreen et al. (1988), Kiørboe et al. (1985a) and quantities of FA/DW of *R. salina* are recalculated from Renaud et al. (1999), Norsker and Støttrup (1994), Brown et al. (1998).

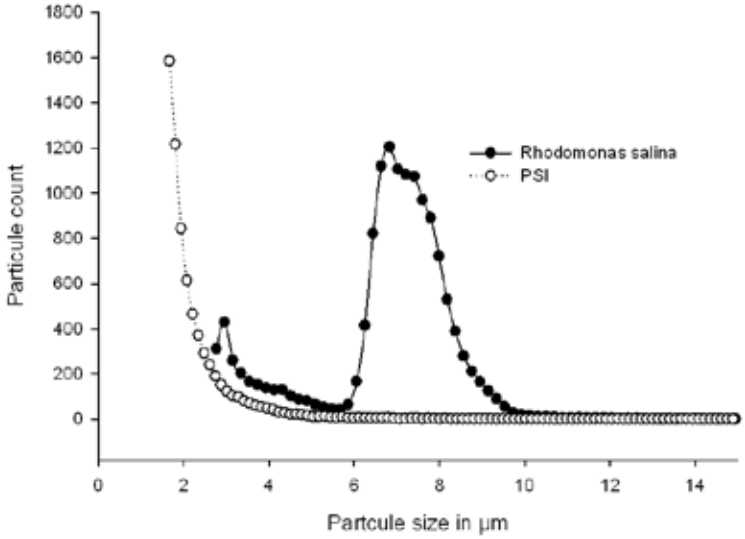

Figure 1: Size spectra of *R salina* (dilution from culture) and PSI in water after sonication (aperture size used were 100 and 70 µm, respectively). The cell size measures were made with a Z3 multisizer Beckman Coulter Corporation.

The HS of the batch of eggs used for the stage development experiments was always > 80%. The stage development experiment did not show any differences in development time in between treatment apart from that offering the PSI alone where hatched nauplii never developed into copepodites and therefore no female incubation was carried out for this treatment. The average time for egg hatching (days) was 0.68±0.11, 0.95±0.31, 0.90±0.21, 0.83±0.13 for Control, P2.5, P5, P10, respectively. The average time (days) to reach copepodite stage was 6.59±0.17, 6.42±0.11, 6.39±0.05, 6.31±0.08 for Control, P2.5, P5, P10, respectively. It took 9.9±0.12, 9.7±0.02, 9.72±0.09, 9.72±0.04 for Control, PSI alone, P2.5, P5, P10, respectively to reach the adult stage. The nauplii in the treatment PSI alone never developed to the copepodite stage and eventually died after a few days.

The three days female incubation revealed an increased egg production from the females who grew in P5 as compared to the Control and the P2.5 treatment females, there was also an increase in egg production from females who grew in P10 treatment compared to the ones raised in the Control

treatment (P<0.05; Figure 2). In the case of P5, the increase in egg production was >50%. The HS was also increased when using the PSI in treatment P5 as compared to the controls (+17%; P<0.05; Figure 2). The bio-volume of females was also increased in the P5 and P10 treatment as compared to the Control (P<0.05; table 2, +9.5% between P5 and Control). The SEP was calculated in two different ways (see Table 2). The SEP calculated from the recalculated bio-volume was lower than when using Berggreen (1988) Length/Weight regression and did not reveal any differences. However, when using Berggreen's regression (1988), an increase SEP was observed in P5 as compared to the Control (P<0.5, table 2).

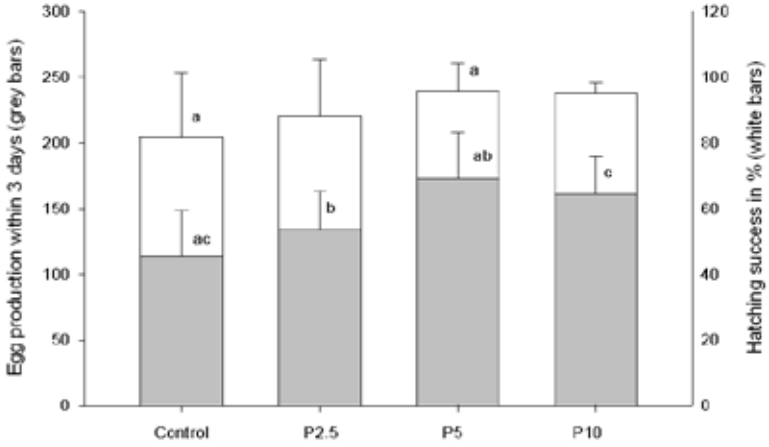

Figure 2: *A. tonsa* egg production per female over 3 days (day2-to Day4; grey bars± SD) and HS of produced eggs (white bars±SD). Treatments with a similar letter (*a,b,c*) are statistically different (P<0.05) from each others.

	Control	P2.5	P5	P10
Bio-volume (Ellipsoid) µm^3	3.25±0.44 10^7 *ac* (n=52)	3.28±0.38 10^7 *b* (n=59)	3.56±0.40 10^7 *ab* (n=86)	3.44±0.39 10^7 *c* (n=100)
Specific egg production (A)	0.45±0.13 (n=11)	0.47±0.1 (n=11)	0.54±0.11 (n=15)	0.47±0.1 (n=13)
Specific egg production (B)	0.60±0.18 *a* (n=11)	0.65±0.14 (n=11)	0.77±0.15 *a* (n=15)	0.72±0.15 (n=13)

Table 2: *A. tonsa* female's bio-volume from different feeding treatments in µm^3± SD (ellipsoid). Specific egg production was calculated in 2 ways: **A**=from the recalculated bio-volume of females (assuming 0.14×10^{-6} µg C µm^{-3}, Kiørboe et al. 1985b) and references therein, or **B**= using the Length-Weight regression line from the same continuous culture of *A. tonsa* fed similar food (Berggreen et al. 1988). Numbers with a similar letter (*a,b,c*) are statistically different (P<0.05) from each others.

Discussion

The particle size of PSI makes it much less available to copepods than *R. salina* due to negligible retention efficiency (Berggreen *et al.* 1988) and we can therefore consider that only a very small but apparently very active fraction of the PSI was ingested. We conducted a grazing experiment with females offered PSI alone to observe if there were any decrease in particle number (volume) but we could not identify any, confirming the results from Berggreen et al. (1988).

The C:N ration observed in PSI proved that this product was of poor biochemical value, there was only a very low proportion of proteins. Also, the fatty acid composition revealed that the PSI lacked PUFA's making it a nutritionally poor diet. The proportions of EPA, ARA and DHA were very small and we can therefore consider the PSI as a very poor food source lipid wise and therefore we can assume that PSI did not generate a trophic effect. These observations of the poor nutritional quality of PSI were also confirmed in the stage development experiment where PSI alone lead to a complete mortality of the nauplii in the bottles while other treatments in which *R. salina* was provided to the copepods showed a normal development. The biochemical composition of *R. salina* has been described many times and is known to be optimal for *A. tonsa* development. This is confirmed by stage development experiments (Berggreen *et al.* 1988, Drillet *et al.* 2008). The sex ratio of the copepods at the end of the incubation was unaffected by the addition of PSI (data not shown). The development time of the copepods was

not affected by the addition of PSI in the bottles neither, however the presence of PSI lead to an increase in body size of the females in the P5 treatment. This is an interesting result showing that the addition of products with probiotic effects could be beneficial for improving the biomass of copepods in cultures. In the present case, the bio-volume was increased by 9.5% which is pretty interesting production wise. Similarly, the egg production was increased in presence of PSI at 5 and 10 mgL^{-1} compared to the Control. This increase went up to 50% in the case of P5 treatment. This corresponds to a significant increase in the production of eggs and it makes it an essential zoo-technical observation which can be used when trying to cultivate copepods in large quantities for aquaculture purposes. The HS of the eggs produced was also increased when produced in the presence of PSI in the environment. This is an important observation for hatchery management when having in mind that the complexity behind HS in copepods is high (Dutz *et al.* 2008) and that there are no easy-recipes to increase HS in copepods at present. The results concerning the SEP are interesting and show the limitation of the extrapolation of data from the scientific literature. In the present study, the length-weight regression from Berggreen (1988) seems not appropriate in the present though it was performed on the same population of *A. tonsa*. The SEP calculated is very high (up to 77% compared to 44% in the reference) and we therefore consider that results from the bio-volume calculation are more likely to make sense in this particular study. The results from the present calculated SEP show that the investment in reproduction compared to the females size is not increased in presence of PSI. It is well documented that a copepods size is strongly correlated with environmental conditions; particularly: temperature, salinity, and the abundance and quality of food (Jones *et al.* 2002, Gaudy and Verriopoulos 2004, Hansen *et al.* 2010). In the present experiments salinity and temperature were identical among treatments but the food items available were different. Considering that the copepods are not likely to eat very small particles, no matter development stage always <10% retention efficiency compared to optimal prey size, and that the nutritional quality of PSI is low, we consider that the benefits from the PSI product are due to probiotic effects solely. As PSI contains only dead bacteria, the action could not be due to the colonization of the copepod gut by *Lactobacilli* as it was observed in shrimp (Ziaei-Nejad et al. 2006). There are however no clear explanations of the mechanisms behind the PSI but one hypothesis is that this product engender a better feed assimilation which can be relocated into growth and reproduction. Among the mechanism that could explain a better uptake of nutrients from the food resides in the activation of digestive enzyme activity as it was shown in *Artemia* by Frouël (2007).

These enzymes could facilitate diet assimilation and decomposition of refractory compounds (Hood *et al*. 1971) and convert all dissolved nutriments, normally unavailable because their size is not appropriate (Verschuere et al. 1999). Also, microbial preparations may supply vitamins (C, B12, retinol etc.), proteins, amino acids and other metabolites (Gapasin *et al*. 1998, Gorospe *et al*. 1996, Yu *et al*. 1988).

In this experiment, it appears that P5 was the optimal treatment for copepods. The doubling of PSI quantity in P10 did not increase its benefit and in some case P10 copepods did worst that the ones at P5. The differences between treatments could alter the feeding behavior of the copepods which pose a plausible scenario for explaining the results obtained. *A. tonsa* is an ambush feeding copepod that can switch to suspension feeding depending on size and availability of prey, with a high concentration of small algal particles favoring suspension feeding (Jonsson and Tiselius 1990). Exhibiting suspension feeding behavior increases the encounter of the copepods with the largest particles of PSI (4-5µm) which may, at the highest concentration increase the ingestion of low quality food and could explain the tendency of loss of effect at P10. Another explanation could be that the particles of PSI could be sticky and glue to the feeding appendages and this could increase the grooming behavior of copepods and decrease their effective feeding time in the water column. However more detailed study should be performed since the algal cultures also contain small particles visualized in the coulter counter spectra (Fig1) that are likely to be bacteria growing in the culture media. The utilization range of PSI and viable bacteria of this size fraction has not been studied.

To conclude, this is the first observation that commercial preparations with probiotic effects could improve the performance of copepods in cultures. Considering all the practical difficulties encountered in the mass cultivation of copepods, this is a novel observation which potentially opens new horizons for researchers innovating copepod cultures for use as live feeds.

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