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Egg hatching rate and fatty acid composition of *Acartia bilobata* (Calanoida, Copepoda) across cold storage durations

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Abstract

To investigate egg storage capacity of the copepod *Acartia bilobata* for aquaculture interest, we tested hatching success rate (HSR) of inclusive eggs (mixture of all egg types) after 4°C storage. The HSR peaked after 14 days storage when incubating at 28°C for 48 hr (85.8 ± 1.6%) and 72 hr (87.6 ± 0.9%), then gradually declined until 1 year (48 hr: 7 ± 0.6%; 72 hr: 19.4 ± 3.9%). Reallocation of fatty acid profile suggests that docosahexaenoic acid (DHA) is correlated with the HSR of *A. bilobata* eggs. Additionally, we investigated the HSR of diapausing eggs (unhatched eggs after 72 hr incubation of the inclusive eggs) after 4°C storage. Their HSR peaked after 14 days storage (48 hr:75.3 ± 3.5%; 72 hr:78.2 ± 2.1%), then gradually declined until 60 days (48 hr HSR:42.1 ± 2.3%; 72 hr HSR:53.0 ± 3.2%). Overall, we illustrated the hatchability of diapausing and quiescent eggs of *A. bilobata* after 4°C storage. The cold storage capacities were low (<60% HSR after 60 days), and it could be limited by the egg DHA content. Our findings provide implications for future studies aiming to improve cold storage techniques of tropical copepod eggs for aquaculture applications.

KEYWORDS
Acartia bilobata, diapausing egg, egg cold storage, fatty acid, quiescent egg

1 | INTRODUCTION

Copepods are the most dominant metazoans in plankton communities. As crucial trophic linkages, they transfer photosynthesized energy from primary producers to consumers in the marine food chain (Turner, 2004). Copepod nauplii are the major developmental stages that are preferentially consumed by fish larvae; therefore, they play important roles in both marine food chain (Beaugrand, Brander, Alistair Lindley, Souissi & Reid, 2003; Sampey, McKinnon, Meekan & McCormick, 2007) and marine larviculture industry (Drillet et al., 2011; Støttrup, 2000). Subitaneous eggs (ready-to-hatch eggs) and dormant eggs (delayed hatching eggs) are two possible resources for naupliar recruitment (Baumgartner & Tarrant, 2017; Glippa, Denis, Lesourd & Souissi, 2014). The majority of nauplii hatches from subitaneous eggs within a few hours or days and directly contributes to the increase in the present population. Some nauplii are derived from dormant eggs and contribute to subsequent population recruitment (Baumgartner & Tarrant, 2017). In fact, dormant eggs form an "egg bank" in the sediments of aquatic environments, and it is considered an ecological strategy for copepods to regulate their population dynamics across seasons (Baumgartner & Tarrant, 2017; Marcus, 1996; Uye, 1985).

Many calanoid copepods are able to produce dormant eggs (Dahms, 1995; Holm et al., 2018). Although the physiological mechanism of copepod egg dormancy is poorly established, two major groups (i.e., diapause and quiescence) have been classified based on their hatching characteristics (Holm et al., 2018; Uye, 1985).

Maternal copepods may generate diapausing eggs when they receive signals of deteriorating conditions such as adverse photoperiod and temperature, and this process may take some period of time to be completed (Baumgartner & Tarrant, 2017; Piercey & Maly, 2000). In particular, the diapausing eggs need a relatively longer time to recover from their refractory phase even when conditions become favourable (Grice, 1981). Quiescence occurs when subitaneous eggs are exposed to suddenly adverse conditions (e.g., low temperature). The quiescent eggs maintain a slowed metabolism in the embryo and are able to accelerate development rapidly once environmental conditions become favourable (Marcus, 1996). Moreover, Chen and Marcus (1997) reported hatching characteristic of the third type of egg dormancy; they called delayed hatching eggs, which were noted as an intermediate dormant type with a shorter refractory phase than the diapausing eggs.

For aquaculture, the characteristics of delayed hatching of the dormant eggs provide feasibility for egg storage. Therefore, the development of copepod egg storage methods is needed to enhance their availability across aquaculture facilities, and it allows the aquaculture producers to accumulate copepod eggs and hatch them at desired time point fitted to the feeding regime of fish larvae. The stored copepod eggs are expected to be easy to use in larviculture just like the common applications of Artemia cysts. As the developments of copepods are temperature dependent (Huntley & Lopez, 1992), the method of artificially cold-induced quiescent eggs has been well developed in a widely distributed calanoid species *Acartia tonsa* for aquaculture purpose (Drillet, Lindley, Michels, Wilcox & Marcus, 2007; Drillet et al., 2006; Holmstrup et al., 2006; Støttrup, Bell & Sargent, 1999). Drillet et al. (2006) reported viability in the eggs of *A. tonsa* (Baltic population) after months of cold storage and suggested that the artificially induced copepod quiescent eggs are promising live prey for fish larvae. Method of cold storage capacity improvement was also exploited with another *A. tonsa* culture originated from the Gulf of Mexico (Drillet et al., 2007). Fatty acid levels of the cold-stored *A. tonsa* quiescent eggs from both populations were evaluated. The changes of fatty acid profiles in the quiescent eggs seemed to be minor during the cold storage (Drillet et al., 2006; Sedlacek, 2008; Støttrup et al., 1999), which suggests their nutritional advantage as live prey for larviculture industry.

Copepod eggs storage techniques have been mostly established for temperate species with specific focus on their quiescent eggs (Drillet et al., 2006; Højgaard, Jepsen & Hansen, 2008; Holmstrup et al., 2006; Støttrup et al., 1999). However, scarce contribution is paid on tropical copepod species. Beyrend-Dur, Dur, Souissi and Hwang (2014) were the first to report that the eggs (unidentified type) of the tropical calanoid copepod, *Acartia bilobata*, collected from sediments of a brackish aquaculture pond in southern Taiwan remain viable after cold storage. This species has great aquaculture potential because of their high productivity (Pan et al., 2014), and the feasibility of a quiescent cold-induction protocol was preliminarily examined (Pan, Souissi, Hwang & Souissi, 2017). In order to better evaluate the potential of *A. bilobata* cold-stored eggs for aquaculture applications, we performed the present study for the following objectives: (a) to identify dormant egg types produced by *A. bilobata* and their patterns of hatchability after different cold storage durations, (b) to examine the fatty acid profiles of the cold-stored eggs after different storage durations.

2 | MATERIALS AND METHODS

2.1 | Copepod and microalgae stock culture

Copepod *A. bilobata* was isolated from a brackish aquaculture pond (salinity 15–20 mg/L) in Tungkang, southern Taiwan (22°28′57.78 N; 120°26′06.71 E) in January, 2014, placed in culture, and transferred to LOG-Marine Station of Wimereux, France, within 3 months of the commencement of the experiments. The copepods were cultivated in 20 L tanks with diluted seawater at salinity 20 mg/L (mixture of distilled water and 1-μm filtered ultraviolet-sterilized seawater). The cultures were maintained at a photoperiod of 12L:12D under a daylight fluorescent light and heated by a water heater (EHEIM thermocontrol 50 W; EHEIM GmbH, Germany) at approximately 28°C. Every 10 days, the copepods (all stages) were gently collected on a 70 μm mesh and transferred to a new culture medium.

A culture of the microalgae *Isochrysis galbana* (Prymnesiophyceae) was obtained from the Roscoff Culture Collection, France (strain no. RCC178). Algal cultures were grown in 2 L flasks containing Whatman GF/C-filtered and autoclaved natural seawater enriched with Walne's medium (Walne, 1970). The cultures were kept in the thermostatic incubator (MLR-351H; SANYO, Osaka, Japan) programmed at 18°C at a photoperiod of 12L: 12D cycle under a daylight fluorescent light. The algae used for copepod feeding was in exponential growth phase (3–4 days after inoculation) and introduced every 2 days in the copepod culture water at an approximate concentration of 10^5 cells/mL, an amount known to be suitable for *A. bilobata* (Pan et al., 2014).

2.2 | Hatching success rate of inclusive eggs after cold storage

Adult *A. bilobata* were collected from the stock cultures using a 120 μm mesh and transferred to fresh 20 L culture water (ca. 800 ind./L) to allow copepods to release their eggs for 24 hr. Copepod eggs were siphoned from the bottom, and the inclusive eggs (mixture of all egg types) were collected by sieving through 120 μm (to retain the copepods) and 70 μm (to collect eggs) meshes. The eggs were rinsed rapidly with distilled water to remove attached substrates and reduce microbes and transferred to a 1 L beaker with diluted seawater (20 mg/L). The eggs were then volumetrically counted on a customized counting plate under a stereomicroscope (SZX9; Olympus, Tokyo, Japan). Groups of approximately 15,000 eggs were placed in 15 mL brown-coloured tubes filled with diluted seawater (20 mg/L). The eggs were then held in a refrigerator at 4°C for seven durations (7, 14, 30, 60, 90, 120, and 365 days). After cold storage, the eggs were sorted from the tubes and counted under the

stereomicroscope. Groups of 100–150 eggs were incubated in 25 mL dishes filled with diluted seawater (20 mg/L). Two incubation periods (48 and 72 hr, $n = 4$ each) were tested for each treatment, and all dishes were incubated in a heated water bath at 28°C and at a photoperiod of 12L:12D cycle. At the end of the incubation, the number of unhatched eggs was counted and the hatching success rate (HSR) was calculated as follows:

$$\mathrm{HSR}(\%) = 100 \times \left(1 - \frac{\text{Number of unhatched eggs}}{\text{Total number of incubated eggs}}\right)$$

In addition, the HSR of the fresh inclusive eggs (not cold stored) was also measured following the aforementioned protocol. The HSRs after 48 and 72 hr of incubations are referred to be 48 hr HSR and 72 hr HSR hereafter.

2.3 | Fatty acid composition of inclusive eggs after cold storage

The inclusive eggs of *A. bilobata* were sampled volumetrically and collected using Whatman GF/C filters by the vacuum filtration (ca. 5,000 eggs/replicate, $n = 2$) after 0, 7, 14, 30, and 60 days cold storage. Filters were lyophilized and subsequently immersed with 5 mL of lipid extraction solvent (chloroform: methanol, 2:1 v:v) in 10 mL glass vials for 36 hr at −20°C. Each sample was then sonicated in an ice bath for 2 hr. The solvent was transferred to new vials and evaporated using a gentle nitrogen stream to a volume of approximately 1 mL, then the extraction was centrifuged in an Eppendorf tube at 10,000 *g* for 3 min. The upper suspension was transferred and then evaporated completely in new glass vials. Transesterification was carried out following the protocol reported by Pan, Sadovskaya, Hwang and Souissi (2018). Briefly, the lipid was transesterificated in methanol-toluene-acetyl chloride reagent for 2 hr at 95°C, and the fatty acid methyl esters were purified and resolved in hexane for gas chromatography–mass spectrometry (GC–MS) analysis.

2.4 | HSR of diapausing eggs after cold storage

After 72 hr incubation to the inclusive eggs in the first experiment, we found that the unhatched eggs stayed morphologically complete during 5 days of additional incubation. Therefore, we identified such eggs as diapausing eggs of *A. bilobata*, and an additional experiment was carried out to investigate the effects of cold storage on their HSR. Around 10^4 fresh inclusive eggs (mixture of egg types, not cold-stored) were incubated in two 2 L beakers; after 72 hr, unhatched eggs (diapausing eggs) were siphoned from the bottom of the beakers and held at 4°C for durations of 7, 14, 30, and 60 days, after which the 48 and 72 hr HSR ($n = 4$) was examined using the same protocol as described above.

2.5 | Data analysis

A one-way ANOVA was used to compare the mean values of the HSRs between different storage durations. Since significant differences were detected among treatments ($p < 0.05$); Tukey's multiple comparison test was used to analyse specific differences between pairs of treatments. All data were analysed using SPSS program, version 17.0 (SPSS, Chicago, IL, USA).

3 | RESULTS

3.1 | HSR of inclusive eggs after cold storage

Figure 1a shows the 48 and 72 hr HSR of the inclusive eggs of *A. bilobata* before and after storage durations. The 48 and 72 hr HSR (avg ± *SE*) of the fresh inclusive eggs (not cold-stored) were 67.7 ± 3.6% and 60.1 ± 0.9%. Interestingly, the HSR increased during the first 2 weeks of cold storage, and significantly highest HSR was found for the 14-day storage treatment (48 hr:85.8 ± 1.6%, 72 hr:87.6 ± 0.9%). After 21 days of cold storage, the HSR started to decrease gradually and the significantly lowest HSR was found after 365 days of cold storage (48 hr: 7.0 ± 0.6%, 72 hr: 19.4 ± 3.9%).

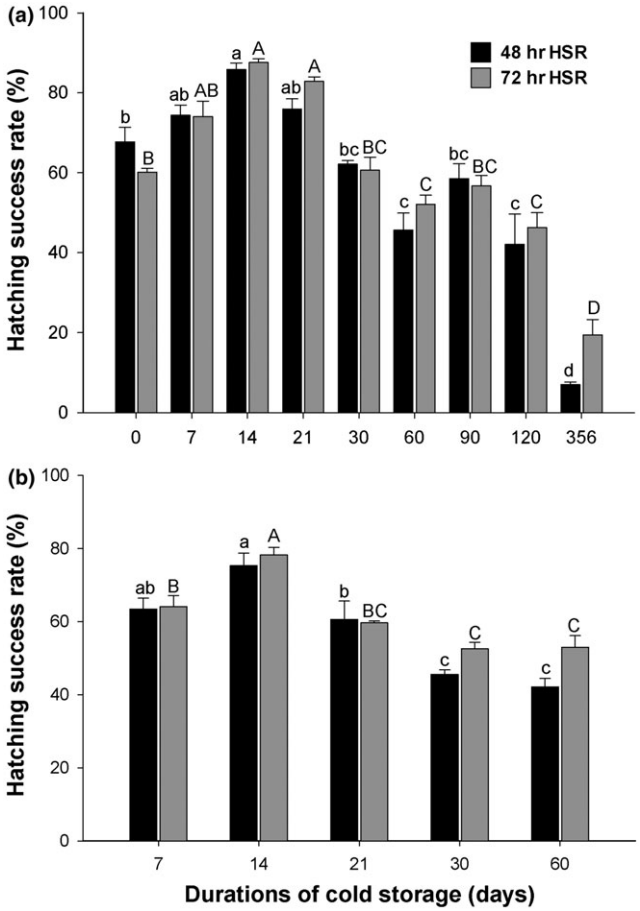

FIGURE 1 Hatching success rate (%) of different *Acartia bilobata* eggs across cold storage durations: (a) inclusive eggs (mixture of all egg types), (b) diapausing eggs. Data are presented as mean ± *SE*, and the different letters above each bar represent significant differences ($p < 0.05$).

3.2 | Fatty acid composition of inclusive eggs after cold storage

The fatty acid compositions (% total fatty acid) of *A. bilobata* inclusive eggs were analysed with duplicate for each treatment, and the results were relatively consistent between duplicate and are presented as average (Table 1). The fatty acid composition did not vary notably over different cold storage durations, where saturated fatty acids (C14:0, C15:0, C16:0, and C18:0) were dominant in the eggs in all treatments. However, the monounsaturated fatty acid (C16:1) and the polyunsaturated fatty acids (C18:2 n-6 and C22:6 n-3) were absent in the eggs of 14-day cold storage treatment in both analytical replicate. In addition, we found negative Pearson correlations between HSR and docosahexaenoic acid (DHA) (C22:6 n-3) composition. The correlation was marginally significant between DHA composition to 48 hr HSR and became significant to 72 hr HSR (48 hr: $r = -0.84$, $p = 0.07$; 72 hr: $r = -0.91$, $p < 0.05$, respectively, Figure 2).

3.3 | HSR of diapausing eggs and estimated HSR of quiescent eggs after cold storage

The 48 and 72 hr HSR of the diapausing eggs after different cold storage durations is illustrated in Figure 1b. Similar to the hatching pattern observed in the inclusive eggs (Figure 1a), the HSR peaked after 14-day cold storage (48 hr HSR:75.3 ± 3.5; 72 hr HSR: 78.2 ± 2.1%) and gradually declined during the cold storage duration up to 60 days. In order to estimate a net HSR of quiescent eggs after a range of cold storage durations, we subtracted the HSR of diapausing eggs (Figure 1b) from the HSR of the inclusive eggs (Figure 1a), and the result is illustrated in Figure 3.

TABLE 1 Fatty acid composition (% total fatty acid) in the inclusive eggs (mixture of all egg types) of *Acartia bilobata* over different cold storage durations. Data are presented as mean value of duplicate

Fatty acid	0 day (fresh)	7 days CS	14 days CS	30 days CS	60 days CS
C14:0	3.35	3.15	2.91	1.24	3.62
C15:0	1.86	1.23	1.54	0.54	1.55
C16:0	37.76	42.02	41.55	37.56	40.21
C18:0	36.45	37.58	48.47	43.73	38.69
Total SFA	79.42	83.98	94.48	83.06	84.07
C16:1	2.47	2.12	0.00	0.00	0.00
C18:1	9.60	9.09	5.52	10.98	10.49
Total MUFA	12.07	11.21	5.52	10.98	10.49
C18:2 n-6	3.29	1.11	0.00	1.44	0.00
C22:6 n-3 (DHA)	5.22	3.70	0.00	4.52	5.44
Total PUFA	8.50	4.81	0.00	5.96	5.44

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; CS, cold storage.

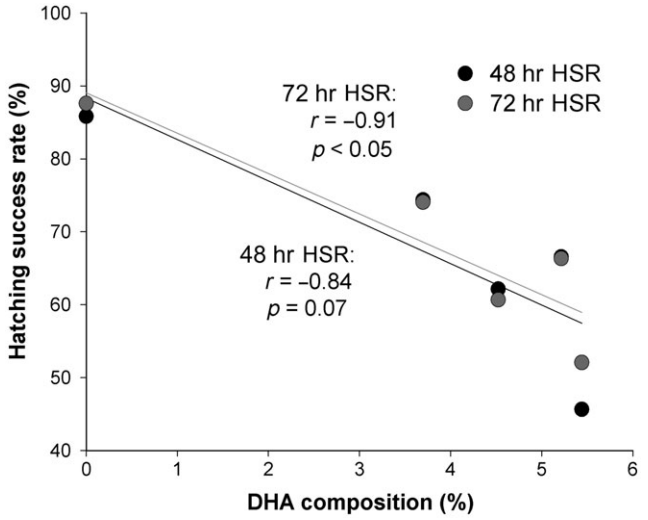

FIGURE 2 Pearson correlation between DHA composition (% of total fatty acid) and the HSR of cold-stored inclusive eggs of *Acartia bilobata*.

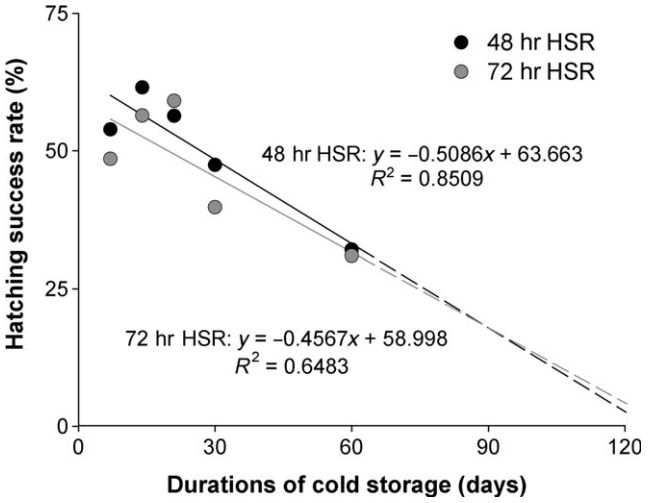

FIGURE 3 Estimated hatching success rate (%) of *Acartia bilobata* quiescent eggs across cold storage durations, and the fitting line is modelled as linear.

4 | DISCUSSION

In the present study, the HSR of *A. bilobata* fresh inclusive eggs (not cold stored) appeared to be low (60%–70%). However, we found a gradual increase in HSR during the first 14 days of cold storage which implies that the cold condition did trigger the hatching of certain *A. bilobata* eggs. Previous studies stated that copepod diapausing eggs need temperature stimulation to terminate their refractory phase, whereas the hatches of delayed hatching eggs do not require temperature stimulus (Chen & Marcus, 1997; Glippa, Souissi, Denis & Lesourd, 2011). Based on the temperature-dependent hatching characteristics, we consider that the copepod *A. bilobata* indeed produces diapausing eggs.

In our previous studies, we used a culture of *A. bilobata* obtained from the Tungkang Biotechnology Research Center of Taiwan, the

TABLE 2 Comparison of 48 hr HSR of the fresh and cold-stored inclusive eggs of *Acartia bilobata* eggs measured in the previous studies (>10 years of laboratory culture) and the present study (<3 months of laboratory culture). Data are present as mean ± *SE*. CS, cold storage

	48 hr HSR (%)					
	0 day (fresh)	7 days CS	14 days CS	21 days CS	30 days CS	References
Long-term laboratory culture	88.5 ± 1.9	89.4 ± 0.8	65.0 ± 3.1	53.7 ± 3.5	62.7 ± 5.4	Pan et al. (2014, 2017)
Newly established culture	67.7 ± 3.6	74.4 ± 2.6	85.8 ± 1.6	75.9 ± 2.6	62.1 ± 0.9	Present study

one was maintained under laboratory condition (as maintained in the present study) for over 10 years (Pan et al., 2014, 2017). Due to the unavailability of the old laboratory culture, a new copepod population was isolated from a brackish aquaculture pond near the area where the old culture was collected. The 48 hr HSR of the fresh inclusive eggs (not cold stored) of the new culture was much lower than the long-term laboratory culture (Table 2), which suggests the variable diapausing egg productions between populations. This phenomenon is identical with the recent report, where Holm et al. (2018) revealed that copepod dormant eggs production is highly variable among individuals and populations. In addition, a diversity of diapausing eggs production has been well demonstrated in different populations of widely distributed calanoid copepods *Eurytemora affinis* and *A. tonsa* (Ban, 1992; Drillet, Goetze, Jepsen, Højgaard & Hansen, 2008; Drillet, Jepsen, Højgaard, Jørgensen & Hansen, 2008; Glippa, Alekseev & Souissi, 2013), and it is considered as a function of genetic differentiation or micro-evolutionary divergence due to geographic isolation (Drillet, Jepsen, et al., 2008; Glippa et al., 2013). For aquaculture applications, the quiescent eggs are much feasible as resources of live prey for fish larvae, because the nauplii hatch faster from quiescent eggs than from diapausing eggs. A reduction in diapausing egg production, that allows higher harvest of subitaneous eggs and consequently facilitates higher quiescent eggs production, is a general preference for aquaculturist. However, the mechanisms to regulate copepod diapausing eggs production remain unknown, which should be addressed for better physiological understanding and further aquaculture application on copepod dormant eggs.

Temperature variation, as a signal of seasonal change in nature, could terminate the refractory phase of the copepod egg diapause and trigger resumption of embryonic development. Based on different ecological strategies, the diapausing eggs of various copepod species hatch after warming or chilling conditions (Ban, 1991; Marcus, 1980; Marcus & Lutz, 1998). However, the HSR of temperature-stimulated copepod diapausing eggs has scarcely been quantified over extended time periods. We found the variable HSR of diapausing eggs after different cold storage periods (Figure 1b), which suggests that the *A. bilobata* diapausing eggs may need various cold storage time to reactivate embryonic development. A hypothesis, which stated that there may be an endogenous bio-timer regulating the termination of copepod diapause, has been raised previously (Baumgartner & Tarrant, 2017). However, the regulatory mechanisms of this bio-timer in copepod diapausing eggs, if they exist, remain not established. Transcriptomic and proteomic studies are needed to verify the hypothesis of a copepod diapause biorhythm (Baumgartner & Tarrant, 2017; Ning, Wang, Li & Sun, 2013) and are expected to provide solutions to artificially modulate the hatchability of copepod diapausing eggs for aquaculture implications.

The 48 and 72 hr HSRs of quiescent eggs were estimated to be 53.9% and 48.5% after 7 days of cold storage respectively (Figure 3). This estimation suggests that around 80% of subitaneous eggs can switch into a quiescent state, yet the viability of quiescent eggs decreased as a function of cold storage time. This decrease is likely to be linked to metabolic maintenance, with energy reserves slowly consumed during cold-induced quiescence (Drillet et al., 2006; Nielsen, Mortensen, Vismann & Hansen, 2006). The transition rate from subitaneous to quiescent eggs in the newly established copepod culture appeared to be lower than the long-term culture (Table 2). It might be expected that the subitaneous–quiescent transition rate also varies genetically among populations (Drillet et al., 2007). Moreover, artificial selection to the individuals with high subitaneous–quiescent transition capacity may take place when the copepod culture experiences a transgenerational egg cold storage protocol during a long-term laboratory culture (Drillet, Goetze, et al. 2008; Drillet et al., 2007).

Changes in lipid levels normally occur during preparation for and maintenance of postembryonic diapause in calanoid copepod species (Campbell, Boutillier & Dower, 2004; Falk-Petersen, Mayzaud, Kattner & Sargent, 2009). However, the lipid of copepod dormant eggs is poorly studied except few laboratory studies have revealed very minor modifications of fatty acid composition in the cold-induced quiescent eggs of *A. tonsa* (Drillet et al., 2006; Sedlacek, 2008; Støttrup et al., 1999). Although we were not able to separate different egg types prior to fatty acid analysis, the data presented here could be mostly contributed by the quiescent eggs because of their dominance. Interestingly, we found obvious declines in C16:1, C18:2 n-6, and C22:6 n-3 in the eggs after 14-day cold storage (Table 1). The proportion of C16:1 and C18:2 n-6 remained low or undetectable in the subsequent cold storage periods, yet the proportion of C22:6 n-3 increased. The reallocation of fatty acid composition in copepod dormant eggs can be an indicator of metabolic activities for embryonic maintenance or development. Moreover, the fatty acid reallocation can be expected as a result of a complex lipid-derived hormone regulation during copepod dormancy (Irigoien, 2004).

Our analysis shows that the percentage of DHA over total fatty acid is negatively correlated with the HSR of the cold-stored eggs (Figure 2). This observation suggests that copepod eggs, like those of other marine invertebrates, use DHA reserves to support successful embryonic development (Rosa, Morais, Calado, Narciso & Nunes,

2003). The absence of C20:5 n3 (eicosapentaenoic acid, EPA), which is also considered crucial fatty acid supporting growth of zooplankton (Guerrero, Jimenez-Melero, Parra, Lopez de la Torre & Melguizo, 2007; Müller-Navarra, Brett, Liston & Goldman, 2000), was seen in both fresh and cold-stored eggs of *A. bilobata*. Dietary fatty acid ingested by female copepod could affect the fatty acid of their eggs (Rayner et al., 2015), and it could be correlated with copepod egg viability (Broglio, Jónasdóttir, Calbet, Jakobsen & Saiz, 2003). The EPA deficiency in the eggs could be the consequence of low EPA proportion in the microalgal diet *I. galbana* (Pan et al., 2018) fed by the maternal *A. bilobata*. The data collected here provide a preliminary exploration, yet the effects of dietary nutrients on the fatty acid compositions of the eggs and their cold storage capacities have to be investigated with molecular tools in the future.

In conclusion, we identified two types of dormant eggs (diapause and quiescence) in the tropical copepod *A. bilobata* based on their hatching characteristics. The production rate of diapausing eggs was high and egg cold storage capacity was low in the *A. bilobata* strain used in the present study, and these parameters are strain specific. Although we confirmed that the DHA content in the eggs is significantly correlated with their HSR, many essential PUFAs were absent in the eggs before and after cold storage. Our report provides a better understanding of biology and physiology of dormant eggs of the tropical calanoid *A. bilobata*. Further studies are still need to improve the HSR and fatty acid profile of the cold-stored *A. bilobata* eggs for their aquaculture applications.

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