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Copepod Embryonic Dormancy: "An Egg Is Not Just an Egg"

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Abstract. Long-lasting embryonic dormancy in invertebrates defies our understanding of what constitutes life because, for example, eggs of some copepods can delay hatching for decades or even centuries. Copepods, often millimeter-sized crustaceans, are some of the most numerous multicellular organisms on earth and are key organisms in most aquatic food webs. Some important free-living marine and estuarine species overwinter or oversummer by arrested embryogenesis in dormancy. The present contribution discusses the complex mechanisms behind embryonic dormancy by compiling knowledge from the 42 calanoid copepods from the superfamily Centropagoidea with well-described embryonic dormancy, which has been of scientific interest for decades. However, the determination of categories of copepod resting eggs—that is, diapause and quiescence, transitions between categories, the mechanisms controlling arrested development by the embryos, and how they interact with their surroundings—is not fully understood. Moreover, a clear link between the presence of the free-swimming population and their resting eggs in sediments is still not convincingly demonstrated. Here I evaluate the relative significance of potential cues driving the production of and the phase shift between egg categories. Understanding the initiation and termination of embryonic dormancy is of great importance for fundamental science—that is, population and food web ecology as well as climate science, aquaculture live feed, and ballast water research. Molecular techniques are developing rapidly, especially within health sciences, thus providing relevant tools applicable for plankton research. Here I suggest that applying molecular methods in addition to traditional physiological approaches in future research will lead to greater understanding of copepod embryonic dormancy, one of nature's wonders.

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Abbreviations: ARA, arachidonic acid; DAPI, 4′,6-diamidino-2-phenylindole; DHA, docosa hexaenoic acid; EPA, eicosa pentaenoic acid.

Introduction

The (epi)genetic and biochemical mechanisms regulating dormancy still puzzle scientists. Some organisms can survive in a state of "suspended animation" by having virtually no metabolism and by consisting only of a structure carrying the molecular information and energy for restarting metabolism. The production of a resting stage, such as a cyst or resting egg, is the most common form of dormancy in aquatic protists and invertebrates. This trait is shared among diverse groups, including dinoflagellates, ciliates, rotifers, nematodes, tardigrades, ostracods, and copepods (Barnes, 1974; Wall and Evitt, 1975; Coull and Grant, 1981; Marcus, 1996; Marcus and Boero, 1998; Clausen *et al.*, 2014). Here I concentrate on embryonic dormancy in calanoid copepods.

Embryonic dormancy is a state of suppressed or retarded embryonic development; in calanoid copepods this occurs through the production of resting eggs. It is not clear whether copepod resting eggs are produced in direct response to environmental changes, for example, photoperiod (Marcus, 1982; Chinnery and Williams, 2003), temperature (Avery, 2005b), epigenetics, or some genetic predisposition. The present contribution seeks to provide a resource that summarizes previous studies of copepod eggs and, in particular, the current knowledge on marine calanoid embryonic dormancy referred to for the first time about 50 years ago by Sazhina (1968) and Zillioux and Gonzalez (1972). It is not my purpose to review all existing literature on the subject but merely to refer to key studies describing the natural occurrence of copepod resting eggs spatially and temporally, the identification of resting eggs, and the physiology of embryos, while discussing the status of these disciplines. After almost half a century of resting egg research, I realize there is still a long way to go before fully understanding the copepod embryonic dormancy phenomenon. Therefore, in the end, I present my suggestions as to how I believe the research field should pursue this interesting "mystery" within biology in the future by implementing novel methods from other disciplines hand in hand with traditional approaches in zooplankton ecology and physiology.

Copepods Exhibit Flexible Life Cycles

Copepods are a group of crustaceans that dominate zooplankton, in terms of both abundance and biomass (*e.g.*, Williams *et al.*, 1994), making them one of the most numerous metazoans in aquatic communities (Walter and Boxshall, 2019). The majority of the approximately 12,000–13,000 species (Humes, 1994; Boxhall and Halsey, 2004) range in length from 0.1 to a few millimeters, but some species can achieve almost 3 cm. Despite their small size, they play a fundamental role in the transfer of matter and energy from microbial and phytoplankton production to higher trophic levels. Moreover, they contribute significantly to vertical flux of organic matter. Copepod biomass ultimately ends up in top predators, for example, birds (Kitaysky and Golubova, 2000), *via* crustaceans (Båmstedt and Karlson, 1998; Hutchings *et al.*, 2017) and, in particular, *via* fish and their larvae (Nielsen and Munk, 1998; Fox *et al.*, 1999; Möllmann *et al.*, 2004). Hence, trophic transfer of copepod biomass into fish populations makes free-living copepods vital for human nutrition. The free-living calanoid copepods typically reside in the pelagic, where they graze phytoplankton and other seston of appropriate sizes and convert the energy to somatic growth and eggs. They spawn their eggs freely in the water column or carry them in embryos or egg sac(s). The eggs hatch either directly from the egg sacs or from detached eggs after the egg sacs are shed.

Copepods can exhibit dormancy at any point in their life cycle, from embryos (embryonic dormancy) to adults (post-embryonic diapause) (Dahms, 1995; Mauchline, 1998; Baumgartner and Tarrant, 2017). What is unique for copepods, compared to, for example, insects, is that dormancy can take place during different developmental stages of the same species (Dahms, 1995); and the same individual can sometimes enter and exit dormancy several times within its lifespan (Falk-Petersen *et al.*, 2009). Dormancy generally reflects adaptations that synchronize reproduction, create a resilient life stage during adverse periods, or both (Dahms, 1995; Danks, 2002). It can play multiple roles and serve various purposes that can be induced by different cues (Dahms, 1995; Danks, 2002). The arrested embryogenesis can last from days to seasons or even many years, suggesting that there is an evolutionary plasticity in embryonic dormancy. Some dormancy traits are heritable (Wyngaard, 1988) together with cues that induce dormancy (Hairston and Dillon, 1990). Selection pressure on the timing of dormancy can lead to rapid evolution of these traits (Hairston and Walton, 1986), observed by phenological patterns in copepods (Katajisto, 2003).

Several studies have reported induction of diapause egg production as adverse environmental conditions are approaching (*e.g.*, Kiørboe and Nielsen, 1994; Guerrero and Rodríguez, 1998; Tachibana *et al.*, 2019). The free-swimming copepod biomass declines at a certain time of the year to a negligible level. Before the drop in biomass, it is suggested that females release diapause eggs that sink to the bottom, are buried in the anoxic sediment, and remain viable over a relatively long time. It is assumed that these resting eggs get re-suspended during the next spring or fall or the following year. They then hatch, inoculate the water column with nauplii, and ultimately build up a new pelagic population (Guerrero and Rodríguez, 1998). Resting eggs, as an analog to seed banks for flowering plants, can be used to cope with winter conditions (Engel and Hirche, 2004; Drillet *et al.*, 2006a). This enables free-living species to disappear from the plankton during the winter (*e.g.*, Uye and Fleminger, 1976; Norrbin, 1991; Kiørboe and Nielsen, 1994; Wesche *et al.*, 2007) and return in the spring (Marcus, 1984). However, resting eggs can also be used to cope with unfavorable summer conditions (Uye, 1985; Marcus, 1989; Tachibana *et al.*, 2019), along with other unfavorable environmental conditions, such as high predation pressure (Hairston, 1996).

The strategy of producing resting eggs is highly flexible, which is obvious through the attempts to categorize them. Besides subitaneous eggs that are ready to hatch (Fig. 1), Grice and Marcus (1981), Uye (1985), and Dahms (1995) suggested two other categories: quiescent and diapause eggs. Later, Chen and Marcus (1997) revised this to three dormant egg categories by including delayed-hatching eggs; and Alekseev *et al.* (2007) even suggested four categories in their book *Diapause in Aquatic Invertebrates: Theory and Human Use*: quiescence and diapause, which are further divided into superpause, mesopause, and oligopause eggs, categorized after the length of their refractory period. All of these categories are based on hatching patterns, that is, whether they have a refractory period and the duration of this period. However, as suggested by Holm (2017), a more appropriate way of viewing resting eggs is to distinguish between quiescent and

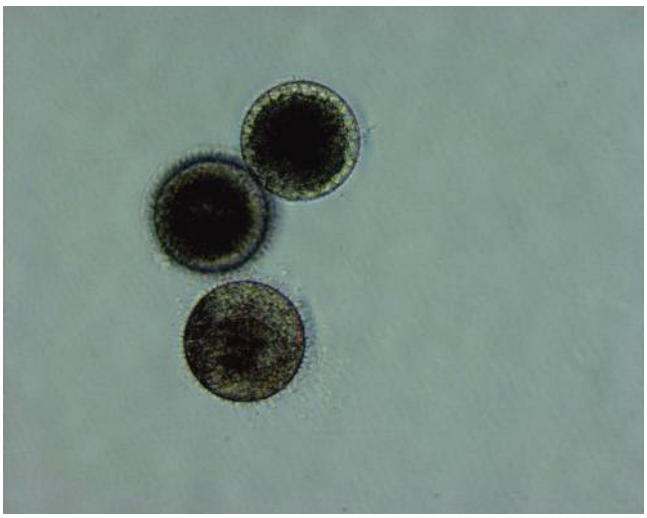

Figure 1. Subitaneous eggs (~80 μm in diameter) from the calanoid copepod *Acartia tonsa* Dana.

diapause eggs, where diapause eggs constitute a continuum of hatching patterns (*i.e.*, length of refractory periods). Following this approach, the category of diapause eggs includes egg types with a range of hatching patterns, and it encompasses delayed-hatching eggs. The length of the refractory period might depend not on the egg itself but on the conditions in the area where it is spawned (Marcus, 1996). Resting eggs are theoretically easily assigned into distinct categories. However, in field and laboratory studies, the information needed to practically distinguish them is often missing. Hence, they are often not categorized as either quiescent or diapause eggs. In the laboratory, resting eggs are determined by knowing the age of the eggs (from the time of spawning) before they hatch (Marcus, 1982; Chen and Marcus, 1997). Thus, depending on the design of a laboratory study and the way in which the results are reported, it may not always be possible to distinguish between quiescent and diapause resting eggs when referring to the experimental literature. Thus, in the present review, I use the broadest definition, which encompasses all categories of "resting eggs" (quiescent, delayed hatching, and diapause; see the next section for further definitions of their traits). Of course, this is not an optimal approach when discussing the spatial distribution of resting eggs in the context of seasonality, because different types of resting eggs may serve different purposes and may provide different benefits for a given population.

Embryonic Dormancy—Copepod Resting Eggs

Engel and Hirche (2004) reported at least 49 marine and estuarine species that produce resting eggs; however, a recent review by Holm *et al.* (2018) revised this to 42 species by excluding anecdotal observations and including only well-documented production of resting eggs. The embryonic diapause trait originated in the most ancient Calanoida during the Triassic or even Permian according to Alekseev and Starobogatov (1996). The copepods exhibiting embryonic dormancy belong to the families Acartiidae, Centropagidae, Pontellidae, Sulcanidae, Temoridae, and Tortanidae, all of which are grouped into the superfamily Centropagoidea (Barthélemy *et al.*, 1998), formerly called Diaptomoidea (Andronov, 1991). Lindley (1992) speculated that the resting egg strategy enabled these copepods to maintain populations in shallow marine and estuarine habitats more successfully than other calanoids and that it even pre-adapted them to colonize freshwater farther inland. However, recently, Belmonte (2018) proposed that resting eggs were not the reason for inland freshwater colonization. The production of diapause eggs by marine species preserves copepod genetic stocks in the sediment and provides the benefits of avoiding predation and crowding (Castellani and Lucas, 2003).

Diapause egg production is triggered by environmental factors, usually before they become detrimental, acting on the female copepod, such as temperature (Castro-Longoria and Williams, 1999; Avery, 2005b), or by token stimuli, such as photoperiod (Marcus, 1982; Chinnery and Williams, 2003); both signal the forthcoming period of adversity. Hatching is primarily controlled by a refractory period (Grice and Marcus, 1981; Uye, 1985; Marcus, 1996). During the refractory period, it is impossible to resume the arrested embryogenesis by any changes in external conditions (Baumgartner and Tarrant, 2017). However, it can be modified by environmental factors, such as temperature (Marcus, 1982). Embryonic diapause is genetically determined and, therefore, under maternal control, where females pass information to their embryos (Hairston and Walton, 1986; Wyngaard, 1988; Ban, 1992; Belmonte and Pati, 2007). Diapause egg production is characterized by an absence of morphogenesis; a greatly decreased rate of DNA, RNA, and protein synthesis; and a reduced or negligible metabolic rate (Grice and Marcus, 1981). In copepods, embryonic diapause is considered facultative (*i.e.*, individuals can either enter a diapause state or continue development) because it has a large component of flexibility (Baumgartner and Tarrant, 2017).

Quiescence is a state of embryonic arrested development (Danks, 1987), which is an immediate response by subitaneous eggs to environmental adversity, perceived by the individual embryo after the egg has left the female (Dahms, 1995; Marcus, 1996). Upon the return of environmentally favorable conditions, embryonic development is resumed (Marcus, 1996). Quiescent eggs can function on a seasonal scale because they can be viable for periods of up to a year at low temperatures (Drillet *et al.*, 2006a). Some of the factors that induce the quiescent state, such as oxygen concentration, can also be seasonal (Rasmussen and Jørgensen, 1992). Therefore, quiescent eggs will occur not only in environments with large, unpredictable variations but also in highly seasonal environments. For instance, Katajisto (2004) observed quiescent *Acartia bifilosa* eggs from the Baltic Sea surviving for 10 months at 4 °C in anoxic laboratory conditions. Moreover, her study showed that sediment-dwelling eggs incubated after 1.8 years of storage retained a 40% hatching success. Hence, Katajisto argued that particular copepod populations in the northern Baltic Sea do not produce diapause eggs because their quiescent eggs are able to overwinter.

A third egg category suggested is delayed-hatching eggs, which are characterized by having an extended period of hatching shorter than that of diapause eggs (Chen and Marcus, 1997; Drillet *et al.*, 2011b). Some researchers interpret delayed-hatching eggs as the embryo being in oligopause or oligodiapause (*sensu* Alekseev *et al.*, 2007). Delayed-hatching eggs, like diapause eggs, are suggested to be maternally preprogrammed (Drillet *et al.*, 2011b).

Most studies have focused on the conditions that induce the production of resting eggs, such as photoperiod, temperature, salinity, pH, oxygen concentration, and crowding (Johnson, 1980; Chinnery and Williams, 2003; Jo and Marcus, 2004; Holmstrup *et al.*, 2006). This might reflect that resting egg

studies have been conducted hitherto in a local context for individual species and not as a global trait.

Geographic Distribution of Copepod Resting Eggs

Copepod species producing resting eggs are distributed worldwide (Holm *et al.*, 2018). However, production of resting eggs has predominantly been observed in relatively shallow waters on the shelf, in coastal zones, and in estuaries, primarily in the northern hemisphere (*e.g.*, Dahms, 1995; Fig. 2A, B). Here, one cannot exclude a certain interpretation bias because not all locations and habitats of the world's oceans have been investigated for the presence of resting eggs.

In areas with large seasonal differences in living conditions, there is a higher risk that conditions at one point fall outside the physiological tolerance limits of a given copepod species. This will promote the production of resting eggs, either in anticipation of this period (diapause) or as a direct response to it (quiescence). Therefore, in the context of environmental characteristics shaping the distribution of the resting egg trait, actual values are not as interesting as the seasonal range in environmental parameters, which potentially shapes the global distribution and function of embryonic dormancy. Despite the multiple purposes of the production of resting eggs, they are produced in areas characterized by large seasonal differences in chlorophyll *a* concentrations (as a proxy for phytoplankton biomass) and temperature, as obtained from the Hadley Centre (http://www.metoffice.gov.uk/climatechange/science/hadleycentre) and GlobColour (2019; Fig. 2A, B). This strongly indicates that in planktonic marine copepods, the production of resting eggs is a strategy in response to seasonality. However, chlorophyll *a* concentration in the water column seems, according to Holm *et al.* (2018), not to explain the presence of resting eggs in the sediments

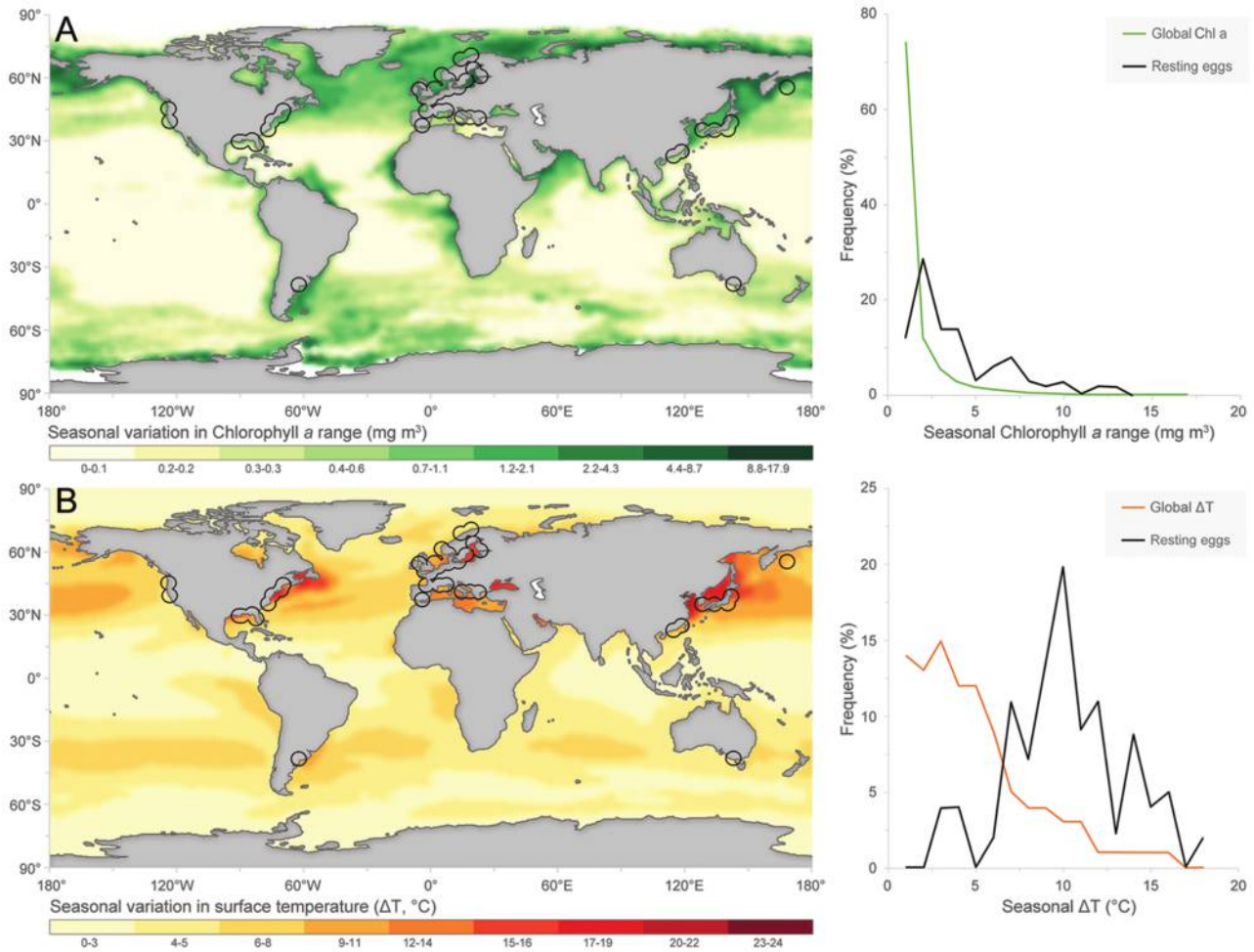

Figure 2. Global distribution of resting eggs in relation to the seasonal variation of (A) chlorophyll *a* (Chl *a*) range and (B) seasonal range in surface sea temperature (ΔT °C) in the areas where resting eggs are found. The variations are based on differences between maximum and minimum monthly mean values from the Hadley Centre (http://www.metoffice.gov.uk/climatechange/science/hadleycentre) and GlobColour (2019), respectively. The right panels depict frequency distribution of the seasonal variation in chlorophyll *a* and surface sea temperature for the areas where copepod resting eggs have been reported. Figure modified from Holm *et al.* (2018).

but merely reflects the likelihood of resting eggs being found in areas with the most common range in chlorophyll *a* concentration (Fig. 2A). Holm *et al.* even suggest that the frequency distribution of resting eggs in relation to chlorophyll *a* concentration simply reflects that areas with a low range in phytoplankton production are more common; and, thus, that chances of resting eggs being found there are higher. Furthermore, they report that resting eggs in particular were more frequent in areas with a strong seasonal range in surface water temperature ($\Delta T = 11.0 \pm 3.3$ °C; Fig. 2B). Based on how common areas with this range in surface temperature are on a global scale (<5%), it suggests that the seasonal range in temperature must be a key factor in the occurrence of resting eggs in marine copepods. Moreover, for many of the studied copepod species, resting egg production takes place near the boundaries of their thermal distribution (both upper and lower limits) (Holm, 2017; Holm *et al.*, 2018).

Many species of marine copepods have wide geographical distributions, covering a large range of environmental conditions (Grice and Marcus, 1981). Hence, they are quite opportunistic. It is relatively common that spatially separated populations of copepods differ in their use of resting eggs and their ability to produce them (*e.g.*, Marcus, 1984; Uye, 1985; Avery, 2005b; Drillet *et al.*, 2011b). For example, *Acartia clausi* in Japan has both an all-year northern pelagic population and a southern population that produces resting eggs. During the summer, when water temperatures exceed the species' thermal tolerance, it disappears from the plankton (Uye, 1985). These regional differences in the ability to produce resting eggs have been ascribed to differences in genetic capacity (Marcus, 1984), but it remains unclear whether this is due to phenotypic plasticity or (epi)genetic adaptation. However, *A. clausi* in Japanese waters is assigned to two different species: *Acartia omorii* and *Acartia hudsonica* (Bradford, 1976; Ueda, 1986). The two species co-occur in Japanese inlet waters (Ueda, 1986), where the previous data for *A. clausi* are considered to include the two species. Therefore, regional differences of *A. clausi* in Japan in the production of resting eggs would seem to be a confusion of two species.

Geographically separated populations of *Acartia tonsa* also differ in their ability to produce diapause eggs, because populations from the Atlantic Coast of the United States do not produce diapause eggs under the same laboratory conditions used to induce production in a Baltic Sea population of *A. tonsa* (Drillet *et al.*, 2008a, b).

Holm (2017) summarized the life cycle of *Labidocera aestiva*, with core distribution along the Atlantic Coast of North America, from Newfoundland in the north to the Gulf of Mexico in the south. At Fort Pierce Inlet, Florida, *L. aestiva* disappears completely from the plankton during winter. There it produces subitaneous eggs during the summer and in the fall, after which it switches to producing diapause eggs (Marcus, 1984). These diapause eggs have synchronized hatching if exposed to a period of chilling (Marcus, 1979); thus, *L. aestiva* exemplifies a species that relies on diapause eggs to persist in the system by creating resilient individuals and synchronizing hatching. In contrast, at Beaufort Inlet, North Carolina, which is at the optimum of the species' thermal distribution, *L. aestiva* is present year-round, but with seasonal fluctuations in abundance (references in Marcus, 1984). This, combined with low and inconsistent diapause egg production, suggests that it does not strongly depend on the production of diapause eggs to persist at this location on an annual basis, despite the conditions not being favorable all year. Because temperature rarely becomes so low that it synchronizes hatching of *L. aestiva* diapause eggs, the eggs can instead function as a bet-hedging strategy at this site. This would ensure long-term persistence in the area, even after catastrophic events that would eradicate the planktonic fraction of the population. Farther south at Fort Pierce Inlet (between the city of Fort Pierce and the barrier islands off of the Florida coast), Turkey Point in southern Florida, and the Louisiana coastal waters, diapause eggs were not produced under the same environmental conditions that induced diapause production farther north (Marcus, 1984). The case of *L. aestiva* thus exemplifies the continuous nature of diapause egg production, where the seasonal strength of the environment seems to determine the dependency of resting eggs on population dynamics when large fluctuations in temperature in a given location take place. In contrast, at intermediate seasonal conditions, with small variation in temperature, diapause eggs function as a bet-hedging strategy. However, in Louisiana's coastal waters, quiescent eggs are found in the sediment, despite the seasonal range in temperature: 10–15 °C compared to 15–20 °C in Delaware Bay (Delaware/New Jersey) and Vineyard Sound (Massachusetts). This might be explained by the winter temperatures in the two areas, because the minimum temperatures experienced at Delaware Bay and Vineyard Sound are <7 °C, whereas the temperature is never <14 °C in Louisiana's coastal waters. Hence, when temperature allows for synchronization of diapause eggs, by inducing quiescence of diapause eggs after having finished their refractory period, they can adapt to seasonal variability. On the other hand, if produced in areas where seasonality is less pronounced but where conditions periodically can become detrimental, albeit unpredictable, diapause eggs can function as a non-seasonal bet-hedging strategy. This would require that their production constitute a constant fraction of egg production and that their hatching be continuous, which is, at present, unknown. Being a flexible trait, there are many variables that can affect the expression of the embryonic dormancy trait and its intensity. Therefore, whether diapause eggs can be used to cope with seasonality depends on the seasonal strength of the predominant cues in the system; but, potentially, it also depends on annual temperature amplitude, that is, seasonal maximum or minimum temperatures (strength of the cue), as depicted in Figure 2B.

Interestingly, there can be large differences in the fraction of eggs that constitute resting eggs produced by the individual

copepod female (Marcus, 1984; Avery, 2005b; Drillet *et al.*, 2011b, 2015). This seems to reflect the unpredictability in the timing of the seasonal phenomena (Avery, 2005b) but may also result from different production strategies. If the fraction of produced diapause eggs remains constant, and if they have a heritable refractory period, which ensures extended hatching, then the production of diapause eggs can serve as a bet-hedging strategy (De Stasio, 2004) that allows the species to cope with temporary unpredictable periods of adversity. However, Avery (2005a) stated that if individuals can modulate their relative investments in subitaneous and dormant eggs, they would realize fitness better than if egg production were expressed by fixed genotypes—those committed to one type of egg or one ratio of egg types. One can also phrase it as "better not to lay one's eggs in just one basket." Certain flexibility in reproduction strategy must provide a higher fitness. The copepod reproductive priorities obviously have implications for understanding the importance and ecological relevance of resting eggs in copepod population dynamics in general.

One can identify some trade-offs in copepod resting egg strategies. An unknown fraction of the resting eggs inevitably ends up being buried deep in the sediment; presumably, these eggs never get the chance to hatch before dying from exposure to anoxia and high H_2S levels (Viitasalo, 1992). Because resting eggs maintain some level of metabolism (*e.g.*, diapause eggs, Romano *et al.*, 1996a, b; delayed-hatching eggs, Hansen and Drillet, 2013; quiescent eggs, Nielsen *et al.*, 2006), they gradually deplete their storage compounds when residing in the sediment; and embryos eventually succumb as a result of energy depletion (Holmstrup *et al.*, 2006). Interactions with the benthos can occur through egg predation and redistribution of eggs by bioturbation in the sediments (Marcus and Schmidt-Gengenbach, 1986; Conway *et al.*, 1994; Albertsson and Leonardsson, 2000). However, some of the predated resting eggs in fact survive gut passage of invertebrate and fish predators (Marcus, 1984; Flinkman *et al.*, 1994).

Distinguishing Characteristics of Resting Egg Categories

When trying to distinguish between different egg categories, it seems natural to start by describing the eggs by their visual appearance. Eggs are extracted from sediments either by sugar flotation or by Ludox methodology (De Jonge and Bouwman, 1977; Onbé, 1978; Chen and Marcus, 1997), or they are provided by laboratory organisms. The egg description is initiated by scrutinizing their geometric forms, sizes, and colors and, thereafter, their external characteristics by eggshell (chorion) morphology. For copepods, there is a vast literature about species-specific and category-specific egg sizes and surface morphologies, and it would be out of scope for the present contribution to review all available articles. Hence, I present a brief overview by describing representative microscopy techniques, several of which are destructive, requiring fixation and advanced sample preparation.

Different egg morphologies have been described for the two categories of eggs: subitaneous and diapause eggs (Sazhina, 1968; Grice and Lawson, 1976; Belmonte and Puce, 1994; Chen and Marcus, 1997; Castro-Longoria, 2001; Onoue *et al.*, 2004; Belmonte and Pati, 2007; Berasategui *et al.*, 2012; several of these authors report distinct differences in surface structures, *i.e.*, spine appearance and, in particular, spine lengths). Belmonte *et al.* (1997), among others (*e.g.*, Diodato *et al.*, 2006), suggested an operational concept using copepod egg-surface morphology to identify subitaneous or diapause eggs. These surfaces are characterized by their respective smooth or spined appearances on the egg chorion (Belmonte, 1992; Belmonte and Puce, 1994; Chen and Marcus, 1997; Castro-Longoria and Williams, 1999; Castro-Longoria, 2001; Castellani and Lucas, 2003). However, further testing (Hansen *et al.*, 2010b) revealed that eggs from the very same copepod female of either *Acartia* sp. (Fig. 1) or *Centropages hamatus* practically hatched simultaneously and successfully, irrespective of whether their egg surface chorion morphology was smooth or equipped with various lengths of spines. Moreover, with *Acartia tonsa*, different egg morphotypes that were quite similar to the ones described in Hansen *et al.* (2010b) were detected; and all of these exhibited the same delayed hatching behavior according to season (Berasategui *et al.*, 2016). Therefore, it is fair to conclude that categorizing eggs based on their surface morphology is not a simple matter. Hence, this trait cannot readily be used as a general identifier for all species (*e.g.*, Kasahara *et al.*, 1974; Ianora and Santella, 1991; Onoue *et al.*, 2004; Diodato *et al.*, 2006; Hansen *et al.*, 2010b; Peck *et al.*, 2015). The purpose of chorion surface spines is suggested to favor flotation, passive transport, and sensory activity and to decrease predation and burial of the resting eggs (*e.g.*, Belmonte *et al.*, 1997).

A detailed description of the egg chorion structure has also been conducted with the purpose of differentiating between egg categories. The histologic literature often describes several layered embryonic envelopes situated under the chitin chorion. In brief, Blades-Eckelbarger and Marcus (1992) describe the ontogenetic formation of cortical vesicles and the egg envelope formation, leading to a final formation of long spiny projections in *Centropages velificatus* eggs. Toda and Hirose (1991) even propose that the multilayer membrane consists of seven or eight layers. Additionally, Hubble and Kirby (2007) described a single-layer chorion compared with a thick three-layered chorion in subitaneous or diapause eggs of *A. tonsa*. Dharani and Altaff (2004) describe a three-layered outer chorion for the subitaneous eggs and a highly complex, thick, and four-layered outer chorion for the diapause eggs of the freshwater species *Sinidiaptomus* (*Rhinediaptomus*) *indicus*. Interestingly, Couch *et al.* (2001) stated that the chorion structure stays constant with the age of the diapause eggs of *Boeckella triariculata*, but it is possible that the thickness of the layers may change with increasing time spent in the diapause state. The overall impression is that egg chorions are

complex structures and that diapause eggs are equipped with much thicker protective multilayer chorions than subitaneous eggs are (Castellani and Lucas, 2003). The thicker chorion is proposed to be necessary for surviving longtime storage in egg banks within sediment environments with hypoxia and extensive H_2S exposure (Nielsen *et al.*, 2006). Although the distinction between the different categories of eggs is conceptual, well-defined morphological and histological information obtained in both field and laboratory studies is often insufficient to precisely categorize them.

Entering the egg and observing the embryo are next steps in understanding the complexity of eggs. The study of embryology has been practiced with light transmission microscopy for *Paracalanus crassirostis*, where drawings are reported from newly laid eggs throughout all embryological phases until nauplii release (Yang, 1977). Later, confocal microscopy revealed several embryonic stages in *Calanus pacificus*, *Calanus marshallae*, *Calanus finmarchicus*, *Metridia pacifica*, and *Centropages abdominalis*. This was made possible by applying DAPI (4′,6-diamidino-2-phenylindole) and PicoGreen staining by osmotically stretching the chorions to enable the dye to penetrate all membranes to dye cell nuclei and external chitinous chorions. The technique visualizes the structures by using a long pass filter (Zirbel *et al.*, 2007). The same DAPI technique combined with epifluorescence microscopy was applied to both subitaneous and quiescent eggs of *A. tonsa*, giving detailed visual insight into the entire embryogenesis process. This revealed that embryos that were entering quiescence before the gastrulation stage would stay in gastrulation during the rest of quiescence, and that they exhibited a slower pace of hatching compared to subitaneous embryos. In contrast, embryos entering quiescence after gastrulation would stay in later embryonic stages during quiescence, and they showed a rapid pace of hatching after quiescence termination (Nilsson and Hansen, 2018).

Another egg category discrimination method could be the differences in the electrical properties of egg cell membranes, as proposed by several authors previously (*e.g.*, Skierczynska *et al.*, 1972; Jaffe and Robinson, 1978; Hagiwara and Jaffe, 1979). Based on microelectrode measurements, these authors discuss properties of resting potential, specific resistance, action potential, and potassium ion permeability of the eggshell membrane. Hagiwara and Jaffe (1979) even discovered that some of these variables actually change during embryogenesis. It is possible that microelectrode measurements could be used to determine differences between subitaneous and resting eggs.

All of the above-mentioned techniques are invasive and destructive. Hindering the study of many species of copepod eggs in the first place is the inability to identify the different categories by using non-invasive methods, such as surface morphological characters. A non-destructive technique for distinction could be developed by testing density differences between egg categories, based on the assumption that diapause eggs have a thicker and denser chorion than subitaneous or quiescent eggs (Marcus, 1984). Uye (1980) already suggested this four decades ago. Typically, settling chambers and/or density centrifugation are used; and, based on Stoke's equation, the falling velocities are converted to densities. Miller and Marcus (1994) determined mean egg densities for *A. tonsa* subitaneous eggs spawned exactly at salinity 15 and 20 °C to be in the range of 1.083 to 1.088 g cm^{-3}, invariant of the development stage of the embryos. Eggs from *Labidocera aestiva* are non-classifiable by microscopic analysis because the chorion surface structures of subitaneous and diapause eggs look similar. Hitherto, the only way to categorize these eggs has been by hatching time. The authors reported egg densities for subitaneous *versus* diapause eggs of 1.081–1.133 g cm^{-3} *versus* 1.010–1.181 g cm^{-3}, revealing an overlap in densities. The authors phrased it as "indirect" evidence that they were different. This difference was ascribed to the chorion ultra-structures but possibly also biochemical differences with respect to, for example, lipid content (Marcus and Fuller, 1986). Wang *et al.* (2005) reported densities for positively identified subitaneous and diapause eggs of *Centropages tenuiremis*. They found mean densities of 1.1176 ± 0.0057 and 1.1481 ± 0.0101 g cm^{-3}, respectively. In conclusion, density differences could be a promising parameter in non-invasive discrimination of egg categories. However, it appears not to be a standalone method because there are frequent overlaps in densities between subitaneous and diapause eggs. One could hypothesize that differences in light interference properties on chorion surface material, such as grooves and spines, could differentiate between egg types, but that remains to be investigated.

More than 20 years ago, Marcus (1996) advocated for a method that would enable the rapid classification of eggs as subitaneous or diapause. She proposed that the effort should include biochemical, physiological, and genetic analysis. I will take up the challenge here and suggest that the way forward is to obtain a reliable method of discriminating between egg categories by combining a suite of established methods and novel non-destructive sorting techniques. Non-destructive techniques obviously are preferable because they would enable further processing of live eggs. I imagine measurements of density, metabolic rate, and, possibly, light interference to differentiate between egg categories. When a package of solid and non-destructive methods is developed, it might be the intention to implement automated (machine learning) techniques for categorization of larger samples of eggs. Additionally, more destructive omics methods such as metabolomics, phosphoproteomics, and transcriptomics, in concert with the above non-destructive methods, need to be applied to differentiate between the egg categories on a molecular level (see *Suggested Pathway to Reach a Mechanistic Understanding of Copepod Embryonic Dormancy*). Together, all of these efforts could unravel what we, at present, cannot discover with our own eyes.

Common Approaches to Study Age and Viability of Copepod Resting Eggs

Most studies on copepod resting eggs are conducted by extracting sediment (from cores) sampled in the field and subsequently incubating it at different environmental conditions that trigger hatching of the sedimented eggs (*e.g.*, Marcus, 1984; Engel and Hirche, 2004; Masero and Villate, 2004, Dahms *et al.*, 2006; Sichlau *et al.*, 2011; Beyrend-Dur *et al.*, 2014). In field studies, age determination of eggs is often done by isotopic analysis of ^{210}Pb and/or ^{137}Cs for estimating the age of the sediment in which the eggs reside (Dahms *et al.*, 2006; Jiang *et al.*, 2006; Katajisto, 2006; Sichlau *et al.*, 2011). This technique is adapted for eggs with an age of years to decades. Determining eggs of shorter ages, such as months-old eggs, could be done by incubating the sediment. Here, eggs that hatch after an extended period are categorized as resting eggs (Grice and Marcus, 1981; Guerrero and Rodriguez, 1998; Glippa *et al.*, 2011). Unfortunately, it is a destructive approach, wherein a given egg can be categorized and determined by species only after it has hatched into a nauplius. Sometimes, nauplii are so difficult to identify that they need to grow and become copepodites before secure species identification is possible.

The abundance of resting eggs in the sediment can be significant, ranging from 10^4 to 10^7 per m^2 (Marcus, 1989; Næss, 1991; Hairston, 1996; Jiang *et al.*, 2004; Dahms *et al.*, 2006; Glippa *et al.*, 2011, 2014; Sichlau *et al.*, 2011). Therefore, this resting egg pool potentially represents an important link between the pelagic and benthic environments (*sensu* Elgmork, 1967; Uye, 1985; Boero *et al.*, 1996; Marcus and Boero, 1998). Generally, pore water of muddy sediments is anoxic below 5 mm from the sediment interface (*e.g.*, Revsbech *et al.*, 1980), and H_2S prevails (Fig. 3). Bioturbation significantly influences the emergence of zooplankton from resting eggs, with effects varying among species; and it may thus affect the dynamics of pelagic populations (Viitasaalo *et al.*, 2007). In undisturbed sediments, with a steady sedimentation of material from the water column and practically without bioturbation or other stochastic events stirring up the sediment, the abundance of viable resting eggs declines exponentially with sediment depth (*i.e.*, age). Such habitats are either more or less defaunated as a result of frequent oxygen depletion (Sichlau *et al.*, 2011), or the sediments are heavily polluted (Dahms *et al.*, 2006). Moreover, only a small fraction of viable resting eggs is found in >20–30-cm-deep sediments (Katajisto *et al.*, 1998; Masero and Villate, 2004). Based on the vertical decline profile, a mortality rate of diapause eggs can be calculated, assuming there is a relatively constant inflow of eggs from the pelagic population (Fig. 4). Using this protocol, this mortality rate was reported to be 0.135 per year (Dahms *et al.*, 2006), 0.1408 per year (Jiang *et al.*, 2004), and 0.35–0.53 per year (Sichlau *et al.*, 2011). Despite a significant mortality rate, there are still diapause eggs left even in the deepest parts of the sampled sediments.

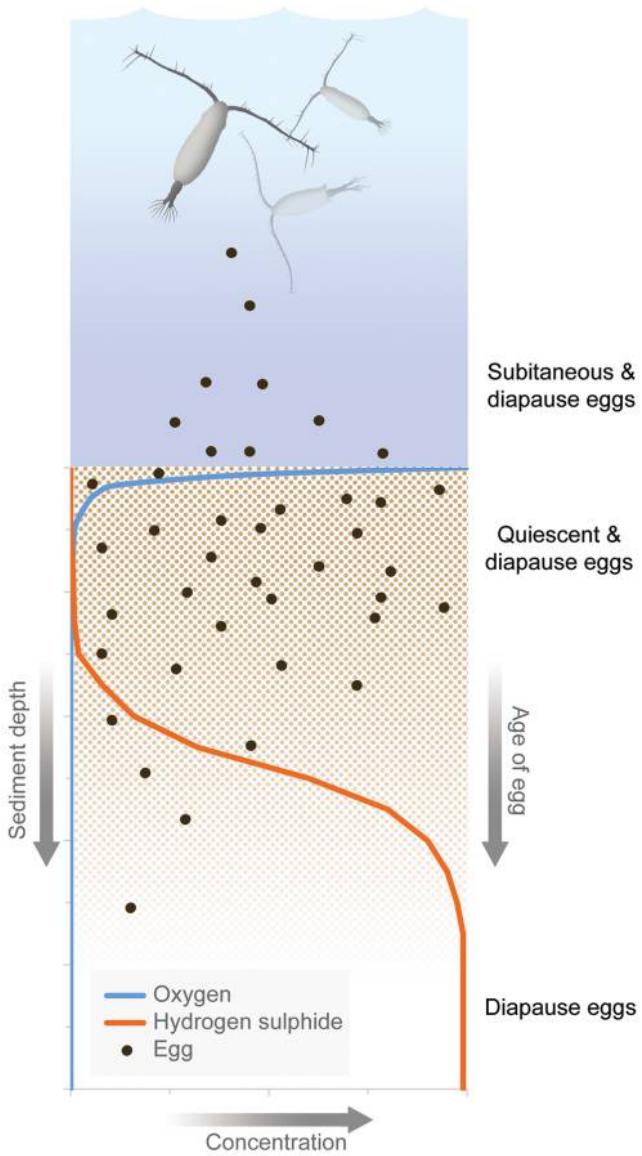

Figure 3. Depth diagram of a sediment profile illustrating the production and sedimentation of eggs from the pelagic and the distribution of copepod eggs from surface sediment (recently spawned eggs composed of subitaneous and quiescent eggs) to deeper as well as anoxic sulphidic sediment (old diapause eggs).

The age of viable resting eggs from the upper 5–10 cm of the sediment is estimated to be 15–19 years (Madhupratap *et al.*, 1996). In deeper marine sediments (20–30 cm), their age has been estimated to be up to 70 years (Marcus *et al.*, 1994; Madhupratap *et al.*, 1996; Dahms *et al.*, 2006; Sichlau *et al.*, 2011); and from freshwater sediments, resting eggs have been dated to be up to 332 years old (Hairston *et al.*, 1995; Jiang *et al.*, 2012). Multiannual storages of reproductive output can reduce variability in recruitment between years with low reproductive success (Baumgartner and Tarrant, 2017) because resting eggs can survive in the sediment for periods

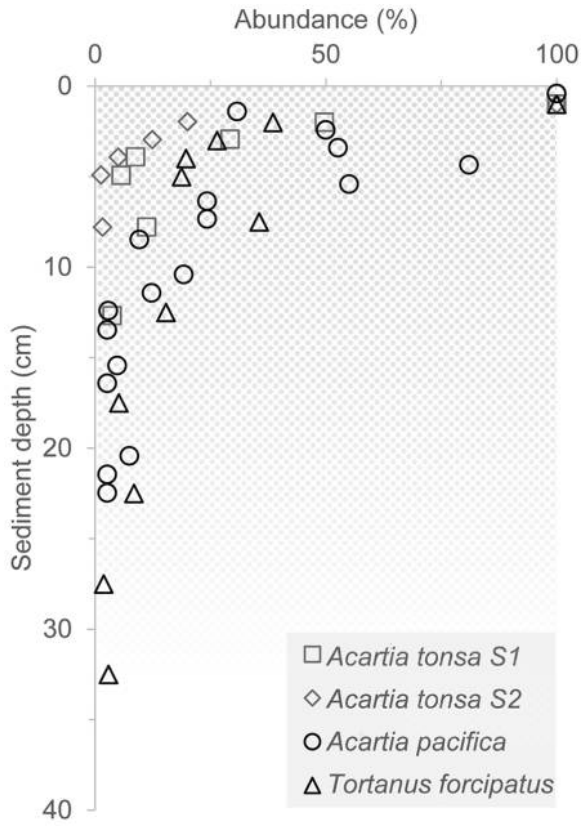

Figure 4. Relative abundance of resting eggs in sediment as a function of sediment depth normalized to the abundance in the top sediment layer. Squares and diamonds indicate sampling stations S1 and S2 in Funen, Denmark (Sichlau *et al.*, 2011); circles indicate Xiamen Bay, China (Jiang *et al.*, 2004); triangles indicate Victoria Harbor, Hong Kong (Dahms *et al.*, 2006).

that far exceed the length of the copepod seasonal cycle. This potentially creates additional micro-evolutionary benefits, such as gene preservation (Marcus *et al.*, 1994; Dahms, 1995), as a result of reduced genetic drift (Hairston and De Stasio, 1988; Marcus, 1996) and storage effect (Hairston, 1996). This might slow the rate of evolutionary change (Hairston and De Stasio, 1988); and, as proposed half a century ago, it might even help explain why some species have widespread distributions with little morphological (and presumably genetic) variation (Nicholls, 1945; Bayly, 1963). However, to which degree the resting eggs affect population dynamics or whether they are "the living dead" (Hairston and Bohonak, 1998) still needs to be determined. Eggs below 2-cm depth in the sediments do not hatch when buried (Kasahara *et al.*, 1975; Uye and Fleminger, 1976). Nevertheless, abundance of resting (quiescent) eggs is often relatively low in the top sediment, presumably because of hatching (Viitasalo, 1992). This indicates that they contribute directly to the pelagic population dynamics and survive for shorter periods than diapause eggs in sediments (Chen and Marcus, 1997).

The fraction of viable "permanently" buried resting eggs must be diapause eggs with a refractory period longer than the optimum period leading to certain mortality. Hence, an exponential decline would be expected in the breaking of diapause and, therefore, in the decline in viable resting eggs in the sediment (Fig. 4). In fact, Drillet *et al.* (2011b) report a unimodal frequency distribution of laboratory hatching success of diapause eggs over eight months (referred to as delayed-hatching eggs) for *Acartia tonsa*. Prior to hatching at 17 °C, the eggs were kept at low temperature (1.5 °C), which induces quiescence. Differences between the fractions of unhatched and hatched eggs at different time steps can therefore be ascribed to the fraction still in diapause. After cold storage and subsequent transfer to normoxic and warmer conditions, hatching success was ≥75% after a storage period of 3–5 months but slowly declined to <10% hatching after 8 months of storage. For a fraction of the eggs, this indicates a necessary period of dormancy to be in the range of about six months, enough to overcome the winter.

Marcus (1979) found that diapause eggs from *Labidocera aestiva* had synchronized hatching after being exposed to a period of chilling. The same has been observed for *Centropages hamatus*, where incubation at high temperatures synchronizes hatching (Marcus, 1989). This potentially reflects diapause eggs having completed their refractory period and having entered the quiescent state because of unfavorable conditions. Upon the return to favorable environmental conditions, the now-quiescent eggs all hatch at the same time, which is manifested by an increase in hatching (*i.e.*, synchronization of hatching). The continuous hatching over a relatively long period is proposed to characterize delayed-hatching eggs (Chen and Marcus, 1997), as reported in the Drillet *et al.* (2011b) study. The similar hatching patterns of delayed-hatching eggs and diapause eggs suggest that they are all one type of egg that can be classified as diapause eggs, as proposed by Drillet *et al.* (2011b).

Copepods have been found to change from producing subitaneous eggs early in the reproductive season to producing diapause or resting eggs toward the end (Marcus, 1979; Avery, 2005b; Berasategui *et al.*, 2012), supporting the interpretation of annual population development (*e.g.*, Kiørboe and Nielsen, 1994). In *Acartia hudsonica*, it has been shown that resting egg production toward the end of the season has a large degree of phenotypic plasticity, because the fraction of diapause eggs being produced varies among individuals (Avery, 2005a, b); but it has also been shown that it has a genetic component (Avery, 2005a). In fact, 42% and 85% of the detected variance could be attributed to variation among individuals (Avery, 2005b). Thus, when seasonal environmental conditions gradually change along a spatial axis, one can expect that there would be transition zones where resting eggs are produced; but average seasonal environmental conditions do not require the species to produce resting eggs to cope with adversity. However, it could also be that diapause eggs here lack a cue for synchronized hatching, thus not making their use as a seasonal adaptation possible. In these transition zones,

diapause eggs could also be used as a bet-hedging strategy that ensures that catastrophic events do not eradicate the species.

Cues Promoting Production of Resting Eggs

Since resting eggs most likely function as a trait that enables copepods to cope with seasonal environmental changes, the females would need reliable cues that signal the forthcoming changes before they become detrimental for their fitness, in order to avoid suboptimal reproduction (Avery, 2005b). Seasonality is due to changes in solar radiation; hence, factors such as photoperiod and temperature are correlated and fluctuate on a relatively predictable seasonal basis. However, biological production of copepod food is not always directly coupled to these variables but is more stochastic. Hence, if diapause timing is completely fixed, there is a potential problem due to mismatches with food availability. It is expected that diapause eggs would allow species to cope with seasonality, because their hatching is genetically predetermined (refractory period) and thereby unaffected by short-term changes.

Many marine copepod species have been shown to increase production as a function of photoperiod and/or temperature (*e.g.*, Sullivan and McManus, 1986; Dahms, 1995; Chinnery and Williams, 2003; Peck and Holste, 2006; Wu *et al.*, 2006; Yoshida *et al.*, 2012; Glippa *et al.*, 2013; Peck *et al.*, 2015). However, multiple other abiotic and biotic factors potentially influencing the production of resting eggs have been identified for copepods in general, but the number of actual factors identified to induce the production of resting eggs in marine copepods is not vast or fully understood (see Dahms, 1995; Fig. 5).

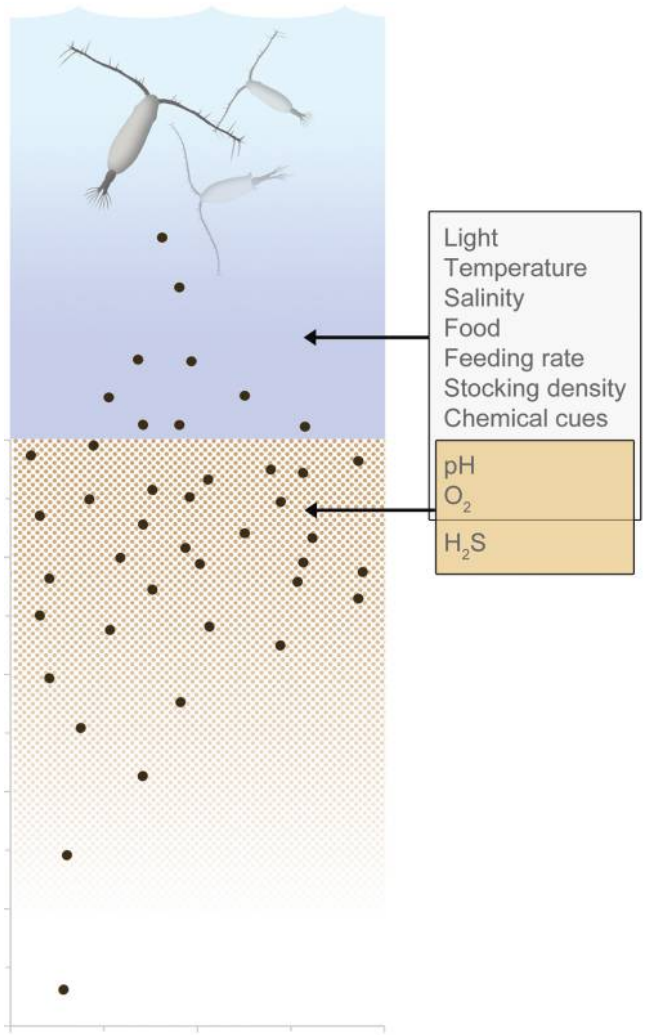

Figure 5. Conceptual representation of the most important environmental cues that female copepods, as well as the different egg categories, receive. The overlapping box represents variables shared by water column and pore water environment where H_2S solely is present in anoxic sediments.

Current Knowledge About Physiology and Biology of Copepod Eggs

Viewing the copepod resting egg as a life-history trait, and in a global context, allows us to better evaluate not only the influence of environmental factors on the occurrence of resting eggs but also the significance and purpose of this trait. Dealing with diapause eggs in the context of drivers (predictable change) and their seasonal range in a spatial context will enable us to make spatial distinctions between populations. It will also help to determine the purpose of resting egg production by spatially separated populations.

Most of our knowledge of egg physiology stems from studying subitaneous and quiescent eggs of euryhaline copepods, because those are easily accessible from field sampling or laboratory cultures. Many fewer reports exist on diapause eggs, presumably because they are more difficult to sample, to extract, and, finally, for many species, to categorize before valid process characteristics can be linked to their actual state. The few studies reporting physiological traits of diapause eggs are from species in which morphological identification of egg categories is manageable. Here I will give a brief overview of copepod egg composition and tolerances of the most prevailing environmental stressors. When possible, I will discuss the strength of the drivers (cues) likely to determine phase shifts in egg production and their quality as tools for egg categorization.

First, copepods are aquatic organisms; as such, their eggs are laid in aquatic media. Therefore, they cannot tolerate desiccation; drying (at 20 °C) kills the resting eggs (Næss, 1991). Second, it is important to notice that the stage in embryogenesis makes a difference in egg viability. In Florida, newly spawned eggs of three copepod species—*Acartia tonsa*, *Centropages hamatus*, and *Labidocera aestiva*—survived anoxic situations far better than fully developed eggs, indicating that they were better equipped to initiate dormancy in early embryogenesis (Marcus and Lutz, 1994). Moreover, survival of *A. tonsa* quiescent eggs in a cold storage situation was longer when they were 5–7 hours old, compared to eggs

that were newly spawned (0–2 hours) or closer to hatching (10–12 hours) when entering quiescence (Drillet *et al.*, 2007). Additionally, newly spawned eggs of *A. tonsa* were more sensitive to low oxygen concentrations than fully developed eggs, whereas no difference was detected for *L. aestiva* eggs exposed to the same treatments (Lutz *et al.*, 1994). Hence, age of the embryo likely matters for later viability as subitaneous eggs and also when entering embryonic arrest. Therefore, it is fair to assume that biochemical composition, as well as embryonic tolerances to various stressors, is dependent on the age of the embryos. However, egg ages and embryonic stages are, unfortunately, very seldom available in the literature reports.

Biochemical composition of copepod eggs

More than 20 years ago, Marcus (1996, p. 146) wrote that "biochemical studies of subitaneous and diapause eggs are noticeably lacking." Ten years later, Drillet *et al.* (2006a, p. 727) stated, "No complete sets of biochemical data are available on copepod eggs in the literature." However, numerous earlier studies have reported on dry weights and the carbon, and even nitrogen, contents of subitaneous eggs. For instance, Kiørboe *et al.* (1985) gave a dry weight of 104 ± 7.8 ng, as well as 45.7 ng in carbon and 9.1 ng in nitrogen, for *A. tonsa* eggs about 80 μm in diameter. Guisande and Harris (1995) measured size and protein, carbohydrate, and lipid content of *Calanus helgolandicus* eggs and concluded that these properties vary with time and were positively correlated with available food concentrations. Since then, Wang *et al.* (2005) reported that biochemical profiles (*i.e.*, dry weight and lipid, protein, and carbohydrate contents) were higher in diapause eggs than in subitaneous eggs of *Centropages tenuiremis*. They also reported that the levels of aspartic acid, glutamic acid, glycine, methionine, isoleucine, leucine, norleucine, lysine, ammonium chloride, and arginine in diapause eggs were significantly higher than in subitaneous eggs. The biochemical discrepancies might be a tool contributing to the destructive differentiation between egg categories. A step further into biochemical profiling of subitaneous and quiescent eggs was taken by Drillet *et al.* (2006a), where detailed profiles of unsaturated fatty acids and free amino acids were determined during cold storage of *A. tonsa* eggs over 20 months. The pool of free amino acids was stable during cold storage, at 51–62 pmol egg^{-1}, as long as the eggs were able to hatch (until 12 months). Moreover, they did not find any differences in total content of amino acids between fresh subitaneous eggs and stored quiescent eggs for 6, 11, and 12 months. They therefore concluded that embryos do not primarily use amino acids as an energy source when in quiescence. The total amount of fatty acids did not decline, and the essential fatty acids eicosa pentaenoic acid (EPA) and docosa hexaenoic acid (DHA) were present in very high proportions, compared to the total amount of unsaturated fatty acids. Moreover, the arachidonic acid (ARA) level was not present in great amounts but increased after three months of storage, which was supported by Støttrup *et al.* (1999). In another study, Drillet *et al.* (2006b) recorded in detail fatty acid and amino acid composition and content of *A. tonsa* eggs over a storage period of nine months. They found a declining trend in the total amount of fatty acids with time, and they reported the temporal development for each of the 28 fatty acids they analyzed. In addition, they confirmed that free amino acids stayed rather constant during storage. Drillet *et al.* (2008b) analyzed the biochemical profile in subitaneous eggs of four different strains of *A. tonsa*. There was a significant difference in DHA/EPA between eggs, where ratios of 0.82, 1.37, 1.34, and 1.48 were detected in the Florida, Danish Institute for Fisheries Research (DIFRES), Kiel, and Alabama strains, respectively. However, the strains had similar amino acid contents in their eggs (6%–8% of dry weight). Generally, proline was the predominant amino acid, with 34%–43% of the total amino acid contents. It looked as though the free fatty acids declined, but not as much as the amino acid pool, during storage or sediment dwelling. Hence, there seems to be value in destructive biochemical analysis for categorization of copepod eggs.

Light

Diapause eggs presumably do not hatch in darkness while they are buried in the sediment. If so, the nauplii will die. Therefore, light may serve as a valuable cue by signaling the transition from diapause to quiescence when diapause eggs leave the sediment. Hence, light intensity and wavelengths are drivers that could potentially trigger hatching in subitaneous and quiescent eggs. Peck *et al.* (2008) nevertheless concluded that egg hatching was invariant of daily light cycle in continuous cultures of *A. tonsa*. However, they also conducted an experiment where cohorts were reared (from nauplii) in constant darkness or in constant light and where eggs produced from these cultures were incubated in either constant darkness or light. In that study, the overall hatching success increased when the eggs were incubated under constant light. Camus and Zeng (2008) reported the highest hatching rate of subitaneous *Acartia sinjiensis* eggs at a light : dark cycle of 24h : 0h. While not significantly different from 18h : 6h, this was comparable with results in Peck and Holste (2006) for *A. tonsa*. Hagemann *et al.* (2016) obtained the highest hatching success at 85.9% at 17 °C in complete darkness for cold-stored quiescent eggs, also from *A. tonsa*. Later, Hagemann *et al.* (2017) observed similar hatching success regardless of the light intensity and wavelength, including when hatching in darkness. Jiang *et al.* (2006) found that photoperiod regimes had no significant effect on the hatching of resting eggs of *Acartia pacifica* from sediments from Xiamen Bay, China. Uye and Fleminger (1976) concluded that eggs from *A. tonsa* and *Acartia* sp. hatched normally in complete darkness, whereas eggs from *Acartia clausi* were adversely

affected by darkness. The production of diapausing eggs in the key species, *Euthynnus affinis*, within the downstream Seine estuary could be induced by short days, but photoperiod was probably not the only driver responsible for the induction of diapausing egg production (Glippa *et al.*, 2013).

Hence, there seems to be no clear pattern in subitaneous, quiescent, or diapause egg hatching *versus* day length between species, and not even within species in the reported results. Photoperiod is quite predictable, unlike light intensity, and contributes to diapause control (*e.g.*, Boyer and Bonnet, 2013). However, both variables determine water and, thereby, sediment temperature. Hence, the integrated photo regime might indirectly determine both production and hatching of resting eggs *via* a temperature signal. It is unlikely that photoperiod alone would determine phase shifts in sediment-dwelling copepod eggs because they cannot receive and interpret any light signals.

Temperature

Resting eggs from at least four calanoid species—*E. affinis*, *A. tonsa*, *A. clausi* (later reclassified to *Acartia teclae*), and *C. hamatus*—could not resist freezing at −15 °C (Zillioux and Gonzalez, 1972; Næss, 1991). Hence, one can assume that copepod eggs generally do not tolerate subzero temperatures below the freezing point of ambient seawater. In the laboratory, subitaneous eggs develop and hatch with a strict positive response to temperature (*e.g.*, McLaren, 1966; McLaren *et al.*, 1969; Uye and Fleminger, 1976; Milione and Zeng, 2008; Hansen *et al.*, 2010a). Storage of quiescent *A. tonsa* eggs for up to 35 weeks was tested at different temperatures, salinities, and oxygen conditions in a full-factorial experiment by Holmstrup *et al.* (2006). The best hatching success was obtained when eggs were stored at low temperatures (<5 °C) under anoxia, resembling conditions in sediment during winter. Temperature not only controls the rate of embryogenesis but also is a universal driver for zooplankton physiological processes. This relationship has been characterized through a meta-analysis of laboratory or subsidiary experimental *in situ* studies of size dependency of grazing and growth in zooplankton of 2 μm to 2000 μm in body size. In total, 42 studies were compiled, revealing a total (mean ± SD) $Q_{10} = 2.80 \pm 0.29$ (Hansen *et al.*, 1997), which is most likely also applicable to embryo development.

Several field studies have shown that resting eggs (presumably diapause eggs) hatch or increase hatching only after a period of either chilling or warming (*e.g.*, Marcus, 1980, 1989; Bay *et al.*, 1986; Avery, 2005b; Glippa *et al.*, 2011). Hence, these resting eggs serve to synchronize hatching, potentially to prevent nauplii from emerging from the sediments without food to support development and growth (Dahms, 1995). This suggests that temperature is important either as a reliable cue for the production of resting eggs or for the termination of dormancy. Numerous studies report that temperature changes often induce or break quiescence in copepod eggs (Dahms, 1995; Drillet *et al.*, 2006a; Holmstrup *et al.*, 2006; Wu *et al.*, 2006; Berasategui *et al.*, 2013).

Resting eggs from *Anomalocera patersoni*, *Acartia hudsonica*, *Centropages abdominalis*, *Centropages hamatus*, and *Acartia bifilosa* were generally found toward the upper limit of these species' thermal distributions (Holm *et al.*, 2018). *Acartia hudsonica* has a thermal distribution up to 30 °C; but in Narragansett Bay on the Atlantic Coast of the United States, it produces resting eggs to avoid temperatures >20 °C (Bay *et al.*, 1986). Thus, temperature not only might serve as a reliable token stimulus or cue for production or termination of diapause but also can be the direct cause of their presence (Chinnery and Williams, 2003). The thermal tolerance of copepods frequently reflects the temperature characteristics of the environment where they live (Mauchline, 1998). When environmental conditions exceed or approach the tolerance limit of a given species, it is expected that the resting egg strategy would be used as a seasonal adaptation to avoid mortality. Sullivan and McManus (1986) suggested that, for *A. hudsonica*, it was short-term changes in temperature that surpassed its tolerance limit, rather than food availability that induced the production of resting eggs (presumably, diapause eggs).

In conclusion, numerous studies have determined that temperature is the most important trigger for inducing hatching of resting eggs, at least in Acartiidae species (*e.g.*, Uye and Fleminger, 1976; Uye *et al.*, 1979; Sullivan and McManus, 1986; Boyer and Bonnet, 2013).

Salinity

Uye and Fleminger (1976) conducted a multifactorial study of several environmental stressors on 3 *Acartia* species; all 3 species tolerated salinities in the range of 17 to 48, always with >50% hatching. Holmstrup *et al.* (2006) showed that quiescent cold-stored (≤5 °C) eggs from *A. tonsa* under anoxia tolerated a salinity range of 10–50 within 20 weeks but performed best within a 10–20 range, with a hatching success of >13%. However, they tolerated very low salinity (zero) for only a short time, decreasing hatching rate with increasing temperature. Holste and Peck (2006) reported asymptotic increasing hatching success of *A. tonsa* eggs from salinity 0–34, with maximum hatching at 81.4%–85.5% between salinities 17 and 25. Svetlichny *et al.* (2010) investigated hatching for *A. clausi* and *A. tonsa* in response to the salinity and temperature of the Marmara and Black Seas. Hatching success in *A. tonsa* eggs spawned at salinity 18 decreased from 77.7% ± 18.5% at 20 °C to 46.7% ± 21.5% at 7 °C and reduced to 10.2% ± 4.7% at 7 °C at salinity 39. However, hatching success of eggs from *A. clausi* from the Black Sea did not depend on temperature; instead, hatching success decreased dramatically from 80%–90% to 3.3%–10% after salinity increased from 18 to 38. These authors reported different hatching dependencies on temperatures but overall decreasing hatching with salinity.

Hansen *et al.* (2012) measured metabolism of individual subitaneous eggs of *A. tonsa* exposed to abrupt salinity changes. The individual (mean ± SD) oxygen consumption was 0.273 ± 0.036 nL O_2 h^{-1} at salinity 34 and 0.282 ± 0.014 nL O_2 h^{-1} at salinity 2. Hence, the respiration rates of individual eggs did not differ significantly, and abrupt changes in salinity therefore did not seem to affect the metabolism of the eggs or was not detectable with the nanorespirometer method. Based on volume changes, Hansen *et al.* (2012) suggested that subitaneous eggs acted as a perfect osmometer, with passive exchange of water and ions over the chorion when abrupt changes in salinity took place. This lack of elevated oxygen consumption and flexible volume of the eggs *versus* ambient salinity can most likely be ascribed to the protective embryonic membrane that can facilitate volume changes in the egg's periviteline space, as suggested by Charmantier and Aiken (1987). The hatching success remained unaffected by such changes in salinity. Interestingly, Charmantier and Charmantier-Daures (2001) also suggested that the crustacean embryo is osmoprotected from external salinity levels and fluctuations by the egg envelope, while at the same time the egg envelope allows for gas exchange. These adaptive traits should promote embryonic survival in the sediment environment (see discussion in Nielsen *et al.*, 2006; Hansen *et al.*, 2013). Additionally, the same membrane structure largely protects the embryo from various anthropogenic chemical stressors—for example, surface disinfectants Buffodine, FAM30, and glutardialdehyde, in addition to rotenone—used to combat parasites in aquaculture (*e.g.*, Næss, 1991; Næss and Bergh, 1994). In contrast, based on their observation of a statistically significant change in density and sinking velocity for eggs that were exposed to different experimental salinities after spawning, Miller and Marcus (1994) indicated that subitaneous eggs, in fact, osmoregulate. Ohs *et al.* (2009) exposed *A. tonsa* eggs to hypersaline water at 4 and 26 °C for just 10 and 20 minutes and recorded hatching success afterward. The viability of eggs exposed to unnaturally high salinities 50, 75, and 100 was not significantly different from controls for all treatment combinations (always >80% hatching), except at 26 °C, when exposed for 20 minutes, which resulted in significantly lower hatching at salinity 100. *Acartia sinjiensis* eggs were incubated at 9 different salinities ranging from 10 to 50; they also showed no difference in mean hatching rate, which was always >80% (Milione and Zeng, 2008).

In conclusion, eggs from these euryhaline calanoid species seem to tolerate a large range of fluctuating salinities, especially *A. tonsa*, with its worldwide distribution (Miller and Marcus, 1994). Hence, salinity seems not to be a likely cue for phase shifts in the production and destiny of copepod resting eggs.

Oxygen

Dormant eggs exhibit low metabolic activity, although the differences in respiration between the different egg types are only vaguely defined (Romano *et al.*, 1996b; Nielsen *et al.*, 2006). It is of imperative significance for survival of resting eggs that they reside in anoxic sediments. This is clearly visualized in the relatively high mortality of eggs in laboratory storage studies where quiescent eggs were cold stored at normoxic conditions over time. Hagemann *et al.* (2016) obtained a 7.5-month storage time of quiescent eggs of *A. tonsa*, although with a steady decrease of hatching success. Drillet *et al.* (2006a) obtained 11 months' storage time for quiescent eggs of the same laboratory strain of *A. tonsa*, with a daily mortality of 0.035–0.13 and declining fatty acid content. Using the same protocol as in the 2 referred studies on eggs from 7 *Acartia* spp. strains from boreal and subtropical locations, Hansen *et al.* (2016) determined daily mortality ranging from 0.0025 (from Gulf of Venice, Italy) to 0.0917 (from Mobile Bay, Mexican Gulf). The mortality rates correlate with egg size, with less mortality of larger eggs; and they also depend on latitude, with eggs from warm-water populations exhibiting the largest mortality rate. These patterns demonstrate depletion of egg storage material over time (metabolism); but they also most likely reflect the populations' selection pressure toward developing embryonic dormancy, which is higher in boreal regions than in more southerly regions. The above-used protocol was recently improved, and eggs stored under strict anoxic conditions showed zero mortality during two months (Jørgensen *et al.*, 2019a). The reported relatively low fraction of hatching, compared with hatching under normoxic situations, supports observations reported by Broman *et al.* (2015). It seems fair to interpret the results as if resting eggs residing in anoxic sediments, first, do not hatch and, second, survive for longer in anoxia than if oxygen is present (*e.g.*, Grice and Marcus, 1981). Hence, oxygen concentration is likely an important cue for phase shifts between diapause-quiescent-subitaneous egg stages.

Only very few studies of oxygen consumption rates are reported for copepod eggs. While oxygen consumption rates have rarely been used as a tool to assess diapause metabolism, this approach has been used in large egg batches (1000–2000 eggs incubated in a closed container) of the copepods *Pontella mediteranea*, *Anomalocera patersoni*, and *Centropages tenuiremis* (Romano *et al.*, 1996a, b; Wu *et al.*, 2009). Winter diapause eggs show a U-shaped metabolic curve with time, with a high rate of early development metabolism, followed by a metabolic downregulation and a lengthy low-metabolism period, before a sharp increase in metabolism occurs, which finally leads to hatching of the eggs (Romano *et al.*, 1996b). In subitaneous eggs, however, the oxygen consumption during ontogenetic development gradually increases by a factor of five, from spawning to hatching, in *P. mediteranea* (Romano *et al.*, 1996b). In the summer-diapausing *A. patersoni*, Romano *et al.* (1996b) observed a different pattern. The temporal egg respiration curve was the opposite of diapause eggs of *P. mediteranea*, where a maximum oxygen consumption rate of 0.002 μL O_2 $embryo^{-1}$ h^{-1} was reached very

late in time, 70 d after spawning, when respiration was twice as high as that recorded during early diapause. This was followed by a slow decline until day 150 at 0.0003 μL O_2 embryo^{-1} h^{-1}, after which hatching was observed at 13 °C. The observed differences in oxygen consumption patterns of diapause eggs between the two species also seemed to involve differences in their metabolism during development time, in addition to metabolic requirements during summer and winter dormancy.

Oxygen consumption rates seem relatively constant throughout embryogenesis in *A. tonsa* (Nielsen *et al.*, 2007). Nielsen *et al.* (2007) recorded oxygen consumption of individual eggs by nanorespirometry with microelectrode technology and reported that oxygen consumption increased exponentially with temperature, from 0.09 ± 0.04 nmol O_2 egg^{-1} h^{-1} at 10 °C to 0.54 ± 0.09 nmol O_2 egg^{-1} h^{-1} at 28 °C, with Q_{10} = 2.51 ± 0.15. Hansen and Drillet (2013) compared oxygen consumption of individual subitaneous and delayed-hatching *A. tonsa* eggs with the same technique. The initial mean ± 95% confidence limits (CL) of oxygen consumption rates of freshly produced subitaneous eggs and 7-day-old delayed-hatching eggs from their Kiel strain were 0.132 ± 0.019 and 0.021 ± 0.013 nmol O_2 egg^{-1} h^{-1}, which showed a statistically significant difference. Between subitaneous eggs of the two different *Acartia* strains, there was no difference in initial oxygen consumption rates (0.132 ± 0.019 and 0.105 ± 0.011 nL O_2 egg^{-1} h^{-1}). The variability of initial oxygen consumption rates for their Kiel strain eggs was within the ranges of 0.219–0.0617 nL O_2 egg^{-1} h^{-1} and for their DTU strain 0.163–0.00792 nL O_2 egg^{-1} h^{-1}, representing factors of 3.6 and 21 from the highest to lowest values, respectively. For the subitaneous eggs, there was no relationship between the initial oxygen consumption measurements and the time duration from spawning to hatching, either for their Kiel strain or for their DTU strain. Additionally, there was no pattern concerning the initial oxygen consumption rates *versus* time to death for the two egg types. The present metabolic measurements between fresh subitaneous and seven-day-old delayed-hatching eggs suggests that there is a difference; such measurements might be useful as a practical non-destructive separation criterion between the two egg categories.

Wu *et al.* (2009) showed that the average oxygen consumption of subitaneous eggs (smooth chorion) of *C. tenuiremis* was lower than for diapause eggs (chorion with long spines) during the pre-diapause stage. The Q_{10} for subitaneous eggs was 2.05, not very different from the one determined by Nielsen *et al.* (2007) for *A. tonsa* eggs. Moreover, Wu *et al.* (2009) confirmed the U-shaped oxygen consumption pattern over time described by Romano *et al.* (1996a); hence, the oxygen consumption after 44 days in diapause reached only 13% of the pre-diapause level, showing a significant metabolic downregulation.

In conclusion, oxygen concentration determines the viability of embryos in dormancy; and changes in the oxygen consumption rate of free-living eggs are certainly a clear indication of a transition between subitaneous and quiescent states. Thus, oxygen consumption rate could be a valuable non-destructive tool for differentiating between all three egg categories.

Hydrogen sulphide

Anaerobic processes in diapause eggs are assumed to be present because of the lack of oxygen in deep sediments while coping with toxic doses of H_2S (Nielsen *et al.*, 2006), which may promote particular metabolites. Hydrogen sulphide is a potent stressor that often is present in anoxic marine sediments where resting eggs reside. Marcus *et al.* (1997) exposed subitaneous eggs from *A. tonsa*, *L. aestiva*, and *C. hamatus* to anoxia and anoxia/hydrogen sulphide for 32 days. Quite a large fraction of the eggs died within two to four weeks, regardless of treatment; however, the remaining eggs in both treatments showed no difference in viability. Marcus *et al.* (1997) suggested that the eggs switched to anaerobic metabolism when exposed to anoxia; however, the authors did not reflect on at what timescale this can last and at what cost. They incubated the eggs at pH 7.9–8.1 and suggested that their H_2S concentrations could be more harmful to eggs at a pH closer to 7, resembling more realistic pore water conditions. Exposing diapause eggs from *C. hamatus* to sulphide for up to 90 days did not result in greater mortality than when exposed to anoxia alone. However, diapause eggs were able to survive anoxia and H_2S for considerably longer than subitaneous eggs (Marcus and Lutz, 1998). In line with this, Invidia *et al.* (2004) concluded that exposure to H_2S was more detrimental to eggs when pH was as low as in pore waters (6–6.5). They also argued for the significance of carry-over effects of H_2S exposure in the egg stage (*i.e.*, life expectancy of the free-living copepods). Nielsen *et al.* (2006) reported for subitaneous *A. tonsa* eggs that short-term (3- to 60-day) exposure to anoxia or anoxia/hydrogen sulphide did not significantly affect the following hatching success of the eggs, but hatching generally declined with increasing length of exposure (long term). Most other studies only ran experiments defined here as short term, for about a month. However, Nielsen *et al.* in fact conducted long-term exposure experiments that more closely mimicked natural situations in sediments. After 60 days of exposure, there were significant differences between the effects caused by anoxia and anoxia/hydrogen sulphide (H_2S concentrations ≥250 μmol L^{-1}). In particular, after 240 days of exposure, there were significant differences in hatching between eggs treated with anoxia and those treated with anoxia/hydrogen sulphide (all tested concentrations were 10–10,000 μmol L^{-1}), where H_2S exposure caused elevated mortality. A short-term experiment, where subitaneous eggs were simultaneously exposed to oxygen and different H_2S concentrations (a non-natural but constructed situation), indicated that H_2S is capable of crossing the eggshell. The metabolic rate of eggs exposed to normoxic conditions was 1.86 ±

0.57 μJ h^{-1}. Eggs exposed to anoxia had a metabolic rate of 0.08 ± 0.02 μJ h^{-1}, indicating a metabolic shutdown, whereas eggs exposed to anoxia and 14.7 mmol L^{-1} H_2S had a metabolic rate of 0.25 ± 0.001 μJ h^{-1}. Hence, elevated metabolism when exposed to H_2S was significantly higher compared to during anoxia. Based on the metabolism experiments and the fact that internal egg pH was ~6 (pore water levels), an unknown H_2S defense mechanism was suggested to be present in *A. tonsa* eggs.

In conclusion, resting eggs are, to a varying extent, tolerant of H_2S, which is a survival strategy when residing in marine sediments. It would be relevant to investigate the mechanism and its efficiency in quiescent compared with diapause eggs. Moreover, since H_2S is always present in anoxic sediments, this could be a signal that controls the phase shift between diapause and quiescence.

pH

Since pH can affect the dynamics of inorganic nutrients that are potentially toxic to copepods and their eggs (Buttino, 1994; Jepsen *et al.*, 2015), addressing pH tolerance is of vital importance for the resting egg discussion. However, not much literature is available on the subject. Invidia *et al.* (2004) studied the tolerance of subitaneous eggs of *A. tonsa* to near anoxia and to H_2S at different pH levels. As described above, pH is vital for the toxicity of H_2S They concluded that short exposure to these treatments did not affect egg viability. However, long exposure to anoxia/hydrogen sulphide was less detrimental than near anoxia alone when pH was in the range of natural seawater. Hansen *et al.* (2017) conducted egg-hatching experiments from batches of subitaneous *Acartia* spp., *Centropages typicus*, and *Eurytemora affinis* eggs at 4 levels of pH: 8.0, 8.5, 9.0, and 9.5. Surprisingly, pH did not have any effect on hatching success of eggs of these species. Moreover, the team reported preliminary results that suggested that hatching by *A. tonsa* eggs took place even at pH 10, as well as at pH 6 (J. K. Højgaard, Roskilde University, pers. obs.). However, when facing the ambient water column environment, an increasing fraction of the nauplii died right after hatching when pH deviated more than one unit from natural pH. It appears that resting eggs tolerate a wide range of pH but that newly hatched nauplii do not. Tolerance to low pH is an adaptation to pore water environments in sediments and gut passage by egg predators, whereas tolerance to elevated pH is an adaptation to hypereutrophic waters with high photosynthesis activity (Tiselius *et al.*, 2008; Hansen *et al.*, 2017). In conclusion, pH is unlikely to be a cue that controls transition among egg stages and certainly not a cue for hatching.

Population density

Ban (1994) found that in *E. affinis* from Lake Ohnuma, located in southern Hokkaido, Japan, high population density (>80 females L^{-1}) induced diapause egg production. Later, Ban and Minoda (1994) demonstrated that, for the same species in laboratory experiments at very high densities (~500 females L^{-1}), an accumulation of their own metabolic products in the medium induced diapause egg production. The molecules carrying a possible chemical cue were not investigated further, though. Jepsen *et al.* (2007) cultured *A. tonsa* at various densities of up to 600 adults L^{-1} in a setup that excluded the effects of their own metabolites. The average egg hatching success was 84.7% ± 4.8%, with no significant differences across the stocking densities. Drillet *et al.* (2015) cultured *A. tonsa* at densities ranging from 10 to >5000 individuals L^{-1}. The increasing adult density did not affect the proportion of delayed-hatching eggs produced (~10% of harvest) but decreased their hatching success significantly. However, the decline of hatching due to density was low (−1.728%) for every increase of 1000 individuals L^{-1}. Camus and Zeng (2009) reported a possible delayed effect on hatching of *A. sinjiensis* eggs produced from copepods kept in culture at high stocking densities. This egg-hatching success pattern was present after 48 hours of incubation but evened out after 96 hours of incubation.

Hence, the natural density of free-swimming marine copepods, which is almost always much lower than the reported experimental densities, does not seem to be an important factor promoting resting egg production; and it does not influence egg-hatching success.

Food availability

It is well known that zooplankton can graze down a major part of the phytoplankton production (*e.g.*, Schmoker *et al.*, 2013). In areas with strong grazing pressure, it would dampen the amplitude of seasonal differences in chlorophyll *a* concentrations, hence masking seasonal fluctuations in the production rate. Copepod egg production is potentially controlled by food concentration (*e.g.*, Kiørboe *et al.*, 1985; Kiørboe and Nielsen, 1994). However, food availability is driven not only by photoperiod and temperature but also by turbulence and nutrient availability (*e.g.*, Kiørboe and Nielsen, 1994). Therefore, food concentration is a less predictable signal compared to photoperiod and temperature. Moreover, only Drillet *et al.* (2011b) have shown that low food availability can lead to increases in the production of resting eggs (delayed-hatching eggs) in a calanoid, *A. tonsa*, which is why food concentration is, so far, not demonstrated to generally be a strong driver of resting egg production.

In conclusion, the relatively few known species of marine calanoids with resting eggs in their life cycle generally tolerate a wide range of abiotic and biotic variables. Hence, they are opportunists that are well adapted to variable environments, such as estuaries. Therefore, the copepods need a cue with a strong signal. The signal strengths of the drivers discussed above leave photo-regime and temperature as the strongest. Personally, I tend to believe that the main cue

activating production of dormant eggs by the pelagic females must be photoperiod and that temperature controls the biology of resting eggs when residing in sediments, as concluded by Holm *et al.* (2018). Moreover, the major cues for subitaneous eggs to enter and leave quiescence seem to be oxygen concentration, H_2S, and temperature. However, the linkage between dormant eggs and the ecological, biological, physiological, and molecular mechanisms leading to production of different copepod egg categories and enabling maternal programming is not yet understood, in large part because of a lack of suitable methodologies.

Unresolved Questions About Copepod Resting Eggs

There are still many unresolved questions, such as how copepods cope with seasonality and adverse environmental conditions with respect to resting egg production. More specifically, it is not known which environmental conditions support the production of resting eggs (hibernating embryos) (Fig. 5), which traits create resistant individuals that can survive periods of adversity, and how the answers to these questions relate to species distribution. Understanding of the epigenetic and genetic mechanisms underlying embryonic dormancy is also limited (Nilsson, 2017). Therefore, I ask the following questions: (1) What are the physical-chemical-biological cues (day length, light intensity, temperature, crowding stress, food shortage, *etc.*); and, in particular, at which intensities and durations do copepods receive these cues in order to translate them into phase shifts from producing subitaneous eggs to producing diapause eggs? (2) How do changes in the environmental stimulate transitions between subitaneous and quiescent egg states? (3) How do the resting eggs (diapause, quiescent) keep track of time (*zeitgeber*) and/or receive and compute external input and "decide" to leave their arrested phase?

To answer these fundamental, but excessive, questions, we need a substantial breakthrough in several physiological aspects of copepod life manifestations. The questions start with the necessary understanding of the mechanisms driving embryonic dormancy, including molecular mechanisms. The questions lead toward the need for more specific efforts. Hence, I propose that we need to (1) develop effective non-destructive tools to distinguish between subitaneous, quiescent, and diapause eggs; (2) detect and unravel which molecular processes control embryonic dormancy in copepods; and (3) obtain the ability to interrupt and control the molecular processes that take place during embryogenic dormancy to understand all mechanisms associated with this phenomenon.

Suggested Pathway to Reach a Mechanistic Understanding of Copepod Embryonic Dormancy

Formerly, standard physiological techniques have been the only realistic approach, as described above, to study embryonic dormancy. These dose *versus* response results are indeed informative but only allow a black box insight into the physiological processes. However, lately, a toolbox of molecular techniques has become widely available (*e.g.*, beyond health science applications). Implementing a suite of these techniques—for example, genomics, transcriptomics, and metabolomics—to support standard physiological techniques in copepod molecular physiology seems promising (see Nilsson, 2017; Amato and Carotenuto, 2018; Tarrant *et al.*, 2019).

Based on transcriptomic analysis throughout embryogenesis, several hormonal profiles were investigated for *Acartia tonsa* (Nilsson *et al.*, 2018). The roles of *ecdysteroid-phosphatephosphatase* (*EPPase*), *ecdysone receptor* (*EcR*), *û fushi tarazu transcription factor 1* (*ûFTZ-F1*), and the *ecdysteroid-regulated early gene E74* (*E74*), which represent different levels of the ecdysone-signaling cascade, were examined. Expression patterns suggested two peaks of the biologically active ecdysteroid 20-hydroxyecdysone (20E). Based on *EPPase* expression, the first peak of 20E likely occurred in concert with the beginning of embryogenesis, originating from yolk-conjugated ecdysteroids. The second peak is suggested to originate from *de novo* synthesized 20E around the embryonic limb bud stage. During quiescence, the expression of *EPPase*, *EcR*, *ûFTZ-F1*, and *E74* either decreased or remained constant over time. This suggests that the ecdysone-signaling pathway plays a key role in the subitaneous development of *A. tonsa* embryogenesis but not during quiescence. Moreover, expression of commonly studied stress-responsive genes during the transition between subitaneous and quiescent embryonic states has been reported for the same species (Nilsson *et al.*, 2014). Expression of *hsp70* remained low during quiescence despite environmental stress. However, *ferritin* expression exhibited a strong increase from days 3 to 5 of quiescence, followed by a decline. After two weeks of quiescence, development of the embryos recovered with the addition of oxygen and increased temperature. The expression of both genes increased: 222-fold for *hsp70* and 52-fold for *ferritin* during the recovery phase toward hatching. Wu *et al.* (2009) reported a profound difference in isoenzymes between subitaneous and diapause eggs of *Calanus tenuiremis*. Additionally, molecular methods for taxonomic identification of resting eggs of copepods have been recently published. The methods work on individual eggs that are removed from sediments and fixed. Their DNA is sometimes extracted using a HotSHOT extraction protocol (Montero-Pau *et al.*, 2008), and regions of the mitochondrial *cytochrome oxidase I* or *16S* genes are amplified using polymerase chain reaction (PCR). The amplified fragments can be directly sequenced or classified visually based on unique restriction fragment profiles developed for each species studied (Montero-Pau *et al.*, 2008; Briski *et al.*, 2011; Lindeque *et al.*, 2013). This approach is 100% objective but requires identification of the egg category in advance, as well as morphological (or presumably genetic) identification of the nauplii after hatching. Hence, there is a growing molecular-based literature on various aspects of

how copepod eggs function. The use of techniques in the cited articles, besides other molecular-based techniques, is highly relevant in order to pursue methods for differentiating eggs between species, and even between egg categories, while recording embryogenesis within species.

Many have tried to selectively stimulate production of diapausing eggs in copepods in the laboratory but have hitherto not provided a complete protocol. This is due to prior studies not linking environmental pressures directly to responses in the females and their embryos, because such studies require considerable effort and resources. It is obvious that we need more detailed information than just external observations and black box reaction norms for various external variables; we seriously need to open up for the wealth of modern molecular tools to enter the interior of the eggs—the embryo. We need to study the embryo itself also at a cellular level and to work on a genetic level, which would assist us in finding nature's secret hitherto associated with embryonic diapause. There is a need for multi-method approaches where physiological responses are detected by classical and modern technologies and/or methods (see Nilsson, 2017; Amato and Carotenuto, 2018; Tarrant *et al.*, 2019). Dormancy can be induced in some animals by steroid hormones and insulin-like peptides (Tan and MacRae, 2018). Protective substances (low and high molecular weight) that maintain molecular structures, such as the disaccharide trehalose (found in, *e.g.*, tardigrades and brine shrimps) (Hashimoto *et al.*, 2016; Tan and MacRae, 2018), as well as molecules that "sense" the environment, must be present. Studies should include stimulation of the production of low-molecular-weight protectives, determined by various omics, using, for example, liquid chromatography-mass spectrometry (LC-MS). High-molecular-weight protectives could be studied using immunochemical determination, RNA and DNA sequencing, quadrupole-time-of-flight (Q-TOF) mass spectrometry, and immunohistochemistry (Hashimoto *et al.*, 2016). Energy-rich (phosphorylated) substances might be studied using phosphoproteomics. More specifically, one could hypothesize that H_2S is used as an info-chemical for the sediment-dwelling eggs to determine their environment. Hydrogen sulphide is able to pass the chorion and is suggested to be detoxified by the embryo (Nielsen *et al.*, 2006). Specific oxidative enzymes that are involved in the breakdown of H_2S should therefore be investigated using transcriptomic, metabolomic, and proteomic analyses to reveal biochemical pathways (Lant and Storey, 2010). One could purify and sequence RNA from subitaneous, quiescent, and diapause eggs in different development stages when characterized after the above-mentioned discrimination approaches for egg categories and from various manipulation experiments. The data could then be used for comparative transcriptomic analysis to provide an overview of the differences in metabolic and regulatory gene expression between the three egg categories exposed to stressors (Nilsson *et al.*, 2014; Koštál *et al.*, 2017). To terminate diapause, eggs of some copepod species require a period of either warming or chilling to trigger hatching (Sullivan and McManus, 1986) or to increase the hatching rate (Uye, 1985; Næss, 1996; Chen and Marcus, 1997). These observations are hitherto also obtained from dose-response experiments and have unfortunately not yet led to a deeper physiological and mechanistic understanding.

I strongly believe that the molecular-oriented research path is the way forward. Hence, now is finally the right time to solve what no one before has been able to—based on a substantial molecular and physiological approach—thus enabling solid fundamental and applied scientific achievements. One can take inspiration from other organisms in which molecular insight is more advanced compared to copepods: for example, the well-studied nematode *Caenorhabditis elegans* and the crustacean brine shrimp *Artemia franciscana*, where a much deeper molecular understanding has been obtained. Evidence is emerging for epigenetic contributions to diapause regulation where small RNAs play a role (see Hand *et al.*, 2016; Reynolds, 2019). This will fundamentally pave the way for the next step, the ambitious goal of describing and later manipulating the link between female copepods and their dormant embryonic offspring. At present, the different life stages, pelagic and benthic, are studied as two different compartments and not as a holistic life cycle concept. In reality, we do not know for certain what happens when we describe annual population dynamics of copepod populations with resting eggs in their life cycles (*e.g.*, Sullivan and McManus, 1986; Kiørboe and Nielsen, 1994; Guerrero and Rodríguez, 1998; Tachibana *et al.*, 2019). We just make our best assumptions when interpreting life cycle characteristics, on the basis of numerical correlations between seasonal abundance of free-swimming adult individuals, resting eggs in the sediment, and past winter or summer blooming of propagules. Hence, it would be interesting to be able to determine the ecological role of quiescent and diapause eggs by quantifying the extent to which they are used to cope with seasonality and/or unpredictable changes in the environment. Furthermore, it is important not only to understand embryonic dormancy strategies to cope with seasonality but also to understand how they respond to stochastic events—both systems with a high level and a low level of seasonality. Quantifying the individual variation due to genetic factors will, according to Avery (2005a), further enable us to assess how populations respond to directional environmental perturbations, such as those that may accompany climate change (Avery, 2005b). This would add to the knowledge on which variables shape the population dynamics of nearshore marine calanoid copepods producing resting eggs and how this might relate to the future environment. To simplify and conceptualize, all available information about abiotic and biotic covariates, as well as the eggs' intracellular molecular characteristics, should be integrated and interpreted by model results. For instance, García-Roger *et al.* (2006) reported a model approach with a rotifer as an example of a zooplankton with embryonic dormancy in its life cycle. They proposed that the abundances of

healthy-looking, hatched, and deteriorated diapausing eggs in the sediment are the result of a coupling between the sediment and the water column. They concluded that egg bank phenology in temporary habitats is the result of selective forces operating in both the water column and the sediment. Therefore, the abundance of the different types of (dormant) eggs in the sediment provides insights into those selective forces.

Relevant Model Species for Studying Copepod Embryonic Dormancy at Molecular Scale

Important criteria for selecting copepod model species with embryonic dormancy are that their population biology and physiology need to be well studied beforehand. Moreover, they have to exhibit widespread geographic distribution and have high tolerance to environmental variables, and we need to have a certain insight about them on a molecular scale. The cosmopolite *Acartia tonsa* (Fig. 6A) is the single most diverse and well-studied species in terms of factors inducing the production of resting eggs, also demonstrated by the numerous studies referred to in the present contribution. Its production of resting eggs can be induced in the laboratory by "unnaturally" strong signals provided by, for example, food availability (Drillet *et al.*, 2011b), crowding (Drillet *et al.*, 2015), abrupt salinity changes (Højgaard *et al.*, 2008; Ohs *et al.*, 2009), and temperature (Arndt and Schnese, 1986; Drillet *et al.*, 2006a; Berasategui *et al.*, 2013; Peck *et al.*, 2015). Recently, the largest genome of any copepod, *A. tonsa*, was assembled (Jørgensen *et al.*, 2019b). This species fulfills all of the stated requirements, which is why I recommend it as a particularly relevant model species. Response patterns similar to abiotic and biotic variables are exhibited by *Eurytemora affinis* (Fig. 6B), and it has been demonstrated to be even more responsive than *A. tonsa* to producing diapause eggs under very high crowding in the laboratory (Ban and Minoda, 1994). *Eurytermora affinis* is an egg sack carrier and is particularly tolerant to salinity changes; and it has invaded many freshwater habitats in North America, Europe, and Asia, primarily within the past 60 years (Lee, 1999, 2016). Its eggs are like the free-swimming stages—extraordinarily robust across

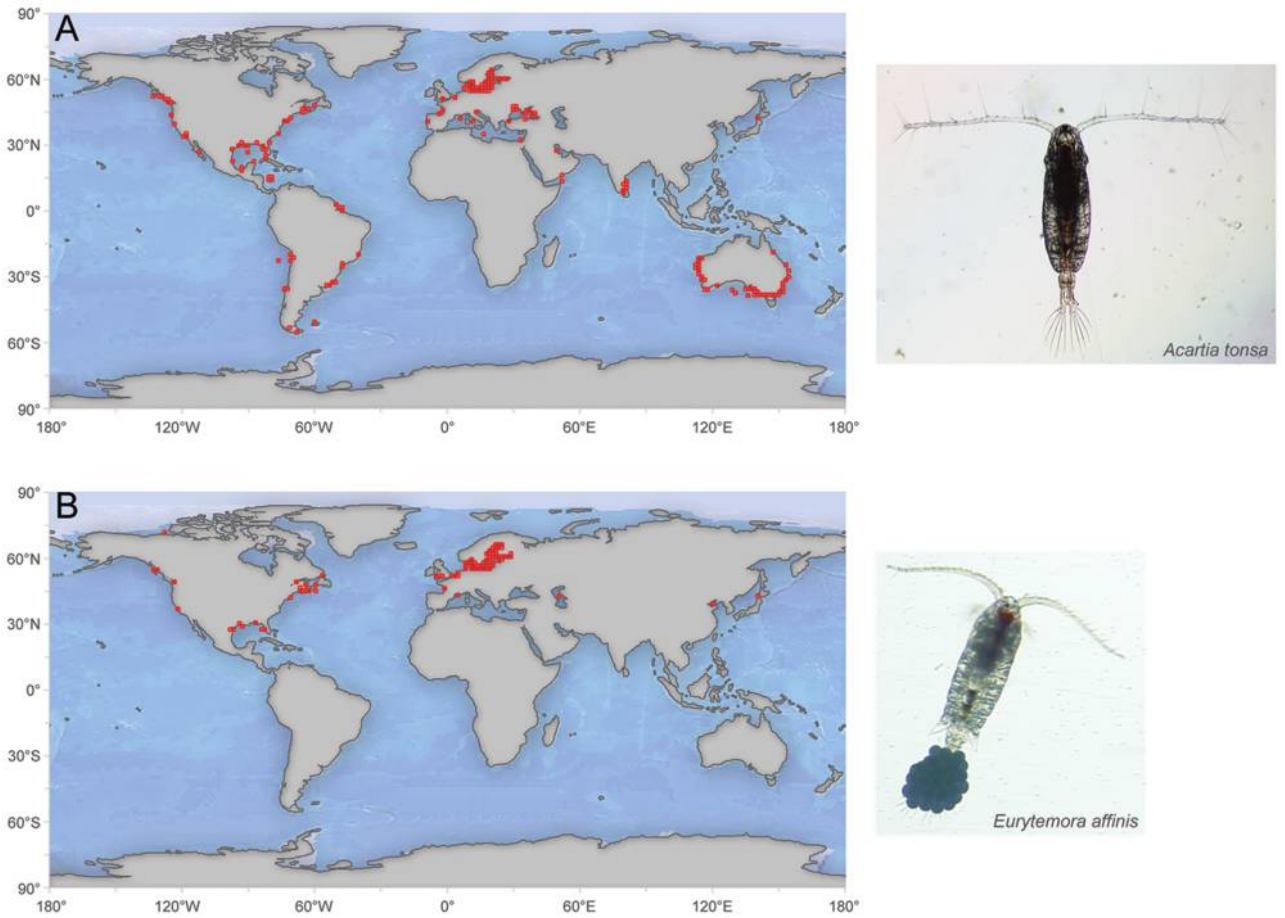

Figure 6. Images of females and geographical distribution of the proposed model species of calanoid copepods. (A) *Acartia tonsa*. (B) *Eurytemora affinis*. The zoogeographic distribution is from the World Register of Marine Species (WoRMS, 2019) and Razouls *et al.* (2015–2019). Photos are courtesy of Dr. Minh Thi Thuy Vu (Roskilde University, Denmark) and Professor Sami Souissi (Université de Lille, France).

a large salinity span (Højgaard *et al.*, 2017). It is also highly tolerant to other stress factors, such as temperature and pH (Hansen *et al.*, 2017). Hence, *E. affinis* is an opportunistic calanoid that I suggest be the other relevant model species. Additionally, the genome of this species has been sequenced; and, together with *A. tonsa*, their genomes, genome sizes, and transcriptomes are available (Rasch *et al.*, 2004; Bron *et al.*, 2011; Evans *et al.*, 2013). These two species enable researchers to work from established scaffolds for further molecular analysis, as already proposed almost 25 years ago by Dahms (1995). Using these organisms in the first place offers a unique chance to pursue a detailed molecular understanding of the mechanisms behind the resting egg phenomenon, with an ambition to later extrapolate obtained experience to all Centropagoidea species with resting eggs in their life cycles.

Future Perspectives and Applications

It is of imperative significance that the linkage between pelagic and benthic components of seasonal copepod population dynamics be thoroughly integrated into our ecological understanding. It is likely that resting eggs represent a significant gene pool partially hidden in marine sediments, ensuring effective gene preservation, in addition to a pool of ready-to-hatch propagules. This bentho-pelagic dimension was proposed more than 20 years ago by, for example, Boero *et al.* (1996) and Marcus and Boero (1998); and, if properly integrated in ecosystem research, it will immensely improve our perception of the significance of a group of key organisms in food web ecology (García-Roger *et al.*, 2006; Broman *et al.*, 2015).

Rearing marine finfish other than salmonids often requires live feed for their larval stages, and here copepods are the optimal solution (Conceicão *et al.*, 2010; Rasdi and Qin, 2014; Nielsen *et al.*, 2017). Copepods have the right size, behavior, and biochemical profile to sustain fish larvae; and they enable far better survival, reduce outbreaks of abnormalities, and maintain original wild-type pigmentation, all crucial for the fastest-growing food industry on Earth—aquaculture (Drillet *et al.*, 2011a). Copepods and their eggs can be mass-produced, even with room for profit (Støttrup *et al.*, 1986; Abate *et al.*, 2015; Sarkisian *et al.*, 2019). Since copepods are fragile organisms, their eggs in quiescence are the only way to store and transport the organisms from producers to hatcheries (Drillet *et al.*, 2006a). Quiescent eggs from *Acartia tonsa* and from *Centropages hamatus* can be cold stored for 7.5–17 months (Marcus and Murray, 2001; Drillet *et al.*, 2006a; Hagemann *et al.*, 2016); and, with a new protocol for *A. tonsa* (Jørgensen *et al.*, 2019a), one could imagine a storage time in years. Hence, the egg storage protocol would be optimized, with virtually no egg mortality. Realizing mass production and effective long-term storage of copepod eggs on a large scale would generate a positive disruption of the traditional live-feed concepts promoting the use of copepods in hatcheries worldwide. Calanoid copepods are nature's choice for marine fish larvae; undoubtedly, they can enrich hatcheries in their effort to optimize existing fish production and can contribute to diversification for the benefit of a "hungry world." Further optimization of storage and shipping of quiescent or even diapause eggs from more copepod strains and species would benefit from fundamental research results obtained on embryonic dormancy.

One of the most important sources of marine invasive organisms is through the spreading by ballast water (Charlton and Geller, 1993; Gollasch, 2007). In ballast water tanks, the environmental conditions are rather challenging for planktonic organisms. Dormant eggs from copepods spread worldwide from one ecosystem to another by ballast water. For instance, it is most likely the reason that one of the model species proposed here, *A. tonsa*, was introduced from American waters in the early twentieth century and is now dominating many European plankton communities (Remy, 1927; Segerstråle, 1957). Therefore, it is of utmost importance to gain knowledge about copepod embryonic dormancy at a molecular level (*e.g.*, Briski *et al.*, 2011) before an effective strategy to combat this worldwide problem can be realized.

Closing Remarks

Today's scientific community has, despite half a century of research, just a superficial understanding of copepod embryonic dormancy. This is primarily due to the fact that most of the previous research has approached the phenomenon by looking at the complex egg structures from the outside for categorization of egg types and by conducting more or less black box dose *versus* response incubations. The research seems quite voluminous, but new discoveries have been limited by the lack of the consistent categorization of eggs. For the majority of species, it is not possible to discriminate between their subitaneous and resting eggs; and, so far, no one has found unity in diversity. There is a need for taking the next step. Now the necessary tools are available to move beyond the descriptive phase by seriously entering the mechanistic phase and studying molecular processes in combination with physiological processes, to really understand why and to what extent an egg is not just an egg.

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