

Diapause within the Context of Life-History Strategies in Calanid Copepods (Calanoida: Crustacea)

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Abstract. Post-embryonic diapause in copepods is an adaptation that allows species in the copepod family Calanidae to thrive in high-latitude environments by transforming a short spring phytoplankton bloom into large numbers of lipid-rich individuals capable of surviving a long period of starvation. The copepods, with their high-energy lipid reservoirs, are a critical food source for higher trophic levels, making the Calanidae a key component of high-latitude marine ecosystems. The physiological ecology of the developmental program remains poorly understood. However, new studies using high-throughput RNA sequencing approaches are giving detailed access to physiological status by generating gene expression profiles for both field-collected and laboratory-incubated individuals. These are beginning to characterize the diapause phenotype, elucidate the transcriptional and physiological progression through the diapause program, and illustrate the effects of organism-environment interactions. This paper reviews gene expression profiling studies on the life cycle and diapause program of *Neocalanus flemingeri* Miller (1988) that were conducted as part of a long-term observation program in the northern Gulf of Alaska. It summarizes recent findings and relates them to the ecology of this species and to that of other calanids.

Introduction

Post-embryonic diapause is widespread in the high-latitude copepods in the families Calanidae and Eucalanidae. These copepods transform a short phytoplankton bloom into a lipid-rich food source that persists beyond the peak in primary production (Comer and Conover, 1968; Kattner *et al.*, 2007; Record *et al.*, 2018). They are near the base of the food chain in environments that support some of the largest and more sustainable commercial fisheries worldwide (Comer and Conover, 1968; Peterson *et al.*, 2014). Global climate change has raised questions regarding the resilience of these ecosystems, given the predicted changes in temperature and the rising frequency of extreme climatic events. Potential replacement of the lipid-rich copepods with less nutritious planktonic species is a major concern because of the likely impact on upper trophic levels (*e.g.*, Sigler *et al.*, 2016). Predicting the effects of climate change on these lipid-rich copepods requires an understanding of both how the environment will change and the effects of that on the life history and the biology of these lipid-rich copepods. The latter requires a better understanding of the physiology of diapause, which occurs at substantial depths of a few hundred to a thousand or more meters below the sea surface. A recent review by Baumgartner and Tarrant (2017) highlighted the limited understanding of the physiology of diapause in copepods, particularly in contrast to what is known about insect diapause (reviewed in Hahn and Denlinger, 2011; Denlinger and Armbruster, 2014). Unlike insects, diapausing copepods are difficult to access, and experimental induction of diapause has not been achieved (Baumgartner and Tarrant, 2017). However, even with these limitations, progress in understanding calanid diapause physiology has come from molecular approaches, such as the quantification of the relative expression of target genes using quantitative reverse transcription polymerase chain reaction and gene expression profiling by

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Abbreviations: CI–CV, immature copepodid stages: copepodite I to copepodite CV; DEG, differently expressed gene; OS0–OS4, stages of oocyte development; OS0, oogonia and previtellogenic oocytes; OS1, young vitellogenic oocytes in early stages of meiosis; OS2, oocytes characterized by ooplasm filled with yolk droplets; OS3, large oocytes with yolk and lipid vesicles; OS4, egg release; RNA-Seq, high-throughput sequencing of messenger RNA; Wk1–Wk5 females, females one week to five weeks after collection.

high-throughput sequencing technologies (Lenz *et al.*, 2014; Tarrant *et al.*, 2014, 2016; Häfker *et al.*, 2018; Roncalli *et al.*, 2018). Here we review recent studies on *Neocalanus flemingeri* Miller (1988) that investigated diapause preparation in the pre-adult stage, profiled active genes in the diapause phenotype, examined the termination of diapause, and traced the progression of the reproductive program after completion of diapause (Roncalli *et al.*, 2018). We summarize those findings here within the broader context of post-embryonic diapause in calanid copepods.

Diapause in the Family Calanidae

Diapausing copepods in the family Calanidae at high latitudes in the northern Pacific, northern Atlantic, and Arctic Oceans belong to one of two genera: *Calanus* or *Neocalanus*. Their abundances typically peak in the spring or early summer because their life cycle is synchronized to the annual phytoplankton bloom. Diapause is facultative in some calanids, although when and what triggers the diapause program remains unknown (Baumgartner and Tarrant, 2017). *Calanus* species inhabiting temperate latitudes (*e.g.*, *Calanus finmarchicus*, *Calanus pacificus*, *Calanus marshallae*) have population cycles that can include multiple generations per year (Conover, 1988). In *C. finmarchicus*, pre-adult individuals (late copepodite, stage CV) become increasingly lipid rich as the summer progresses, suggesting that by June or July the majority are on the diapause program (Marshall and Orr, 1955; Miller *et al.*, 1991; Meise and O'Reilly, 1996). Disappearance of the CV copepodites from the upper hundred meters correlates with the accumulation of individuals at depth (Miller *et al.*, 1991). After ontogenetic migration to deep waters, the CV copepodites enter a period of low metabolism and ar-

rested development (Hirche, 1996). At the northern end of their range (*e.g.*, the Norwegian Sea), *C. finmarchicus* populations complete a single generation per year; and all copepodites are presumably on the diapause program (Tande, 1982; Tarrant *et al.*, 2016). One-year life cycles are typical for the three subarctic Pacific *Neocalanus* species, while the arctic/subarctic *Calanus glacialis* has a one- or two-year life cycle; and the arctic *Calanus hyperboreus* has a two- to four-year life cycle (Hirche, 1997; Kosobokova, 1999; Arnkværn *et al.*, 2005). While a direct development program is absent or very rare in these species in their natural habitat, individuals in long-term cultures do not diapause, suggesting that the diapause program is nevertheless facultative (Hirche, 2013).

The majority of calanids enter diapause in the pre-adult stage (CV), and maturation into the adult stage occurs at depth and soon after emergence from the dormant phase (Table 1). However, exceptions to this pattern occur in both *Calanus* and *Neocalanus* (Table 1). A small proportion of *C. finmarchicus* enters diapause in the CIV stage (Tande, 1982), while *C. glacialis* and *C. hyperboreus* diapause in multiple copepodite stages (CIII–CV) and in the adult stage (Hirche, 1991; Hirche and Niehoff, 1996). In the subarctic *Neocalanus flemingeri*, the primary stage that enters diapause is the adult female; but individuals in a variant population (“large form”) diapause in the CIV copepodite stage (Miller and Clemons, 1988; Miller and Terazaki, 1989; Kobari and Ikeda, 2001).

Cost of Reproduction in Diapausing Calanids

Diapause is a strategy that postpones reproduction to optimize the probability of survival and fitness of the next generation. In insects, lipid stores accumulated during the preparatory

Table 1

Summary of diapause and reproductive strategies in copepods in the family Calanidae inhabiting temperate to polar oceans in the northern hemisphere

Species	Primary habitat	Diapause stage	Life span	Type of breeder	Sources
<i>Calanus helgolandicus</i>	Eastern Atlantic	CV	Months	Income*	1, 2, 3
<i>Calanus finmarchicus</i>	North Atlantic	CV	Months to 1 year	Capital/income, income	1, 4
<i>Calanus glacialis</i>	Subarctic Atlantic	CV	1–2 years	Capital/income, income	1, 4
<i>Calanus hyperboreus</i>	Subarctic Atlantic, Arctic	Multiple stages	2–4 years	Capital	1, 4
<i>Calanus pacificus</i>	Pacific	CV	Months	Income*	5
<i>Calanus marshallae</i>	Pacific	CV	Months	Income*	1, 6
<i>Neocalanus flemingeri</i> (small form)	Subarctic Pacific	Adult female	1 year	Capital	7
<i>Neocalanus flemingeri</i> (large form)	Western Pacific	Adult female, CIV	1–2 years	Capital	7, 8, 9
<i>Neocalanus plumchrus</i>	Subarctic Pacific	CV	1 year	Capital	7
<i>Neocalanus cristatus</i>	Subarctic Pacific	CV	1 year	Capital	7

The most common diapausing stage is listed for each species. Estimates of life spans are based on laboratory studies and field observations and include both “direct” and “diapause” developmental programs (differences in life span between directly developing vs. diapausing copepods are expected). Breeding type refers to the source of energy used to meet cost of reproduction, where income is current food resources, income/capital is the combination of stored energy and new food resources, and capital is stored energy. Classification of breeding type includes diapausing and direct developing individuals, but information is incomplete for some species and subspecies (marked by an asterisk).

Sources: 1, Baumgartner and Tarrant, 2017; 2, Rey-Rassat *et al.*, 2002; 3, Bonnet *et al.*, 2005; 4, Niehoff *et al.*, 2002; 5, Runge, 1984; 6, Peterson, 1988; 7, Mackas and Tsuda, 1999; 8, Miller and Terazaki, 1989; 9, Kobari and Ikeda, 2001.

phase fuel months of diapause; and after emergence, they contribute resources to reproduction even though feeding has resumed (Zhou and Miesfeld, 2009). A similar dependence on stored lipids after emergence from diapause occurs in copepods.

Most calanoid copepods are iteroparous and income breeders; egg production is low when food abundances are low but increases rapidly in response to new food resources (e.g., Checkley, 1980; Hirche, 1989; Mauchline, 1998; VanderLugt *et al.*, 2009). Income breeding occurs in the genus *Calanus* in individuals developing directly (Table 1); egg production rates are related to food levels (Hirche *et al.*, 1997; Niehoff, 2004), consistent with field observations of high egg production rates during phytoplankton bloom conditions (Niehoff *et al.*, 1999). However, in diapausing individuals, remaining lipid stores contribute significantly to egg production initially (Hirche and Kattner, 1993; Miller *et al.*, 1998; Niehoff *et al.*, 2002). Early stages of oogenesis can be completed before any evidence of significant feeding. *Calanus glacialis* can produce some eggs in the absence of new resources, while completion of oogenesis in *Calanus finmarchicus* requires at least some new food resources (Tourangeau and Runge, 1991; Niehoff *et al.*, 2002). This mixed income and capital breeding strategy occurs in early spring when food availability is variable because the timing of the phytoplankton bloom varies.

While their reproductive strategies differ, *Calanus hyperboreus* and all three *Neocalanus* species are exclusively capital breeders (Table 1). Reproduction in *C. hyperboreus* occurs during the winter and is fueled by lipids accumulated during the previous year's phytoplankton bloom (Niehoff *et al.*, 2002). After the reproductive period, *C. hyperboreus* females resume feeding during the spring phytoplankton bloom and for one or more years repeat the cycle of diapause followed by egg production during the winter (Hirche, 1997, 2013; Niehoff *et al.*, 2002). In contrast, the three north Pacific *Neocalanus* species have non-feeding adults, and reproduction is linked to end of life. The pre-adult stage (copepodite CV) migrates to deep water and remains at depth for the rest of its life (Miller *et al.*, 1984; Miller and Clemons, 1988; Mackas and Tsuda, 1999; Tsuda *et al.*, 1999).

Case Study: *Neocalanus flemingeri* Miller (1988)

Life cycle and ecology

Neocalanus flemingeri Miller (1988) was recognized as a species separate from *Neocalanus plumchrus* in the 1980s (Miller, 1988). While zooplankton surveys do not always differentiate between the two species, their life histories differ in several ways (Tsuda *et al.*, 1999). In the Gulf of Alaska, *N. flemingeri* is a rich food source for larval and forage fishes and invertebrates (Cooney *et al.*, 2001; Coyle and Pinchuk, 2003, 2005; Sigler *et al.*, 2016). With a single generation per year, this species has synchronized its life cycle to the annual spring phytoplankton bloom (Fig. 1). Naupliar stages

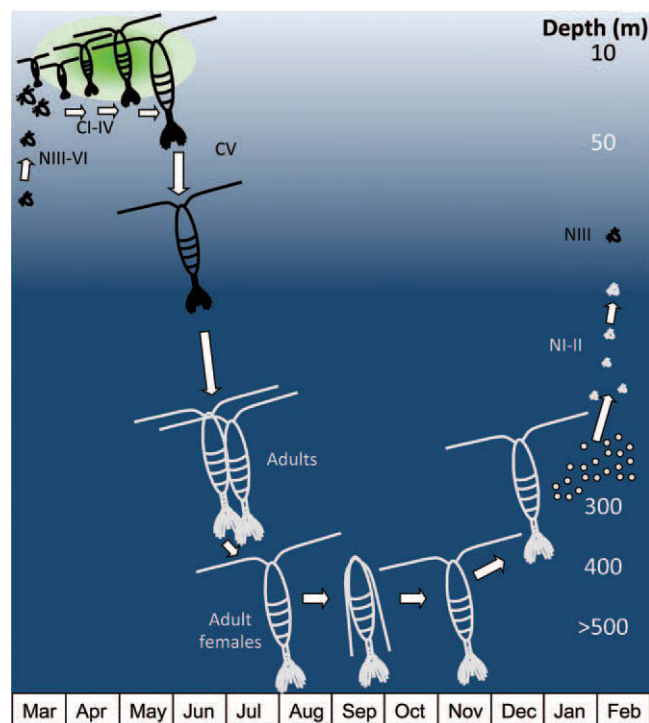


Figure 1. Schematic diagram of the life cycle of *Neocalanus flemingeri* in the Gulf of Alaska. Feeding naupliar (NIII to NVI) and copepodite (CI to CV) stages are shown in black; non-feeding stages (naupliar stages NI and NII and male and female adults) are shown in light gray. Green oval indicates phytoplankton bloom. Depths in meters (not to scale) are indicated on the right side. Temporal progression is indicated by months on the bottom (March–February). Timing of life-history cycle and vertical distribution is based on Miller and Clemons (1988), Cooney *et al.* (2001), Coyle and Pinchuk (2003), and Liu and Hopcroft (2006). Timing of annual phytoplankton bloom is based on Coyle and Pinchuk (2005), Strom *et al.* (2006), and Waite and Mueter (2013).

appear in the upper water column in March (Miller and Clemons, 1988; Kobari and Ikeda, 2001). Between March and May, progression through the copepodite developmental stages and accumulation of lipid stores coincide with increased primary production and the annual phytoplankton bloom (Liu and Hopcroft, 2006; Strom *et al.*, 2006, 2016). Region-wide increases of biomass in *N. flemingeri* are typically 20-fold or greater (Coyle and Pinchuk, 2003). In May, the population is dominated by the pre-adult CV stage; and ontogenetic migration to depth (≥ 400 m) starts in early June, although the time of descent may vary depending on location and environmental conditions (Miller and Clemons, 1988; Tsuda *et al.*, 1999; Kobari and Ikeda, 2001). At depth, CV individuals molt into the adult stage and mate. By early July, few males remain, and females enter into diapause (Kobari and Ikeda, 2001). Females in advanced stages of gonadogenesis become abundant starting in December, coinciding with a move to a shallower depth (250–500 m instead of >500 m; Fig. 1; Miller and Clemons, 1988). Peak egg production occurs in January and February (Miller and Clemons, 1988; Kobari and Ikeda, 2001). Each female produces multiple clutches of eggs (up to 6) that are released over a 1- to 2-month period, with an average

lifetime fecundity of >900 eggs per female (Saito and Tsuda, 2000). By March, the number of adult females has decreased by an order of magnitude, and those that remain are mostly “spent” (= post-reproductive) (Miller and Clemons, 1988). *Neocalanus flemingeri* females spend their entire adult life span of seven to nine months at depth.

The species inhabits a region characterized by complex hydrography leading to a heterogeneous environment that includes gradients in nutrient availability, in primary production, and in phytoplankton standing stocks (Weingartner *et al.*, 2002, 2009; Strom *et al.*, 2006, 2007, 2016; Coyle *et al.*, 2013; Waite and Mueter, 2013). Off-shelf oceanic waters are dominated by small phytoplankton species, while larger diatoms and heterotrophic ciliates are more common in coastal waters, as determined by size-fractionated chlorophyll *a* and microscopy (Strom *et al.*, 2016). However, these patterns in productivity are highly dynamic because of the mixing of currents and the formation of eddies (Weingartner *et al.*, 2009; Waite and Mueter, 2013). Depending on the location and/or water mass, individual *N. flemingeri* may experience one to two orders of magnitude difference in food resources (Mackas and Coyle, 2005; Coyle *et al.*, 2013). While *N. flemingeri* occurs throughout the region, it feeds selectively on large food particles (>20 μm), such as ciliates and diatoms (Dagg *et al.*, 2009). Thus, development through four feeding naupliar stages and five copepodite stages, while accumulating lipids, occurs in an environment in which primary production is variable and preferred food particle sizes can be uncommon—in particular in the offshore areas that are deep enough for diapause (>400 m; Figs. 1, 2; Miller and Clemons, 1988).

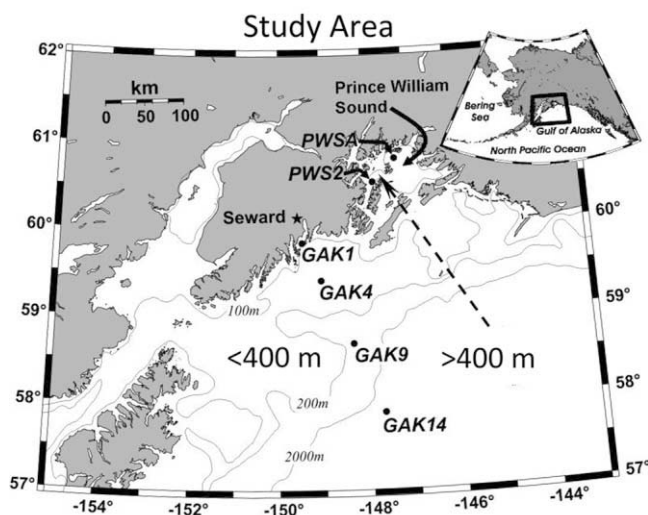


Figure 2. Map of the northern Gulf of Alaska marking the location of collection stations sampled for pre-adult *Neocalanus flemingeri* individuals (copepodite stage CV) in May 2015 for gene expression profiling. Depth labels indicate areas that are either deep enough for diapause (>400 m) or not (<400 m) (note 200- and 2000-m contour lines). Dashed arrow indicates location of a deep trough in Prince William Sound (PWS), where diapausing females were collected in September 2015. Inset shows location of study area within the Gulf of Alaska (GAK).

Preparation for diapause in a heterogeneous environment

Global gene expression patterns in combination with functional analysis generate a snapshot of the transcriptional profile for an organism at the time of collection and preservation. In the spring of 2015, we used gene expression profiling of individual stage CV individuals as a proxy for how physiological status and state of diapause preparation varied across an inshore-offshore environmental gradient in food resources (Fig. 2; National Center for Biotechnology Information [NCBI] BioProject PRJNA496596; Roncalli *et al.*, 2019). During a six-day period (May 5–10, 2015) *Neocalanus flemingeri* was collected from six stations in conjunction with the Seward Line long-term observation program (Sousa *et al.*, 2016). The stations ranged from a fjord (Prince William Sound) to an off-shelf location along the Seward Line (GAK14; Fig. 2). Similarly to other years, there was a strong gradient in phytoplankton abundances measured as chlorophyll *a* (mg m^{-3}) from Prince William Sound (PWS2, PWSA) to the outer shelf in the Gulf of Alaska (GAK9, GAK14). This gradient was particularly pronounced in the large-sized cells (>20 μm ; Fig. 3A; Roncalli *et al.*, 2019), which are preferred by *N. flemingeri* (Dagg *et al.*, 2009).

High-throughput sequencing of messenger RNA (RNA-Seq) generated between 10 million and 20 million short-sequence reads (150 base pairs) per individual, which were mapped against a *N. flemingeri* reference transcriptome (Roncalli *et al.*, 2019). Gene expression profiles were then compared with each other by using a clustering program (hclust; Müllner, 2013). Individuals grouped mostly by collection site, indicating that transcriptional profiles of individuals from the same station were more similar to each other than were those between stations and/or regions. These differences were consistent with the hypothesis that the CV copepodites were responding to environmental differences through physiological acclimation.

Accumulation of lipid stores is a signature process of diapause preparation in both insects and copepods (Hahn and Denlinger, 2007; Johnson *et al.*, 2008), and this process is characterized by the upregulation of genes involved in lipid synthesis and transport (Hahn and Denlinger, 2011; Denlinger and Armbruster, 2014; Tarrant *et al.*, 2016; Tan *et al.*, 2017). Functional analysis of gene expression patterns suggested that individuals differed in their preparation for diapause: genes involved in lipid metabolism and development were over-represented (= enriched) among the differentially expressed genes (DEGs). Examples of how relative expression of a lipid synthesis gene (*FABP6*, fatty acid binding protein 6), a lipid degradation gene (*HOAD*, 3-hydroxyacyl-coa dehydrogenase), and a protein degradation gene (*Endopeptidase Clp*) differed among stations are shown in Figure 3B–D. These expression profiles suggest that individuals collected in Prince William Sound were building lipid stores, as expected during diapause preparation. In contrast, the individuals from the most offshore stations showed not only low expression of lipid synthesis

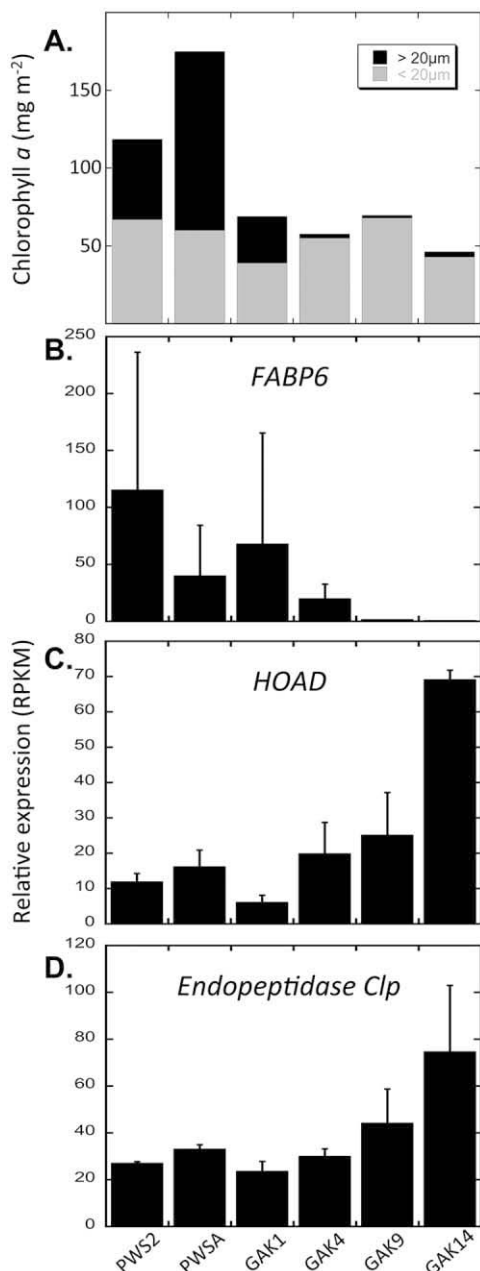


Figure 3. Size-fractionated chlorophyll *a* levels and relative expression of selected *Neocalanus flemingeri* protein-encoding genes involved in lipid metabolism and protein degradation. (A) Integrated chlorophyll *a* concentrations (mg m⁻²) between subsurface and 50 m showing 2 size fractions: small (<20 μm, gray) and large (>20 μm, black). Chlorophyll *a* concentrations were provided by S. Strom (Western Washington University) and are part of the Seward Line long-term observation program (Sousa *et al.*, 2016). (B) Lipid synthesis: fatty acid binding protein (*FABP6*). (C) β oxidation (lipid catabolism): 3-hydroxyacyl-CoA dehydrogenase (*HOAD*). (D) Protein degradation: *Endopeptidase Clp*. Relative gene expression was measured in pre-adult *N. flemingeri* individuals (copepodite stage CV), and replicate samples for each station were averaged. Relative expression is given in RPKM (reads per kilobase per million reads). Error bars are standard deviations (data available through National Center for Biotechnology Information [NCBI] BioProject PRJNA496596; NCBI, 2018; Roncalli *et al.*, 2019). GAK, Gulf of Alaska; PWS, Prince William Sound.

genes but also upregulation of genes involved in lipid and protein degradation (Fig. 3B–D). Their pattern of gene expression suggests that in these CV individuals, lipid accumulation was depressed and energetic demands were being met by catabolizing stored energy sources. Thus, accumulation of lipid stores in preparation for diapause was occurring in Prince William Sound, where chlorophyll *a* levels in the >20-μm size fraction was high, but not under the low chlorophyll *a* conditions present at the offshore stations at the time of collection. The differences in gene expression are the first measurements linking environmental conditions to a key aspect of diapause preparation: lipid metabolism. These observations suggest that preparation for diapause in *N. flemingeri* may be compromised in the high-nutrient, low-chlorophyll waters characteristic of the oceanic waters in the Gulf of Alaska. This is consistent with Mackas and Coyle's (2005) analysis, which suggests that copepods typically experience nonoptimal conditions, which in turn are likely to lead to variations in their distribution, survivorship, and condition.

Diapause phenotype

Diapausing individuals of *N. flemingeri* are found below 400 m in the Gulf of Alaska and other oceanic regions across the subarctic Pacific (Miller and Clemons, 1988; Miller and Terazaki, 1989; Tsuda and Mackas, 1999; Kobari and Ikeda, 2001). They are also abundant in the deep areas in western Prince William Sound (Cooney *et al.*, 2001), where adult females were collected in late September from 400–700 m (e.g., station PWS2, Fig. 2). Mechanical, chemical (temperature), and photic sensory stimulation during collection effectively activated the termination of diapause in *N. flemingeri* within an hour or less. This is clear from the change in posture and behavioral responsiveness; initially, the females are suspended vertically, with their antennules (A1; first antennae) pointing down and their urosome bent somewhat dorsally (Fig. 4A–C). The pereopods are adducted against the prosome, and the females are behaviorally nonresponsive. Within an hour, the A1 individuals are deployed laterally (Fig. 4D); and, while the females continue to be inactive, they respond with weak escapes to physical stimulation. Emergence from diapause has been described for *Calanus finmarchicus* and includes similar transition from this diapause posture and lack of behavioral responsiveness to increased behavioral activity and molting (Grigg and Bardwell, 1982; Hirche, 1983).

We characterized the transcriptional profiles of *N. flemingeri* females at collection ("time zero") using RNA-Seq technology (Roncalli *et al.*, 2018). Compared with the post-diapause females, relative gene expression of time zero females showed many similarities with diapausing insects. That is, low expression was observed for genes involved in transcription, translation, DNA metabolism, cell division, and protein turnover. Specifically, low expression was measured in annotated genes identified as *helicase*, *DNA polymerase*, *ribosomal protein*,

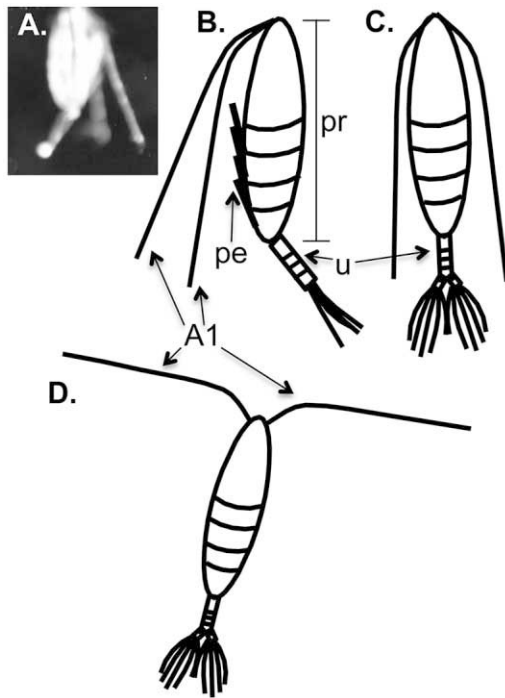


Figure 4. Image and schematic drawings showing the posture of adult female *Neocalanus flemingeri* upon collection from depth in September and after a short incubation in the laboratory (one or two hours). (A) Photographic side view showing vertical alignment and position of pereopods, urosome, and antennules (first antennae) of the diapause posture. (B, C) Schematic drawing of side and dorsal views of an adult female in the diapause posture. (D) Schematic drawing of female in dorsal view showing deployed antennules (this posture was observed in females within a few hours of collection and during incubation in the laboratory). A1, antennules (first antennae); pr, prosome (bar indicates length of prosome); pe, pereopods; u, urosome.

serine protease, *E3 ubiquitin*, *G2 cyclin*, *innexin 2*, and *neurogenic delta* (Roncalli *et al.*, 2018).

The diapause phenotype is characterized by an increased life span in insects and the regulation of “aging genes” (*e.g.*, Denlinger and Armbruster, 2014; Kučerová *et al.*, 2016). These genes have been identified as having “pro-longevity” or “anti-longevity” effects, and many are regulated during diapause (Ragland *et al.*, 2010). In *N. flemingeri*, we observed downregulation of several anti-longevity genes (*e.g.*, *edem 1*, *sirtuin 6*) in combination with the upregulation of pro-longevity genes (*e.g.*, *embryonic lethal abnormal vision*, *cheerio*) in time-zero females (Roncalli *et al.*, 2018).

Upregulation of genes involved in the cellular stress response is another characteristic of diapausing insects (Hahn and Denlinger, 2011; Sim and Denlinger, 2011; Košťál *et al.*, 2017). Genes encoding proteins involved in the protection against oxidative stress (superoxide dismutase) and ferritin were upregulated (Fig. 5); however, those encoding heat-shock proteins were not (not shown; Roncalli *et al.*, 2018). Similar to other diapausing arthropods, the gene encoding PEPCK (phosphoenolpyruvate carboxykinase) was upregulated. This enzyme is a key catalytic enzyme that provides a source

of glucose while also being involved in tolerance to low oxygen levels. In another recent study, the diapause phenotype was characterized for *C. finmarchicus* pre-adults (copepodite stage CV), using a target gene approach (Häfker *et al.*, 2018). One key finding was the absence of a diel cycle in circadian clock genes in deep-CV individuals between August and March. However, expression levels of genes such as *clock* (*clk*), *period1* (*per1*), *timeless* (*tim*), *cryptochrome2* (*cry2*), and *clockwork orange* (*cwo*) remained high between August and November, in spite of the fact that at least two of these genes are downregulated at night in May (*clk* and *cwo*) (Häfker *et al.*, 2018). In addition, Häfker *et al.* found high expression of *ferritin* (*fer*), *hemocyanin* (*hc*), *arginine kinase* (*argk*), and *HOAD* (*hydroxyacyl CoA dehydrogenase*) and low expression of *PEPCK* (*phosphoenolpyruvate carboxykinase*) in diapausing stage CV *C. finmarchicus* between August and November. While differences in the experimental design of the two studies limit comparisons, this does suggest the possibility of species-specific differences in gene expression patterns during diapause, as has been found among diapausing insects (*e.g.*, Košťál *et al.*, 2017).

Transition from diapause termination to reproduction

Diapausing *N. flemingeri* females can be collected from depth in good condition, with high long-term laboratory survival rates as they transition from diapause to egg release and end of life over a 10-week period (Roncalli *et al.*, 2018). The physiological changes from diapause to egg production were tracked in laboratory-incubated females by using gene expression profiling (Roncalli *et al.*, 2018). Prior to preservation for gene expression analysis, females were examined microscopically for morphological changes related to the progression of oogenesis through five oocyte developmental stages (OS0–OS4; Niehoff, 2007).

The first sign of diapause termination was the behavioral change in posture (Fig. 4), which occurred within minutes to hours of collection. Changes in transcriptional physiology were investigated by identifying differentially expressed genes, followed by “enrichment analysis.” This analysis identifies biological processes associated with specific gene ontology terms that are over-represented among the DEGs by comparing the number of expected DEGs with the number of observed DEGs within functional categories. Figure 5 provides a summary of the changes observed in the females between collection and egg release, which started 7.5 weeks after collection. Upregulated (upward arrow, gray boxes, Fig. 5) and downregulated (downward arrow, white boxes, Fig. 5) biological processes are based on differential gene expression between time zero females and those from all other weeks. Large gray arrows (Fig. 5) indicate processes that were differentially expressed in females from a single week.

Females one week after collection (termed Wk1 females) exhibited gene expression changes indicating that (1) the

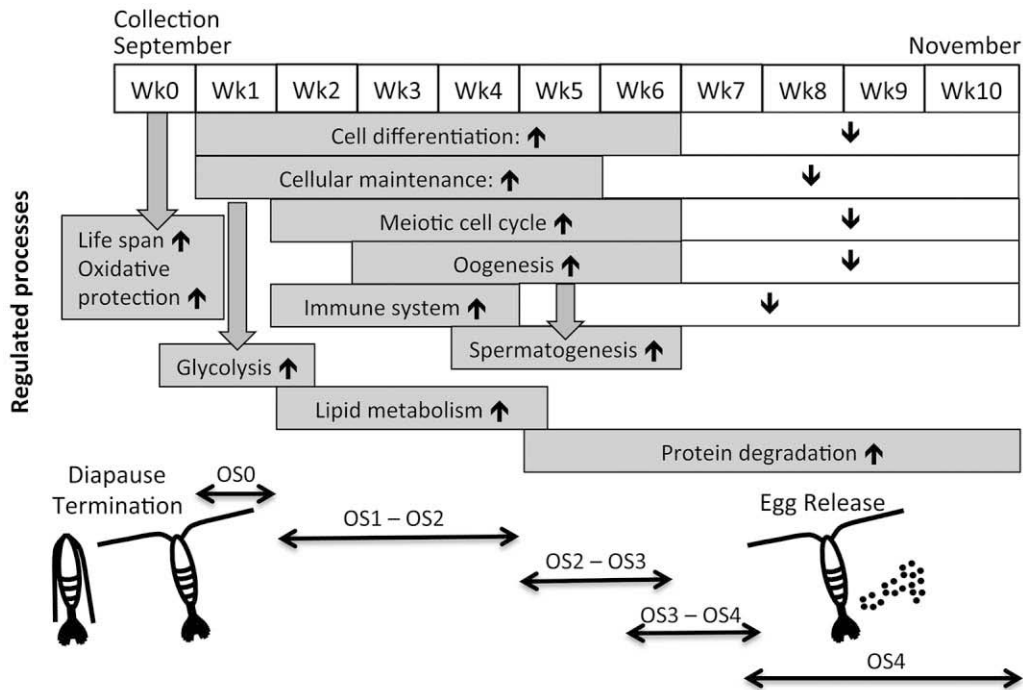


Figure 5. Diagrammatic representation showing regulated biological processes during the developmental progression from collection (Wk0) to egg release and end of life span (Wk10). Labels on the top show the time line from collection (Wk0, September 20, 2015) to Wk10 (November 26, 2015). Boxes below list biological processes that were either upregulated (upward arrow, gray boxes) or downregulated (downward arrow, white boxes) in females harvested at weekly or semiweekly intervals starting at collection. The lower panel shows the proposed oocyte developmental stages (OS0–OS4) matched to microscopic observations and differential gene expression patterns. Nomenclature follows stages based on histological studies of calanoid copepods: OS0, oögonia and previtellogenic oocytes; OS1–OS2, oocytes in vitellogenesis 1; OS3–OS4, oocytes in vitellogenesis 2; OS4, mature oocytes (Niehoff, 2007).

reproductive program had been activated, (2) metabolic activity had increased, and (3) genes involved in cellular maintenance were upregulated (Fig. 5; Roncalli *et al.*, 2018). Activation of the reproductive machinery was characterized by the upregulation of genes involved in germline formation and cell differentiation, coinciding with the upregulation of genes involved in glycolysis and innate immunity (Fig. 5).

Starting in Wk2 females, upregulated processes included several that were more specifically associated with reproduction and oogenesis (Fig. 5; Roncalli *et al.*, 2018). Another transition in gene expression between Wk1 and Wk2 females was the upregulation of lipid metabolism (Fig. 5). Notable transitions between Wk4 and Wk5 females included downregulation of genes involved in the immune system and upregulation of genes involved in protein degradation, which persisted thereafter (Fig. 5). Upregulation of genes involved in spermatogenesis occurred in Wk5 females (Roncalli *et al.*, 2018). In *Drosophila melanogaster* these regulated genes encode proteins that are involved in sperm development (*Ago3* and an *ATP-dependent helicase*), which are usually male specific. A third transcript encodes a *testis serine protease*, which regulates sperm-egg interaction. While the exact function of these genes is unknown in copepods, one interpretation of the observed expression pattern is that the stored sperm in

N. flemingeri undergo a type of dormancy. In response to physiological changes in the female, sperm become activated and complete their development in the female as oocyte development reaches the OS3 stage.

In the transition between oocyte developmental stages OS2 and OS3, enrichment analysis suggests a trade-off between the cost of reproduction and cellular homeostasis: biological processes such as innate immunity and cellular maintenance (*e.g.*, muscle function) became downregulated starting in Wk5 females (Fig. 5; Roncalli *et al.*, 2018; VR and PHL, unpubl. data). As development advances to the fourth and fifth stage of oocyte development (OS3 and OS4), genes and processes that were upregulated during the early stages of oogenesis became downregulated (Fig. 5). This gene expression pattern suggests that in *N. flemingeri* the early stages of oocyte development (OS0–OS2) are terminated before the release of the first clutch of eggs. In contrast, multiple stages of oocyte development are observed concurrently in income-breeding copepods that are actively producing broods (Niehoff, 2007).

Summary

Large copepods are key members of high-latitude marine ecosystems; they translate short peaks of primary production

into long-lasting lipid-rich bodies that are consumed by other invertebrates, fishes, marine mammals, and seabirds (Conover and Corner, 1968; Kattner *et al.*, 2007; Jónasdóttir *et al.*, 2015; Record *et al.*, 2018). Lipid storage occurs in complex life histories that include a seasonal diapause and a dependence on “capital” energy to partly or completely fuel reproduction 2 to 10 months after the previous spring phytoplankton bloom. Furthermore, non-feeding nauplii (NI and NII) depend on maternal lipids; and in species such as *Neocalanus plumchrus*, these lipids fuel a prolonged dormancy in the nauplii (Fujioka *et al.*, 2015). Whether these copepods can persist in their environments with predictions of higher temperatures and an increase in extreme climatic events remains an open question. In a recent review, Baumgartner and Tarrant (2017) highlight existing knowledge gaps in the understanding of copepod diapause, especially in comparison with studies on terrestrial insects.

Focusing on one species, the copepod *N. flemingeri*, we find that high-throughput gene sequencing has provided basic information on the physiological transitions from diapause to egg production. Gene expression patterns during oogenesis were similar to those described for the model fruit fly (*Drosophila melanogaster*), which has been studied in detail, providing links between gene expression and progression through the reproductive program. Furthermore, transitions between phases of oogenesis were correlated with changes in the expression of genes involved in maternal investment in cellular function and sources of energy. Similar gene expression patterns may occur in other copepods, although members of the genus *Calanus* continue to feed as adults. In that genus, reproduction after diapause is fueled by a combination of stored and new resources (Niehoff *et al.*, 2002).

In high-latitude environments, lipid-rich copepods such as *N. flemingeri* synchronize their life cycle to the annual phytoplankton bloom. The active growing season for the copepod is short (about three months; Kobari and Ikeda, 2001). Gene expression profiles of pre-adult CV individuals collected in early May suggest that diapause preparation progresses at different rates along resource gradients in the northern Gulf of Alaska. The environment typically includes differences in the timing of the bloom and order of magnitude differences in the availability of large phytoplankton cells, a preferred food (Mackas and Coyle, 2005; Strom *et al.*, 2006; Waite and Mueter, 2013). While the copepods at the off-shelf station were active and appeared healthy, the transcriptional signals measured in 2015 suggest that resources may have been insufficient to build lipid stores necessary for diapause and reproduction. However, sampling was limited to a five-day period, thus providing a snapshot of a single time point. More temporal information would have been needed to determine whether off-shelf resource-limited conditions persisted through the remaining growth period. The ecophysiological data for *N. flemingeri* have provided context for the plasticity in life-history traits observed both within and between species (Baumgartner and Tarrant, 2017). *Neocalanus flemingeri*, similarly to other calanid cope-

pods, has evolved under conditions of spatial and temporal variability in food availability. Its ability to acclimate physiologically may be a key adaptation underlying its success and potential for persistence in a changing environment.

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