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Reduced up-regulation of gene expression in response to elevated temperatures in the mid-Atlantic population of *Calanus finmarchicus*

Irina Smolina [a,*], Rachel Harmer [b,1], Penelope Lindeque [b], Galice Hoarau [a]

[a] Faculty of Biosciences and Aquaculture, Nord University, 8049 Bodø, Norway
[b] Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL13DH, UK

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ABSTRACT

Climate change is affecting numerous species worldwide, including dominant and important copepods of the genus *Calanus*. Despite the growing body of studies that examine effects of climate change stressors on *Calanus* species, comparative intraspecific studies are lacking. Importantly, acclimatization and genetic adaptation can modify the stress response, thus leading to a differential response of separated populations to the same stressor. The molecular and physiological responses of a *C. finmarchicus* population from the mid-Atlantic, with an *in situ* temperature of 8.5 °C, were investigated under experimental thermal conditions of 0 °C, 5 °C, 10 °C, 15 °C, and 20 °C for durations of 3 h and 6 days. This experimental set-up mirrored previously published experiments conducted on *C. finmarchicus* at the northern limit of its distribution allowing a comparison between two populations. The greatest physiological response, assessed as fecal pellet production, was seen after 3 h exposure at 10 °C and 15 °C, and after 6 days exposure at 5 °C, 10 °C and 15 °C. Molecular response was assessed by the change in expression of 5 selected genes: *hsp70_2*, *dnaja1*, *nap 1l1*, *rps11*, and *gdh*. Only two out of the five genes (*gdh* and *nap1l1*) showed significant up-regulation with increased temperature and duration of exposure. These findings differ from the results obtained in the northern population where all 5 genes were differently expressed. Overall, the results suggest population-specific response to temperature in *C. finmarchicus*, however determining the source of such variation (genetic adaptation or acclimatization) requires more detailed studies.

1. Introduction

Planktonic marine copepods of the genus *Calanus* (Crustacea: Copepoda: Calanoida: Calanidae) largely dominate the zooplankton biomass in the global ocean (up to 70%) (Conover, 1988; Head et al., 2003; Uye, 2000). They play a key role in energy transfer from the primary production level to higher trophic levels (Beaugrand, 2009; Falk-Petersen et al., 2007), with many ecologically and commercially important fishes (e.g., Gislason and Astthorsson, 2002; Søreide et al., 2008; Uye, 2000), as well as invertebrates, birds and marine mammals (Michaud and Taggart, 2007; Skjoldal et al., 2004; Weslawski et al., 1999) depending on *Calanus* as a food source. In the North Atlantic Ocean, *Calanus finmarchicus* (Gunnerus, 1770) is the dominant *Calanus* species where it plays a central role in trophic dynamics (Falk-Petersen et al., 2007; Hirche and Kosobokova, 2007), and has been the focus of several basin-scale research programs (Gifford et al., 2010; Melle et al., 2014; Tande and Miller, 2000). Distribution of *C. finmarchicus* ranges from the Scotian Shelf and Gulf of Maine in the south to the Barents Sea, Greenland Sea and Buffin Bay in the north. The highest population densities of *C. finmarchicus* are found along the south-west to north-east axis of the North Atlantic where temperatures range between 4 °C and 9 °C (Planque and Batten, 2000; Sundby, 2000). Since the 1960s a consistent northward shift of *C. finmarchicus* distribution has been detected (Beaugrand et al., 2002; Chust et al., 2014). In the North Sea this decrease in the abundance of *C. finmarchicus* has been associated with an increase of the more southern species *C. helgolandicus*, which has a lower lipid content and different phenology than *C. finmarchicus* (Reid et al., 2003; Bonnet et al., 2005). Such replacement has had negative consequences for the recruitment of commercially important fish stocks such as cod, *Gadus morhua* and salmon, *Salmo salar* (Beaugrand and Reid, 2003; Olsen et al., 2011), as well as for the biological carbon pump (Beaugrand et al., 2010). Furthermore, with the continuing change of climate, a reduction in *C. finmarchicus* abundance and a further northward shift are predicted (Reygondeau and Beaugrand, 2011a; Villarino et al., 2015). Such distributional changes of *Calanus* species are expected to lead to significant changes in food-web dynamics and secondary production, particularly at the southern and northern edges of distribution, since co-occurring *Calanus* species differ in energy-rich lipid content and phenology (Conover, 1988; Falk-Petersen et al., 2007, 2009).

* Corresponding author.
E-mail address: irina_smolina@hotmail.com (I. Smolina).
[1] Present address: Institute of Hydrobiology and Fisheries Science, University of Hamburg, Olbersweg 24, 22,767 Hamburg, Germany.

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In recent years, much attention has been given to organisms' responses to climate change. Experimental manipulation is one way to study a species tolerance to various components of the climate, and to forecast consequences at both the species and ecosystem level. Accordingly, the responses of several *Calanus* species to various climate stressors such as temperature (Grote et al., 2015; Hildebrandt et al., 2014; Kjellerup et al., 2012; Smolina et al., 2015), ocean acidification (Hildebrandt et al., 2014; Pedersen et al., 2013; Runge et al., 2016; Weydmann et al., 2012), and harmful algal blooms (Lauritano et al., 2012, 2015; Roncalli et al., 2016) have already been studied. Within these studies considerable attention has been devoted to temperature, since it affects every aspect of the zooplankton life, from cellular processes to behaviour, development and fitness (Clarke, 2003; Huntley and Lopez, 1992), as well as distributional shifts of *Calanus* (Chust et al., 2014; Helaouët et al., 2011; Reygondeau and Beaugrand, 2011a). Recently, a first insight into the molecular response of *C. finmarchicus* from the northern limit of its distributional range (*in situ* temperature of 0 °C) to elevated temperatures was obtained (Smolina et al., 2015). The study showed that this population had a strong transcriptomic response to an increase in temperature (from 0 °C to 15 °C) involving up-regulation of genes related to protein folding, transcription, translation and metabolism, and suggested 5 °C as an optimum temperature. Physiological experiments on *C. finmarchicus* from the same area support this suggestion (Hjorth and Nielsen, 2011; Kjellerup et al., 2012), while studies from warmer areas suggest 10–12 °C as an optimal temperature for *C. finmarchicus* (Harris et al., 2000; Møller et al., 2012). Given these observations and the fact that the distribution of *C. finmarchicus* is characterised by geographically varying ranges of temperature, salinity and light conditions (Melle et al., 2014), it is likely that response to environmental factors in *C. finmarchicus* is population-specific.

Studies comparing the response of two or more populations of marine invertebrates to climatic stress are limited, but those that exist tend to support a population-specific stress response (e. g., Cottin et al., 2015; Gleason and Burton, 2015). For example, population-specific responses in copepods have been shown for populations of *C. helgolandicus* (Lauritano et al., 2012) and *Acartia hudsonica* (Colin and Dam, 2007) fed a toxic algae diet, *Pseudocalanus acuspes* exposed to ocean acidification (Thor and Oliva, 2015), and *Tigriopus californicus* exposed to elevated temperatures (Hong and Shurin, 2015; Schoville et al., 2012). Such variation in population response to environmental stressors could be due to acclimatization via phenotypic plasticity or genetic adaptation (Kelly et al., 2012; Sternberg and Thomas, 2014), and this knowledge gap is limiting our capacity to accurately predict species responses to climate change.

The effects of temperature increase on *C. finmarchicus* distribution are documented by Chust et al. (2014) and a further northward shift is predicted, particularly at the southern edge of distribution where seawater temperatures might exceed the thermal niche for *C. finmarchicus* for several months in a year by 2100 (Helaouët et al., 2011; Reygondeau and Beaugrand, 2011a). Thus, knowledge on physiological and gene expression plasticity among populations of *C. finmarchicus* to elevated temperatures may be of particular interest and importance to reduce uncertainties of climate change impacts. Here, a population of *C. finmarchicus* from the mid-Atlantic was subjected to a series of thermal conditions that mimic experiments performed earlier with *C. finmarchicus* sampled from the Arctic (Smolina et al., 2015). The response was assessed at the physiological (fecal pellet production, a proxy for grazing rate) and molecular (gene expression of 5 selected genes) levels. The first aim was to assess the response of a *C. finmarchicus* population from the mid-range of its distribution (8.5 °C *in situ* temperature) to the temperature tolerance range of the species (−1.8 °C–21 °C; Hirche, 1987), and the second aim was to compare the response of this mid-Atlantic population to experimental thermal conditions to the response of a population from the northern limit of *C. finmarchicus* distribution.

2. Material and methods

2.1. Sampling and experimental set-up

Sampling of copepods was conducted on 28 March 2013 at station 127 (South of Iceland, 62° 49.27′ N, 021° 21.74′ W) during the Eurobasin Trans-Atlantic Cruise MSM 26 onboard RV Maria S Merian. Samples were collected by vertical haul with a 300 μm WP2 net in the upper 400 m, where water temperature was 8.5 °C. Live *Calanus finmarchicus* females were picked from the net sample and acclimated in 2 L bottles of GFF-filtered (Whatman) seawater with *Thalassiosira weissflogii* as prey (10 μg chl L^{-1}) for >48 h at ambient sea surface temperature. Acclimation and temperature experiments were performed onboard of the research vessel.

Temperature experiments were conducted in individual bottles incubated in water baths with five temperatures: 0 °C, 5 °C, 10 °C, 15 °C and 20 °C. A temperature of 0 °C was maintained using ice in an insulated lidded box inside a controlled-temperature room set at 5 °C. Temperatures 10 °C, 15 °C and 20 °C were maintained in water baths with aquarium heaters and circulation. Water bath temperatures were monitored using temperature Hobo Data Loggers (Onset Computer Corporation) set to log at 5 min intervals. Experimental bottles were filled with GFF-filtered seawater and acclimated to experimental temperature overnight.

Females of *C. finmarchicus* were exposed to five temperatures for 3 h (short-term temperature condition, STC) and 6 days (long-term temperature condition, LTC) using 20 individuals per treatment in individual 250 mL bottles. Individual females were transferred into bottles using forceps and the bottles were inoculated with *T. weissflogii* to a final concentration of 5 μg chl L^{-1}. Every 48 h or at experiment termination, females were gently poured out of bottles into a submerged sieve with 50 μm mesh. Dead individuals were noted and live ones were transferred with forceps into a new experimental bottle that was acclimated to the temperature and contained *T. weissflogii* at 5 μg chl L^{-1}. The sieve contents were rinsed into 6 well plates to collect fecal pellets for quantification of grazing rates as a proxy of physiological response. In addition, spawning females were noted. At termination of each experiment, live females were anaesthetized by gentle submersion in a beaker of carbonated water. Once they had stopped swimming the copepods were transferred into individual 1.5 mL tubes with RNAlater (Qiagen) and kept in the fridge overnight followed by storage at −80 °C prior to transcriptomic analysis.

2.2. Extraction of RNA/DNA and genetic species identification

Extraction of RNA and DNA was performed simultaneously from each individual using the E.Z.N.A. DNA/RNA Isolation Kit (Omega Bio-Tek) according to manufacturer's instruction. To ensure correct species identification females of *C. finmarchicus* were genotyped for six insertion/deletion (InDel) nuclear markers according to Smolina et al. (2014).

2.3. Synthesis of cDNA and quantitative real-time PCR

All RNA samples were quantified using the Qubit RNA Assay kit (Life Technologies) with a Qubit 2.0 Fluorometer (Life Technologies), and integrity of selected RNA from each extraction was verified by agarose gel electrophoresis. Twelve RNA samples (where available) for each treatment, as well as two controls (positive control and no reverse transcriptase control) were individually reverse-transcribed to cDNA in 10 μL reactions using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions, with a starting amount of 40 ng of total RNA.

The qPCR reactions were performed for five genes of interest that showed the most significant changes in regulation in the northern population of *C. finmarchicus* in response to elevated temperatures (Smolina et al., 2015): *hsp70_2* (heat shock protein 70 cognate 2), *dnaja1* (DnaJ

homolog subfamily A member 1), *nap 1l1* (nucleosome assembly protein 1l1), *rps11* (40S ribosomal protein S11) and *gdh* (glutamate dehydrogenase); and two reference genes: *cdc42* (cell division control protein 42 homolog) and *eif1ax* (eukaryotic translation initiation factor 1A) that were identified as the most stable genes under elevated temperature conditions in *C. finmarchicus* (Smolina et al., 2015). The qPCR reactions, melting curve analysis of amplified products and calculations of the PCR amplification efficiency (E) and regression coefficient (R^2) were performed as described in Smolina et al. (2015). Expression quantities of the five genes were normalized using geNorm 3.5 (Vandesompele et al., 2002) and two reference genes.

2.4. Statistical analysis

Physiological response represented by fecal pellet production was estimated as production rate per 24 h, i.e. number of pellets per female per day. Therefore, number of fecal pellets obtained from STC (3 h) was multiplied by 8, while total number of fecal pellets from LTC (6 days) was divided by 6. Statistical analysis of data on fecal pellet production rate (FPR) and gene expression was performed using R version 3.1.0 (R Development Core Team, 2011). Normal distribution and homogenous variance of data were assessed visually by frequency histograms and Q-Q plots. Since not all the data satisfied parametric assumptions, nonparametric methods were applied. Effects of the exposure duration (STC vs. LTC) and experimental temperatures (0 °C, 5 °C, 10 °C, 15 °C and 20 °C) on FPR and expression of selected genes were analysed separately. The effect of stress duration was assessed with a Mann-Whitney *U* test, first for a combination of all stress temperatures, then at each temperature separately with *p*-value correction for multiple comparisons using the FDR method (Benjamini and Hochberg, 1995). The effect of experimental temperature was investigated separately for STC and LTC experiments using a Kruskal Wallis test. In the case of significant temperature effects, values were compared pairwise among all temperatures with a nonparametric analog of Tukey test in the R package "nparcomp" (Konietschke, 2012).

3. Results

3.1. Physiological indicators

No mortality was observed during STC, while during LTC the lowest mortality (5%) was observed at 5 °C, and increased with temperature to a maximum of 45% at 20 °C. Mortality at 0 °C, 10 °C and 15 °C was 15%, 10% and 25% respectively. The majority of mortality occurred in the first four days of the experiment, with mortality rates decreasing in the last 48 h during the 6-day experiment.

Overall, the duration of the temperature experiment had no significant effect on fecal pellet production rate (FPR) ($P > 0.05$). Fecal pellet production rate was significantly affected by temperature in each experiment. During STC FPR values were significantly higher at 10 °C and 15 °C than at 0 °C and 5 °C ($P < 0.05$) (similar to the previous study; Smolina et al., 2015), while during LTC FPR values were significantly higher at 5 °C, 10 °C, and 15 °C than at 0 °C ($P < 0.01$, Fig. 1A). Values of FPR at 20 °C were intermediate compared to other temperatures during both STC and LTC. Notably, only a very small percent of females spawned during STC (maximum 17% at 5 °C), while during LTC the percentage of spawning females rose from 50% to 100% with a temperature increase of 0 °C to 15 °C and then dropped to 9% at 20 °C.

3.2. Gene expression performance

All genes selected for qPCR, were successfully amplified, with a reaction efficiency between 90% and 94% and regression coefficient between 0.995 and 0.999. The duration of temperature experiments had a significant effect on expression values of only one gene (*gdh*) out of five (Fig. 1). Expression of *gdh* was significantly up-regulated during LTC at 15 °C compared to STC ($P = 0.01$). During STC none of the investigated genes showed any significant change in expression in response to temperature (Fig. 1). However, during LTC, the expression levels of two genes out of five were affected by temperature with *nap1l1* having a significantly lower expression level at 0 °C than at 10 °C ($P < 0.05$, Fig. 1B) (acclimation temperature), and *ghd* being down-regulated at 0 °C compared to 10 °C and 15 °C ($P < 0.05$, Fig. 1C).

4. Discussion

Marine organisms can adjust to temperature change in their natural environment (Somero, 2005), and it has been suggested that a short-term wide range of temperature variation may be more easily tolerated than long-term small changes in the mean temperature (Hong and Shurin, 2015). Overall, short-term tolerance and physiological survival have broader thermal limits than long-term successful species performance, which is controlled by the metabolic balance between energy gains and losses (from physiological homeostasis and basal metabolism

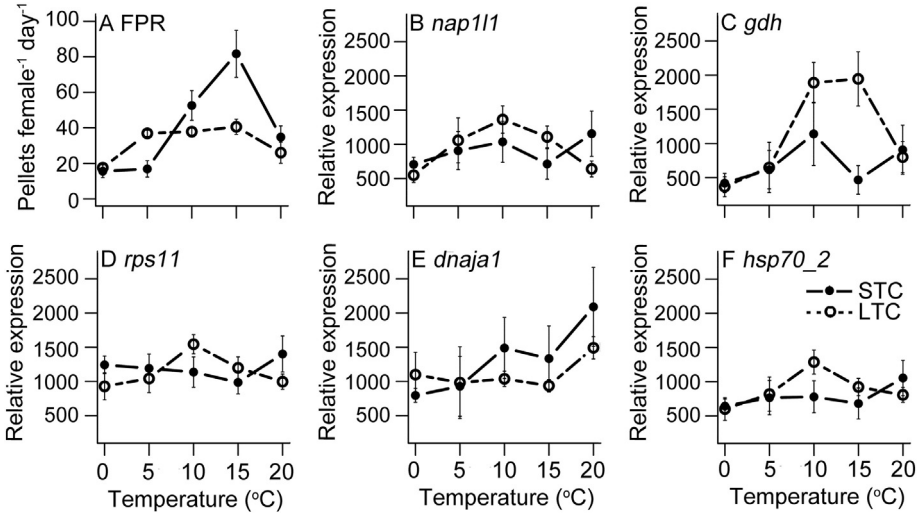

Fig. 1. Response to experimental temperature conditions in the mid-Atlantic population of *Calanus finmarchicus* to different experimental duration and temperatures. A) fecal pellet production rate (pellets female^{-1} day^{-1}), B)–F) relative expression of selected genes. Duration of temperature conditions: STC – short-term temperature condition (3 h), and LTC – long-term temperature condition (6 days). Values are shown as mean ± standard error. Asterisk indicates significantly different response values between STC and LTC. Different letters indicate significantly different response values between temperatures during STC (lower-case) and LTC (upper-case).

to movement) (Alcaraz et al., 2014). Our results showed that *Calanus finmarchicus* from a mid-Atlantic population could adjust well to the wide temperature range, which the species can encounter, with physiological and molecular performance being weakly affected by the intensity and duration of temperature increase. These findings differ from previous experiments on *C. finmarchicus* sampled from the Arctic where expression of all five genes investigated was affected by temperature and/or duration of experimental conditions (see Fig. 2 for synopsis). In both studies physiological response, assessed as fecal pellet production, showed a classic unimodal response in relation to temperature increase; with a constant increase in the process output until a critical thermal maximum was reached, after which there was a monotonic drop (reviewed in Alcaraz et al., 2014).

The thermal tolerance range of an organism can be extended through changes in gene expression, particularly through up-regulation of heat-shock protein genes (Feder and Hofmann, 1999). Induction of heat-shock proteins under thermal stress is a common defense strategy to stabilize and refold denatured proteins as well as to degrade and replace proteins that cannot be repaired (Feder and Hofmann, 1999; Hofmann and Todgham, 2010). No significant change in the expression of the two *hsp* genes studied here (*hsp70_2* and *dnaja1*) were observed in the mid-Atlantic population of *C. finmarchicus* in contrast to the northern population (Fig. 2; Smolina et al., 2015). Nevertheless, it is too early to conclude that this population is not stressed at 20 °C, particularly given its high mortality and low percent of spawning females after 6 days at this temperature. Constitutive expression of these *hsps* could be explained by multiple homologues within *hsp* families in *Calanus* spp. and their differential regulation under various stresses (Aruda et al., 2011; Smolina et al., 2015; Voznesensky et al., 2004). Therefore, additional whole transcriptome profiling would be beneficial and may result in detection of both population-specific and population-universal molecular processes in response to stress. One such population-universal response was demonstrated by the up-regulation of *gdh*, a gene encoding the mitochondrial enzyme glutamate dehydrogenase, which was detected in the both *C. finmarchicus* populations under elevated temperatures. This enzyme is known to play a key role in the metabolism of free amino acids, however, its function in the thermal stress response of *C. finmarchicus* is still not clear.

Population specificity, similar to that shown by the *hsp* genes in *Calanus* has been observed in the expression of stress-associated genes in response to elevated temperatures in southern and northern populations of the copepod *Tigriopus californicus* (Schoville et al., 2012), the killifish *Fundulus heteroclitus* (Whitehead and Crawford, 2006), the seagrass *Zostera marina* (Bergmann et al., 2010; Franssen et al., 2011), and the seaweed *Fucus* (Jueterbock et al., 2014; Pearson et al., 2009; Smolina et al., 2016). Such variation in a species ability to cope with diverse environmental pressure has been shown to be a result of phenotypic plasticity (Lardies and Bozinovic, 2008) and/or local adaptation (Yampolsky et al., 2014). At this stage it is difficult to identify which mechanism plays the main role in temperature response in *C. finmarchicus*, however evidence suggest that both mechanisms may be at play. Local adaptation refers to the genetic differentiation among populations resulting in increased mean fitness in the local environment (reviewed by Savolainen et al., 2013) and it may play a significant role even in the case of planktonic species in the open ocean, leading to population-specific responses to environmental stressors, including global climate change (Peijnenburg and Goetze, 2013; Sanford and Kelly, 2010). Evidence of genetic differentiation among populations of *C. finmarchicus* has been detected both within and between basins of the North Atlantic (Smolina, 2015; Unal and Bucklin, 2010). Such genetic differentiation may be responsible for significant regional differences in numbers of generations per year, timing of reproduction, seasonal patterns of abundance, vertical distribution, and other life history traits that are observed among *C. finmarchicus* populations (Melle et al., 2014; Planque et al., 1997). Locally adapted populations have been documented for the copepods *Tigriopus californicus* (Schoville et al., 2012), *Acartia hudsonica* (Colin and Dam, 2007), and *Calanus helgolandicus* (Lauritano et al., 2012), however direct evidence for adaptation among *C. finmarchicus* populations to temperature or other environmental factors is missing. In addition, investigation of *C. finmarchicus* abundance vs. sea surface temperature relationships in the North-East Atlantic from1960 to 2010 suggests that thermal adaptation has not mitigated the impacts of ocean warming on reduction of the distributional range of this key species (Hinder et al., 2014).

On the other hand, phenotypic plasticity, and acclimatization in particular, may play a substantial role in thermal stress responses,

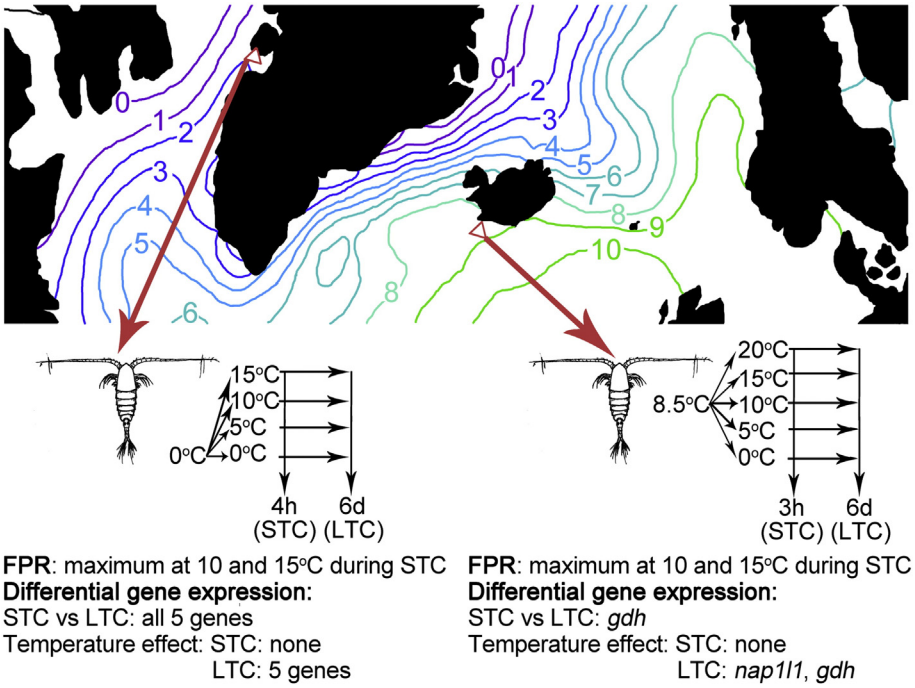

Fig. 2. Synopsis of the results obtained in this study and compared with previous results obtained in published work on *Calanus finmarchicus* sampled from the Arctic (Smolina et al., 2015). Depicted isotherms represent mean sea surface temperature over period January 2011 – April 2013 (data access and visualization via http://www.esrl.noaa.gov on 22 March 2016). Key: STC – short-term temperature condition; LTC – long-term temperature condition; FPR – fecal pellet production rate (pellets female^{-1} day^{-1}).

especially in ectothermic marine organisms where temperature acclimation can affect temperature tolerances (Jiang et al., 2008; Somero, 2005). For example, an increase in acclimation temperature may expand the thermal tolerance, as seen for several copepods including *Calanus sinicus* (Jiang et al., 2008), *Eurytemora affinis* (Bradley, 1978), *Acartia tonsa*, and *Acartia clausi* (González, 1974). Therefore, acclimation to an *in situ* temperature of 8.5 °C may explain higher tolerance to experimental temperatures (in terms of fewer genes being up-regulated) in the mid-Atlantic population of *C. finmarchicus* compared to the Arctic population with an in situ temperature of 0 °C (Smolina et al., 2015). Similarly, a study on effects of temperature on respiration in *C. finmarchicus* showed that for individuals acclimated to 4 °C and 10 °C, respiration falls after 10 °C and 15 °C respectively (Halcrow, 1963). In addition, seasonal acclimatization in the natural environment is suggested to allow organisms to sufficiently adjust their physiological and biochemical mechanisms under gradual temperature change with the summer-captured copepods *Labidocera euchaeta* and *Calanus finmarchicus* having significantly better performance under elevated temperatures than the spring-captured ones (Halcrow, 1963; Jiang et al., 2008).

Both genetic adaptation and phenotypic plasticity will be important in adaptive responses to climate change (e.g., Munday et al., 2013), and the potential for both must be investigated in *C. finmarchicus* to forecast its responses to climate change. While our findings show reduced up-regulation of temperature-responsive genes in the *C. finmarchicus* from a warmer habitat, more sophisticated experimental studies are necessary to shed light on the contribution of genetic adaptation and phenotypic plasticity in the response of *C finmarchicus* to temperature. To achieve this goal, it would require performing a long-term common garden experiment with many populations across the North Atlantic. Yet, this presents logistical and practical challenges in the case of copepods with a broad distribution range and has been addressed only in very few cases (e.g., Hong and Shurin, 2015; Thor and Oliva, 2015). In addition, at least the second generations of organisms reared under similar conditions should be preferred, to avoid long-lasting effects of early-life exposures, maternal effects and transgenerational effects (Porcelli et al., 2015). Another important intraspecific factor that may influence variation in response to environmental stressors is life stage. For instance, fluctuation in stress impact on *C. finmarchicus* varies among developmental stages in response to temperature (Kvile et al., 2014), carbon dioxide concentration (Cripps et al., 2014), oil toxicity (Jager et al., 2016), as well as the breadth of the ecological niche changes during development (Reygondeau and Beaugrand, 2011b). Thus, the majority of studies on the responses to thermal stress are female-biased and additional stage-specific investigations are needed to understand population dynamics and persistence.

Ongoing climate change, particularly seawater warming, is affecting and will continue to affect predominant copepods of the genus *Calanus*, resulting in distributional changes with consequences for higher trophic levels (Beaugrand and Reid, 2003; Chust et al., 2014; Olsen et al., 2011; Reygondeau and Beaugrand, 2011a). While a previous study has already revealed differences in the thermal stress response between closely related *Calanus* species (Smolina et al., 2015), the degree of variation in responses of different *Calanus* populations is largely unknown. Overall, this work presents a first attempt to investigate plasticity of the molecular response to temperature change in *Calanus* and the findings suggest that the response to temperature in *C. finmarchicus* may vary depending on the populations studied and their local environmental conditions. Nevertheless, more detailed studies are needed to uncover the role of genetic adaptation to local conditions and acclimatization via phenotypic plasticity in response to rising sea temperature.

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