Live discrimination of *Calanus glacialis* and *C. finmarchicus* females: can we trust phenological differences?

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**Abstract** Two key players in the Arctic and subarctic marine ecosystem are the calanoid copepods, *Calanus finmarchicus* and *C. glacialis*. Although morphologically very similar, these sibling species have different life cycles and roles in the Arctic pelagic marine ecosystem. Considering that the distribution of *C. glacialis* corresponds to Arctic water masses and *C. finmarchicus* to Atlantic water masses, the species are frequently used as climate indicators. Consequently, correct identification of the two species is essential if we want to understand climate-impacted changes on *Calanus*-dominated marine ecosystems such as the Arctic. Here, we present a novel morphological character (redness) to distinguish live females of *C. glacialis* and *C. finmarchicus* and compare it to morphological (prosome length) and genetic identification. The characters are tested on 300 live females of *C. glacialis* and *C. finmarchicus* from Disko Bay, western Greenland. Our analysis confirms that length cannot be used as a stand-alone criterion for separation. The results based on the new morphological character were verified genetically using a single mitochondrial marker (16S) and nuclear loci (six microsatellites and 12 InDels). The pigmentation criterion was also used on individuals (*n = 89*) from Young Sound fjord, northeast Greenland to determine whether the technique was viable in different geographical locations. Genetic markers based on mitochondrial and nuclear loci were corroborative in their identification of individuals and revealed no hybrids. Molecular identification confirmed that live females of the two species from Greenlandic waters, both East and West, can easily be separated by the red pigmentation of the antenna and somites of *C. glacialis* in contrast to the pale opaque antenna and somites of *C. finmarchicus*, confirming that the pigmentation criterion is valid for separation of the two species.

**Introduction**

*Calanus* dominate the zooplankton in the Arctic marine ecosystem and constitute the link between the primary producers and the rich stocks of fish, birds and marine mammals (reviewed by Falk-Petersen et al. 2009). In the subarctic North Atlantic and Arctic, three species of *Calanus* coexist: the Arctic *Calanus hyperboreus* and *C. glacialis* and the North Atlantic *C. finmarchicus*. All three species have life cycles adapted to Arctic conditions including seasonal ontogenetic migration and accumulation of lipids during spring and summer, as well as hibernation and arrested development during winter (Conover 1988; Madsen et al. 2001). Despite being morphologically very similar (Frost
their life cycles, size and in particular lipid content are quite different (Swalethorp et al. 2011).

In spring, when the breakup of the sea ice triggers the formation of the spring bloom (Dünweber et al. 2010), the Calanus species ascend from the deep water. In Disko Bay, western Greenland, C. finmarchicus and C. glacialis ascend to the surface layer at the same time in spring (Madsen et al. 2001, Swalethorp et al. 2011). However, the two species represent different phenology; C. glacialis commences spawning before the onset of the spring bloom utilizing stored lipids (Hirche and Kattner 1993) (capital breeder sensu Varpe), while C. finmarchicus, which can be characterized as an income breeder, needs to feed on the bloom (Plourde & Runge 1993) in order to initiate spawning a few weeks after C. glacialis (Madsen et al. 2008, Swalethorp et al. 2011).

The core distribution of Calanus glacialis and C. finmarchicus corresponds to Arctic and Atlantic water masses (Falk-Petersen et al. 2009), respectively; therefore, they are used as climate indicators. Based on model simulations, for the Arctic pelagic ecosystem, it is predicted that climate change in particular will cause a northward shift of the Atlantic C. finmarchicus (Slagstad et al. 2011) and will increase the area where the sibling species C. glacialis and C. finmarchicus co-occur and reproduce.

The model results are experimentally supported by Kjellerup et al. 2012 who, based on extensive temperature experiments with C. glacialis and C. finmarchicus, found that a slightly warmer ocean will potentially cause a shift in the composition of the zooplankton community from a dominance of C. glacialis towards a more prominent role of the smaller less energy-rich C. finmarchicus. Such shifts will, according to Falk-Petersen et al. 2007, have major implications on the food web, since C. finmarchicus support an alternative pelagic food web than the more lipid rich C. glacialis. Therefore, if the energy flows through the Arctic marine ecosystem in a warmer future should be quantified and modelled realistically, correct identification of C. finmarchicus and C. glacialis in field samples, especially live collections used for experiments investigating phenology, is essential.

Recent publications have questioned the prosome length criterion used to separate Calanus finmarchicus and C. glacialis (Undstad and Tande 1991; Hirche et al. 1994) due to an overlap of the prosome length range of the two species (Lindeque et al. 2006, Parent et al. 2011). Separation of the two species has recently been additionally challenged by the documented hybridization between the two species in the northwest Atlantic (Parent et al. 2012).

However, in situations where morphological characters do not provide sufficient variation to identify species level, then taxonomic discrimination can be achieved using genetic characters. Such techniques have been developed for copepods of the genus Calanus using microsatellite markers (nuclear, bi-parentally inherited: Provan et al. 2009; Parent et al. 2012) and restriction fragment length polymorphism (RFLP) PCR of mitochondrial 16S rDNA (mtDNA, maternally inherited: Lindeque et al. 1999). This later technique has successfully been used in numerous past studies (Lindeque et al. 2004, 2006; Parent et al. 2012; Gabrielsen et al. 2012). Molecular techniques, relying on analysis of the DNA, quite obviously preclude the identification of live individuals. It is possible that adult females are individually incubated and left to spawn such that the eggs are used for molecular identification, thereby confirming the identity of the mother. However, this is labour-intensive, unsuitable for large studies, relies on the females spawning eggs, adds to the handling and stress of the animals and still does not solve the problem of potential hybrids. It is therefore necessary to have a quick and reliable identification technique in order to sort live females for experimental procedures, thereby allowing investigation and documentation of phenological differences in response to environmental variables such as temperature and food (Kjellerup et al. 2012).

Accordingly, the aim of this study was to present new morphological criterion for discrimination of C. glacialis and C. finmarchicus live females based on ‘redness index’ verifying its applicability with molecular methods and compare its performance with prosome length criterion.

Materials and methods

Sampling

Western Greenland

Copepods were sampled on the 2, 8 and 13 May 2011 during the spring bloom in Disko Bay, western Greenland. Sampling was done from R/V Porsild (Arctic station, Copenhagen University) at a monitoring station used in previous studies (Levinsen et al. 2000; Madsen et al. 2001; Hansen et al. 2012) (69°14′N, 53°23′W) using a WP2 net with a non-filtering cod end (200-μm mesh size). After sampling, the contents of the cod end were quickly transferred into buckets with seawater and placed in thermo boxes filled with seawater and ice.

East Greenland

Following the protocol described above, copepods were sampled from the motor boat Åge V. Jensen I on the 10 August 2012 in Young Sound fjord, Daneborg, northeast Greenland, at sampling station A (74°18′N, 20°15′W, Rysgaard et al. 1999) and in the Greenland Sea station GH 5.
Morphological identification

Within 5 h of collection, adult females collected in West Greenland were returned to the research station and sorted in seawater-filled Petri dishes kept in ice-filled trays under a dissecting microscope. Based on size and redness, approximately 50 *C. glacialis* and 50 *C. finmarchicus* (Fig. 1) females, including those with ambiguous characters, were picked from each sample, i.e. the females were taken in the order they were encountered in the raw sample until 50 of one species (*C. finmarchicus*) were collected. Thereafter, only the other species as well as females that demonstrated ambiguous pigmentation (FDaP) were picked until 100 individuals were collected in total from each sample (Fig. 2). The females were incubated for egg production and processed as described below. In East Greenland, 89 females were sorted, taken in the order they were encountered in the raw sample. None of the East Greenland females were incubated for egg size or morphology or analysed for redness, but individually stored in 75 % non-denatured ethanol and 0.2 μm filtered seawater in 5-ml PCR tubes at 5 °C until molecular analysis. No females showing ambiguous morphological characters were observed in the East Greenland samples.

**Egg morphology**

Females picked from the West Greenland samples were placed individually in 24-well tissue culture trays (NUNC™Multi wells) containing 3 ml of 45 μm filtered seawater and incubated at constant temperature (5 °C) for 36–40 h to allow the females to produce eggs. Eggs produced by each female were carefully collected directly from the 24-well culture tray by a Pasteur pipet and placed on a glass slide in a small volume of water. A cover slip with four Vaseline corners was placed on the top of the glass slide before being photographed using a Nikon 90D camera attached to an Olympus BX51 microscope by an eyepiece adaptor [NDPL-1(×2)]. A clear discriminator of live eggs is the outer membrane of *C. glacialis* eggs, which is absent in *C. finmarchicus* (Werner and Hirche 2001). Egg diameter was measured as the diameter between the inner membrane of the egg using the image analysis software ImageJ (v. 1.38w) (http://rsb.info.nih.gov/ij/) by calibrating the software using photographs with known distances.

**Prosome length**

After incubation of the West Greenland samples, each female was placed in a Petri dish with as little water as possible to position animals at the same level and photographed on an Olympus SZX12 microscope using a Nikon 90D camera attached to an Olympus BX51 microscope by an eyepiece adaptor [NDPL-1(×2)]. A clear discriminator of live eggs is the outer membrane of *C. glacialis* eggs, which is absent in *C. finmarchicus* (Werner and Hirche 2001). Egg diameter was measured as the diameter between the inner membrane of the egg using the image analysis software ImageJ (v. 1.38w) (http://rsb.info.nih.gov/ij/) by calibrating the software using photographs with known distances.

**Fig. 1** Typical specimens of *a* *Calanus finmarchicus* and *b* *C. glacialis* illustrating the diagnostic morphological (size) and pigmentation criteria (redness) used to separate females and eggs of the two species. The black scalebar is 1 mm and 0.1 mm for females and eggs, respectively. Photograph: Sanne Kjellerup

**Fig. 2** Example of two females that demonstrated ambiguous pigmentation (FDaP). *a* A female with two pale genital somites; *b* a female displaying one pale and one red genital somite. Besides the appearance of the somites, both females display morphological traits similar to *C. glacialis* both in size and in redness of antennae. Photograph: Sanne Kjellerup
90D camera with an eyepiece adaptor [NDPL-1(×2)]. Each female was then washed in GFF-filtered seawater and stored in 75% non-denatured ethanol and 0.2 μM filtered seawater in 5-ml PCR tubes at 5 °C until molecular analysis. Prosome length was measured using the image analysis software ImageJ (v. 1.38w) (http://rsb.info.nih.gov/ij/) by calibrating the software using photos with known distances.

**Redness index**

To create an objective tool to evaluate the coloration of the females, a redness index was defined and determined using ImageJ with the plug-in ‘Threshold Color’ (http://www.dentistry.bham.ac.uk/landinig/software/software.html). For each female, the genital segment was cut out of the picture and the percentage of red pixels of the genital somites out of the entire genital segment was quantified. We chose the HSB colour model and selected red areas using the following values: hue 0–23 and 0–255, saturation 75–255 and brightness 1–255. In a second step, we measured the complete area of the genital segment. The images were converted to 8-bit grey scale, and the selected areas were measured using the ‘Image/Adjust/Threshold’ and the ‘Analyze/Measure’ commands. This analysis was repeated for the antennae.

**Genetic identification**

All samples from West and East Greenland were analysed ‘blind’ without prior knowledge of either prosome length or pigmentation. Both mitochondrial (mtDNA) and nuclear (nDNA) markers were used to genetically characterize each individual.

**Mitochondrial marker**

Individual *Calanus* were identified to species according to the RFLP signature of their mitochondrial 16S rDNA following PCR amplification, restriction digestion and agarose gel electrophoresis, using the molecular identification technique described by Lindeque et al. (1999, 2006) with minor modifications. The final technique is described below. Individual animals were removed from ethanol and cut in half with a sterile scalpel blade. Excess ethanol was removed from the half individual by dabbing on absorbent paper and rehydrated in 200 μL of MilliQ water in a 96-well plate for approximately 6 h at room temperature. Following rehydration, the water was removed and replaced with 22.75 μL MilliQ water and 10 μL of 5× Flexi GoTaq DNA polymerase buffer (Promega UK). The sample was homogenized by inserting a hypodermic needle (19G) into a pellet pestle hand-held homogeniser (Anachem Ltd.,) and incubated overnight at 4 °C. The remaining PCR components were then added: 5 μL 2 mM dNTPs (Promega UK Ltd.), 10 μM of primers 16SAR (5′-CGC CTGTTATAACAAACAT-3′; Palumbi and Benzie 1991) and 16SB2r (5′-ATTCAACATCGAGGTCAAAAC-3′; Lindeque et al. 1999), 2 μL 25 mM MgCl₂ and 1.25 U GoTaq DNA polymerase (Promega UK Ltd.,). Amplifications were carried out in a G Storm or VWR thermocycler. The cycling parameters included an initial denaturation step at 94 °C (5 min) followed by 40 cycles of 94 °C (1 min), 45 °C (2 min), 72 °C (1 min). After annealing at 45 °C (2 min) was followed by an extension phase at 72 °C (5 min) and storage at 10 °C. Aliquots (5 μL) of the amplification reaction were analysed by gel electrophoresis (1%) to check amplification efficiency. Restriction digests were performed on a 7.5-μL aliquot of each amplification by the addition of 0.75 μL bovine serum albumin (1 mg ml⁻¹), 0.25 μL 5 M NaCl and 2.5 U of each restriction enzyme Dde I and Vsp I (Promega UK Ltd.,). Incubations were performed at 37 °C for >4 h. The digestion products were separated by electrophoresis through a 2% agarose gel and visualized by UV transillumination.

**Nuclear markers**

Individual *Calanus* were also genotyped using six microsatellite loci (EL696609, EL585922, EH666870: Provan et al. 2009; FK868270, FK670364, FK867682: Parent et al. 2012) and twelve recently developed insertion/deletion (InDel) nuclear markers (Smolina et al. 2014). These loci are diagnostic between *Calanus finmarchicus* and *C. glacialis* (Online Resource 1: Table S1), and as co-dominant nuclear markers, they can allow the identification of hybrids (Online Resource 1: Fig S1, Table S2). DNA was extracted from the remaining half of each individual using the EZNA® Insect DNA Kit (Omega Bio-Tek, USA) according to manufacturer’s instruction. PCRs were performed using a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, USA) in a total volume of 5 μL. PCR mix contained approximately 3 ng of DNA, reverse and fluorescently labelled forward primers with a final concentration of 0.25 μM each and 1X AmpliTaq Gold® PCR Master Mix (Applied Biosystems, USA). Cycling parameters included an initial cycle of 95 °C (60 s), 54 °C (20 s), 72 °C (25 s), followed by five cycles of 95 °C (30 s), 54 °C (20 s), 72 °C (20 s), 30 cycles of 95 °C (20 s), 54 °C (20 s), 72 °C (20 s) and a final extension at 72 °C (10 min). All 18 loci were run on a 3500XL Genetic Analyzer (Applied Biosystems, USA) following the manufacturer’s protocol. Alleles were scored using GeneMapper 3.7 (Applied Biosystems, USA) and checked by eye.

Admixture of nuclear genotypes was analysed using STRUCTURE (v. 2.3.4) (Pritchard et al., 2000). STRUCTURE uses a Bayesian algorithm to identify $K$ ($K = 2$ for...
C. finmarchicus and C. glacialis) clusters of genetically homogenous individuals and to assign each individual to one of the two clusters or jointly to both clusters if their multilocus genotype indicates hybridization. We used the following parameters: ancestry model = admixture; frequency model = correlated; burn-in = 2,000,000; MCMC length = 1,000,000 after burn-in. The output of STRUCTURE is an admixture coefficient, defined as the proportion of membership to the C. glacialis or C. finmarchicus cluster for each individual.

The power of admixture analyses to detect hybridization in Calanus was evaluated by simulation following Coyer et al. 2007. We used the genotypes from 30 individuals of each species for East Greenland and 50 for West Greenland to generate 100 artificial genotypes for F1, F2 and backcrosses using HYBRIDLAB (v 1.0) (Nielsen et al. 2006). The analysis was done for the microsatellites, the InDels and both markers together, and the admixture of artificial genotypes was analysed with STRUCTURE using the parameters presented above. Genetic differentiation between the two species was estimated with Fst using geneTIX 4.02 (Belkhir et al. 2001).

Results

Morphological identification

Out of 300 females sampled in West Greenland, 288 were identified to species based on size and pigmentation by eye using a microscope (before the analysis of prosome length and redness criteria from photographs). One hundred and thirty-nine of these were attributed to C. glacialis and 149 to C. finmarchicus. There were 12 individuals that could not readily be ascribed to either species and appeared to demonstrate mixed characteristics, especially ambiguous pigmentation criteria (FDAP).

Of the 89 females from East Greenland, 60 were identified as C. glacialis and 29 as C. finmarchicus based on size and pigmentation by eye using a microscope. No females showing ambiguous morphological characters were observed in the East Greenland samples.

Egg morphology

Analysis of egg size and morphology from the females collected and incubated from West Greenland showed the eggs of the two species to have different average diameters, 151 ± 12 μm, n = 86 and 177 ± 11 μm, n = 54 for C. finmarchicus and C. glacialis, respectively. In addition, the surfaces of the eggs of the two species are very different. C. finmarchicus eggs have a smooth surface, while C. glacialis eggs have spines on the outer membrane (Fig. 1). Of the 12 females that demonstrated ambiguous pigmentation (FDAP), only three produced eggs, all eggs had the same size and morphological trait as C. glacialis eggs.

Prosome length

The prosome length distribution of females from West Greenland (n = 288) showed a bimodal pattern with a mean length for C. finmarchicus and C. glacialis of 2.7 ± 0.16 and 3.5 ± 0.27 μm, respectively (Fig. 3). The 12 females that did not display the defining pigmentation criteria (Figs. 1, 2) had a prosome length representative of C. glacialis females 3.4 ± 0.31 (Fig. 3, Table 1). Based on prosome length alone, there is an overlap between species in the range 2.79–3.07 mm representing the smallest C. glacialis and longest C. finmarchicus, respectively. Within this range, we find 47 of the 300 females sampled: thirty-eight being C. finmarchicus, 8 C. glacialis and 1 FADP corresponding to 25, 6 and 8 % of the three groups, respectively.

Redness

Since females analysed were photographed alive and some females kept turning and folding their antenna along the prosome, redness could only be quantified on 218 and 219 females for genital somites and antenna, respectively. It was possible to ascertain the redness values for 109 C. finmarchicus from West Greenland using ImageJ analysis of pictures. The redness values for the genital somites of C. finmarchicus were 0.06 ± 0.19 % for the somites and 0.013 ± 0.19 % for the antennae, respectively. By contrast, the redness values for C. glacialis (n = 100) from West Greenland were much higher; 25.51 ± 9.08 % for

Fig. 3 Prosome length distribution of females of Calanus finmarchicus, C. glacialis and females that with ambiguous pigmentation (FDAP). The plots with horizontal error bars show mean length ± SD for each of the three groups.
the somites and 11.7 ± 9.23 % for the antennae. The 12 females that demonstrated ambiguous pigmentation (FDaP) based on the identification by eye under a microscope would not ‘normally’ be identifiable to species level and consequently not used for experiments by our group. Redness analysis on the FDaP was possible for the genital somites in 10 of 12 females and for 9 of 12 females for the antenna. The FDaP showed low redness % especially on the genital somites where most (seven females) had less than 1.2 % red pixels. For the antenna pigmentation, the FDaP were also at the low end of ‘normal’ for \textit{C. glacialis} (Table 1, Fig. 4). Consequently, 4 % of the females could have been misidentified, but on the other hand avoided in experiments due to their ambiguous pigmentation.

\textbf{Genetic identification, mtDNA markers}

Of the 300 individual females analysed from West Greenland, all but one were unambiguously identified to species level by rFlP-PCR. One of the samples appeared decomposed in the ethanol and could not be successfully amplified by PCR. The resulting DNA fingerprints or restriction profiles of the remaining samples assigned 149 individuals to be \textit{C. finmarchicus} and 150 individuals to be \textit{C. glacialis}. This identification matched exactly with the morphological identification.

Genetic analysis of the 89 individuals from East Greenland based on the RFLP signature of their mitochondrial 16S rDNA identified 60 to be \textit{C. glacialis} and 29 to be \textit{C. finmarchicus}. This also matched exactly with the morphological-based identification.

\textbf{Genetic identification, nuclear markers (microsatellites and InDels)}

Genotypes were obtained at 18 nuclear diagnostic loci for 283 of 300 individuals from West Greenland and for 89 of 90 individuals from East Greenland. For the 18 missing individuals, not enough good quality tissue was left for DNA extraction.

Genetic differentiation between species was highest using the InDels (Table S1). Simulations showed that the microsatellites alone do not have sufficient power to fully discriminate between the introgressed individuals and parental species. The highest discrimination power was achieved using all the co-dominant nuclear markers together (microsatellites and InDels) (Fig S1, Table S2). These nuclear markers confirmed species status assessed using morphological and mtDNA identification. All individuals were clearly (probability >99 %) assigned to either species; no hybrids were detected. Genetic analysis revealed all females with ambiguous characteristics (FDaP) to be \textit{C. glacialis} (Table 1), and these individuals were also not significantly different in size ($P > 0.005$, Tukey’s test) or in antennae redness ($P > 0.005$, Dunn’s test) to ‘normal’ \textit{C. glacialis}.

\textbf{Discussion}

Identification and separation of formalin-fixed ethanol preserved or fresh \textit{Calanus finmarchicus} from \textit{C. glacialis}, in particular the younger stages, based on size criterion (Unsad and Tande 1991, Hirche et al. 1994) is questionable (Lindeque et al. 2006; Parent et al. 2011) due to an overlap in prosome length between the different species at any particular developmental stage. In recent studies, the use

\begin{table}
\centering
\begin{tabular}{lcccc}
Species & Mean prosome length (mm) ± SD (n) & % of reproducing females & Redness index (% of red pixels) for genital somites ± SD (n) & Redness index (% of red pixels) for antennae ± SD (n) \\
\hline
\textit{C. finmarchicus} & 2.68 ± 0.16 (149) & 57.3 & 0.06 ± 0.19 (109) & 0.013 ± 0.06 (109) \\
\textit{C. glacialis} & 3.52 ± 0.27 (139) & 24.5 & 25.51 ± 9.08 (100) & 11.7 ± 9.23 (100) \\
FDaP & 3.43 ± 0.31 (12) & 27.3 & 4.91 ± 8.21 (10) & 8.87 ± 12.36 (9) \\
\end{tabular}
\caption{Mean prosome length, percentage of reproducing females, redness index for genital somites and antennae of \textit{C. finmarchicus}, \textit{C. glacialis} and females that demonstrated ambiguous pigmentation (FDaP).}
\end{table}
of molecular identification techniques has confirmed that *Calanus* species are consistently misidentified to species level when using the morphological character of prosome length as a distinguishing characteristic. This has been proven for *Calanus* over a wide geographical range including the Irminger Basin (Lindeque et al. 2006), the Canadian Arctic and Atlantic coasts (Parent et al. 2011) and Svalbard (Gabrielsen et al. 2012). However, the extent of overlap varies within the area where the species co-occur; Parent et al. 2011 showed that the extent of size overlap was primarily associated with the variable *C. finmarchicus* mean size which is dependent on temperature during the development. Even for a single-species population, Lindeque et al. 2006 concluded that a bimodal distribution of size could be sampled, possibly as a result of *C. finmarchicus* mixing from different regions and hence developing under different temperatures as suggested by Parent et al. 2011. Our investigation corroborates that prosome length of adult females from Disko Bay cannot be used as a stand-alone criterion for separating *Calanus finmarchicus* from *C. glacialis* due to an overlap in size (Fig. 3).

Recently, Parent et al. (2011) questioned to what extent the Atlantic *C. finmarchicus* and the Arctic *C. glacialis* can be separated to species. Though they overlap in length, they do differ in size, pigmentation (Kjellerup et al. 2012), lipid content, lifecycle (Swalethorp et al. 2011) as well as overall distribution (Lindeque et al. 2004; Hirche and Kosobokova 2007). The present study has for the first time through molecular analysis proven that the pigmentation/redness criterion documented in this study can successfully be used to identify and separate live adult females of the two sibling species *C. finmarchicus* and *C. glacialis* in both West and East Greenlandic waters. Verification of these field characters visible by eye greatly facilitates setting up experiments, e.g. to determine how the different species will react to an increase in temperature (Kjellerup et al. 2012), and thereby describe and model species-specific physiological response to climate change and evaluate the increase in prevalence of different temperatures as suggested by Parent et al. 2011. Our investigation corroborates that prosome length of adult females from Disko Bay cannot be used as a stand-alone criterion for separating *Calanus finmarchicus* from *C. glacialis* due to an overlap in size (Fig. 3).

During our previous investigations of phenology of *Calanus* species from Disko Bay, only *Calanus* females unambiguously fulfilling the pigmentation criteria were included in experiments, dubious females as those in Fig. 2 were ignored. In the present investigation, 12 females, all found in West Greenland (4 %), showed ambiguous pigmentation criterion. According to the signature of their maternally inherited 16S mtDNA and egg morphology, these females displayed *C. glacialis* characters. However, in a recent study on natural hybridization between *C. finmarchicus* and *C. glacialis*, Parent et al. (2012) pinpointed Disko Bay as an area of potential frequent hybridization due to the temporal and spatial overlap of the two reproducing females (Madsen et al. 2001, 2008, Swalethorp et al. 2011). Our molecular identification relied on species-specific variation at restriction sites in the 16S mitochondrial gene and on 18 diagnostic nuclear loci. The combination of molecular markers used in the present study give us unprecedented power to discriminate between parental species and hybrids (Online Resource 1). Interestingly, no hybrids were detected in either West or East Greenland samples.

To date, the pigmentation criterion presented here for adult female *C. finmarchicus* and *C. glacialis* from Disko Bay has been used and confirmed by differences in phenology, i.e. the timing of egg production and the egg morphology of the two species in the southern part of Greenland (Arendt et al. 2010) and eastern part of Greenland (Nielsen et al. 2007). Now, these morphological characteristics have been confirmed by molecular analysis, and we hope that the new pigmentation criteria presented here can be helpful, applied and confirmed in future studies covering the North Atlantic and Arctic to generate knowledge on the plasticity of the phenology of these two key species throughout their distribution area and in particular in the regions where they co-occur.

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