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Red Pigmentation Can Be Used to Reliably Distinguish Between Live *Calanus finmarchicus* and *Calanus glacialis* Females in the Fram Strait

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Copepods from the genus *Calanus* provide an important lipid-rich food source in the Arctic marine foodweb. Despite extensive research on *Calanus finmarchicus* and *Calanus glacialis*, accurately identifying adults to species level remains challenging due to similar morphologies. Although these species co-occur in many regions, the distribution of *C. finmarchicus* and *C. glacialis* correspond to Atlantic and Arctic water masses respectively and are frequently used as climate indicators. Correct identification is therefore vital for understanding the phenotypic plasticity of these species and the impacts climate change will have on *Calanus*-dominated marine ecosystems. In this study, prosome length and percentage of red pigmentation (redness) of genital somites, the antennae, and throughout the whole body were determined for 139 females of *C. finmarchicus* and *C. glacialis* from the Fram Strait. Molecular analysis of a 16S rDNA barcode confirmed that the best morphological features for resolving the identity of these two species were the redness of the antennae and the redness of the genital somites. Overall accuracy of using antennae redness and genital somite redness to discriminate between the two species were the same, yet each of these explanatory variables had different specificity; *C. finmarchicus* were more accurately identified by the absence of redness in the genital somites, whereas *C. glacialis* were more accurately identified using antennae redness. Given the ecological importance of these congeners, these findings contribute to a better understanding of the reliability of using morphological characteristics to identify *Calanus* to species level, especially when sorting live specimens for climate-related ecological experiments.

Keywords: copepod, identification, barcode, molecular systematics, prosome length

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INTRODUCTION

In the North Atlantic and Arctic marine ecosystems, copepods of the genus *Calanus* dominate the zooplankton community biomass (Jaschnov, 1970; Fleminger and Hulsemann, 1977). These energy-rich animals play a vital role in food webs consuming primary producers and microzooplankton and passing this energy up to higher trophic levels including ecologically and commercially important

species of fish, birds and whales (Varpe et al., 2005; Karnovsky et al., 2008; Falk-Petersen et al., 2009). Calanoid copepods also play a critical role in regulating the Earth's climate, by removing carbon dioxide from the sea surface through their consumption of phytoplankton and transporting this carbon to depth through vertical migration and respiration, and the production of fast-sinking carbon-rich faecal pellets (Wilson et al., 2008).

Three species of *Calanus* co-exist in the sub-arctic North Atlantic and Arctic: *Calanus hyperboreus*, *Calanus glacialis* and *Calanus finmarchicus*. The reproductive range of *Calanus helgolandicus* also overlaps with *C. finmarchicus* and *C. glacialis*, but this warm-temperate water species is largely restricted to southern areas of the North Atlantic, the Celtic Sea and North Sea. *C. hyperboreus* is a deep-water Arctic species, found predominantly in the Arctic Ocean and Greenland Sea (Hirche et al., 1994). *C. glacialis* is considerably smaller than *C. hyperboreus* and is found mostly in the Arctic shelf-seas (Frost, 1974; Conover, 1988). *C. finmarchicus*, overlapping in size with *C. glacialis*, is a North Atlantic species (Jaschnov, 1970), often coexisting with *C. glacialis* due to the advection of Atlantic water masses into the sub-Arctic. Considering that the spatial distribution of *C. glacialis* and *C. finmarchicus* correspond to the Arctic and Atlantic waters that they respectively originate from, they are regarded as indicator species for those water masses, and indeed climate indicators (Unstad and Tande, 1991; Hirche and Kosobokova, 2007; Falk-Petersen et al., 2009). Model simulations predict that ocean warming and sea ice loss will cause an 'Atlantification' of the Arctic Ocean (Wassmann et al., 2006; Slagstad et al., 2011) and hence a northward shift of *C. finmarchicus*. The model is further supported by analysis of long-term data (Møller and Nielsen, 2020) and experimentally by Kjellerup et al. (2012) who found that a warmer ocean will increase the area in which these sibling species co-exist and reproduce, and likely support a shift in dominance from *C. glacialis* to *C. finmarchicus* in the sub-Arctic. Indeed, a recent field campaign has highlighted that not only are *C. finmarchicus* expatriated to the Arctic but are capable of completing their life-cycle once there, allowing local recruitment (Tarling et al., 2022). Any change in the spatial distributions of these congeners can be used as a tool to monitor climate change effects in northern hemisphere marine ecosystems (Falk-Petersen et al., 2007).

Despite the fundamentally similar role that the different *Calanus* species play in the marine ecosystem, their size, lipid content, life cycles and phenology all differ, albeit to different extents (Swalethorp et al., 2011; Trudnowska et al., 2020). For example, *C. glacialis* is recognised as a capital breeder (Hirche and Kattner, 1993) and is more lipid rich than *C. finmarchicus*, which is considered to be an income breeder, with the potential for capital breeding when food conditions are poor (Plourde and Runge, 1993; Mayor et al., 2009). In addition, *C. glacialis* is essentially an ice-associated species, whereas *C. finmarchicus* is predominantly an open ocean species (Wold et al., 2011). As such it has been suggested that *C. finmarchicus* and *C. glacialis* support different Arctic food webs (Weslawski et al., 2000; Karnovsky et al., 2008; Falk-Petersen et al., 2009), and therefore a shift in *Calanus* composition may have major implications on the energy flow through the food web (Falk-Petersen et al., 2009).

To fully understand how climate change and a warmer future will impact Arctic marine food webs, correct identification of these species is essential (Nielsen et al., 2014). *C. hyperboreus* can reliably be distinguished from both *C. glacialis* and *C. finmarchicus* due to its larger size, whereas *C. glacialis* and *C. finmarchicus* are morphologically very similar (Frost, 1974) and are often misidentified. Traditional methods for distinguishing between *C. finmarchicus* and *C. glacialis* include assessing the curvature of the 5th pair of swimming legs – a method that is not only ambiguous, complex and stressful to the animal, but which has also proven to be unreliable, even when undertaken by skilled taxonomists (Lindeque et al., 1999; Choquet et al., 2018). An easier method of identification is based on prosome length, since *C. glacialis* are typically larger than *C. finmarchicus* (Unstad and Tande, 1991; Hirche et al., 1994; Weydmann and Kwasniewski, 2008). However, this approach is also limited because of prosome length overlap of these congeners in regions such as the Greenland Sea, the Irminger Basin, Svalbard and the Norwegian coast (Lindeque et al., 2006; Parent et al., 2011; Gabrielsen et al., 2012; Nielsen et al., 2014; Choquet et al., 2018; Trudnowska et al., 2020). When morphological variation cannot unambiguously identify species, genetic variation may be used for taxonomic discrimination. *Calanus* species exhibit considerable base-sequence divergence in the mitochondrial 16S rRNA gene (Bucklin et al., 1995). Molecular techniques have been used for the identification of copepods from the genus *Calanus* including species-specific PCR (Hill et al., 2001); amplification of a region of the 16S rRNA gene using PCR followed by restriction fragment length polymorphism (RFLP) analysis of the amplified product (Lindeque et al., 1999; Lindeque et al., 2004; Lindeque et al., 2006); nuclear microsatellite markers (Provan et al., 2009; Parent et al., 2012; Choquet et al., 2017; Trudnowska et al., 2020) and nuclear insertion/deletion markers (Smolina et al., 2014). The method developed by Lindeque et al. (1999, 2004) has provided a definitive method to unambiguously identify any developmental stage of *Calanus* to species, and has been successfully used in multiple, large-scale studies (Lindeque et al., 2004; Lindeque et al., 2006; Gabrielsen et al., 2012; Parent et al., 2012; Tarling et al., 2022).

A drawback to all molecular approaches is that they cannot be used to identify animals prior to conducting physiological and ecological experiments that provide insight into the phenological plasticity of different species in response to temperature, food, and other environmental variables. Such experiments are required to fully understand the impacts of climate change on north Atlantic and Arctic food webs, and hence there remains a need to quickly and reliably identify individual live animals with minimal disturbance.

This study builds on the work of Nielsen et al. (2014) to determine the reliability of using prosome length, redness of antennae, somites or the whole animal, to identify live adult females *Calanus* spp. from the Fram Strait. This region of sympatry for *C. finmarchicus* and *C. glacialis* (Von Appen et al., 2015) represents a transition zone between the central

Arctic Ocean and the northern North Atlantic where the West Spitsbergen Current transports warm Atlantic Water into the Arctic Ocean and the East Greenland Current carries cold polar water towards the South West (de Steur et al., 2009).

MATERIALS AND METHODS

Sample Collection

Zooplankton samples were collected in the Fram Strait during late summer 2019 (JR18007, 04/08/2019 to 28/08/2019) aboard RRS James Clark Ross using a motion-compensated Bongo net (200 µm mesh), fitted with a cod end with mesh window, from a depth of 200 m to the surface from 'Ice Station 2' (78°20'33.8"N 4°42'04.5"W) and 'D3' (79°35'59.6"N 7°19'57.6"E). The collected animals were transferred into a controlled temperature laboratory set at ambient seawater temperature (1°C).

Morphological Characteristics

Live adult female *Calanus* were carefully picked into 0.2 µm filtered seawater using swan-necked forceps and a dissecting microscope (Wilde M5) under dim light. A total of 139 adult female *Calanus* were individually photographed in a custom-made Petri dish with visible gradations of known dimensions. Images were captured using an iPhone 7 connected through a microscope adaptor (×12 objective lens, ×10 eyepiece lens), before the photographed individual was transferred into a numbered 1 mL glass vial containing 95% ethanol until molecular analysis. The image analysis software Image J (v. 2.0w; https://imagej.nih.gov/ij/) was used to measure the prosome length by calibrating the software with known distance. Redness of the antennae and genital somite (**Figure 1**) was measured by cropping the images as tightly as possible around these features, masking any other parts of the organism with white pixels so as not to include them, and measuring red pixels as described in Nielsen et al. (2014); Nielsen et al. (2014b), using thresholding values of: Hue 0-23, Saturation 75-255, Brightness 1-255.

Molecular Identification

Individual *Calanus* were genetically characterised to species level based on the Restriction Fragment Length Polymorphism (RFLP) signature of their mitochondrial 16S rDNA using the molecular identification technique described by Lindeque et al. (1999); Lindeque et al. (2006) with modifications detailed below. This was carried out blind, i.e. without reference to the redness and prosome length results.

Individual copepods were removed from sample tubes containing 95% ethanol using a hypodermic needle and were cut in half using a sterile scalpel, half was returned to the ethanol and the other half was processed. Excess ethanol was removed by dabbing on absorbent paper, before rehydration in 150 µL DNA grade water (Fisher Scientific) in a 96-well plate for 6-12 hours at room temperature. After rehydration, the water was removed and replaced with 34.5 µL DNA water and 5µL of 10 x Taq DNA polymerase colourless buffer (Qiagen). The samples were homogenised using a hypodermic needle (19G $1^{1/2}$) inserted into a pellet pestle hand-held homogeniser (Sigma-Aldrich) and incubated at 4°C overnight. Following incubation, the remaining PCR components were then added: 5 µL 2 mM dNTPS (Qiagen), 2.5 µL of primers 16SB2R (5′-ATTCAACAT CGAGGTCACAAAC-3′; Lindeque et al., 1999) and 16SAR (5′-CGCCTGTTTAACAAAACaT-3′; Palumbi and Benzie, 1991) and 0.5 µL of Taq DNA polymerase (Qiagen). Amplifications were performed in a G-Storm, GRI 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)

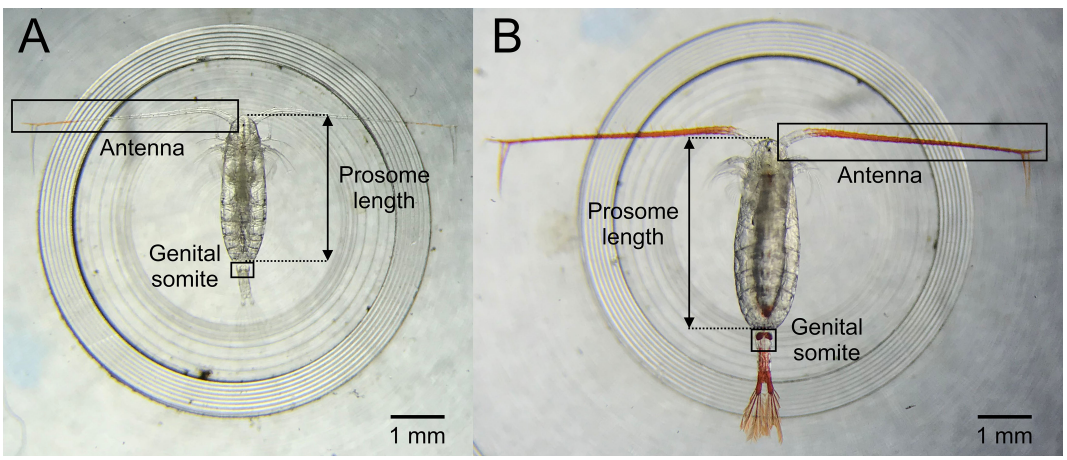

FIGURE 1 | Example images of **(A)** *Calanus finmarchicus* and **(B)** *Calanus glacialis* showing the diagnostic features used (prosome length and redness criteria of antenna and genital somite) to distinguish between females of the two species.

and 94°C (1 min). A final annealing phase at 45°C (2 min) was followed by an extension phase at 72°C (3 min) and storage at 4°C.

To check for amplification efficiency, aliquots (5 μL) of the amplification reaction were analysed by gel electrophoresis (2%). Restriction digests were performed on a 10 μL aliquot of each amplification by the addition of 13 μL DNA water, 3 μL 10 x Tango Buffer and 2 μL of each restriction enzyme (Dde1 and Vsp1, Thermo Scientific). Incubations were performed at 37°C for 4 hours in a PCR machine. 10 μL aliquots of the digestion products were separated by electrophoresis through a 2% metaphor agarose gel (VWR) which had been pre-chilled for 30 minutes at 4°C to improve resolution. The banding patterns were visualised by UV transillumination (SynGene) and the restriction digests analysed.

Data Analysis

The accuracy with which *C. finmarchicus* and *C. glacialis* could be discriminated using prosome length, genital somite redness, antennae redness and whole animal redness was examined using logistic regression with binomial errors (Crawley, 2007). The explanatory power of each of these variables was assessed using a variety of metrics: explained deviance (analogous to R^2 in a linear regression model); the Akaike Information Criteria (AIC) (a measure of fit after penalizing the model for the number of independent variables); the overall accuracy of the model (its ability to correctly predict the species of *Calanus*); the area under the receiver operating characteristic (ROC) curve (the higher the value, the better the prediction power of the model). Confusion matrices are presented to illustrate the number of correct/incorrect species identifications generated using each individual explanatory variable. All statistical analyses were conducted in the R programming environment (version 3.6.1; R Core Team, 2019).

RESULTS

Morphological Characteristics

The prosome length distribution of female *C. finmarchicus* (n = 90) and *C. glacialis* (n = 49) showed a bimodal pattern, with considerable overlap between the two species (**Figure 2**). *C. glacialis* alone also showed a less pronounced but slight bimodal distribution (**Figure 2**). The mean ± SD prosome lengths of *C. finmarchicus* and *C. glacialis* were 2.56 ± 0.24 mm and 3.11 ± 0.38 mm, respectively. The redness (area %) of the genital somites, antennae and whole animal for both species are presented in **Figure 3**. Respective mean values ± SD were 0.14 ± 1.32, 0.33 ± 0.68 and 0.21 ± 0.35 for *C. finmarchicus* and 9.06 ± 8.31, 3.05 ± 1.80 and 0.43 ± 0.44 for *C. glacialis*.

Molecular Identification

All 139 individual adult females were identified to species level by RFLP-PCR. Based on the restriction profiles, 49 individuals were assigned to *C. glacialis* and 90 individuals to *C. finmarchicus*.

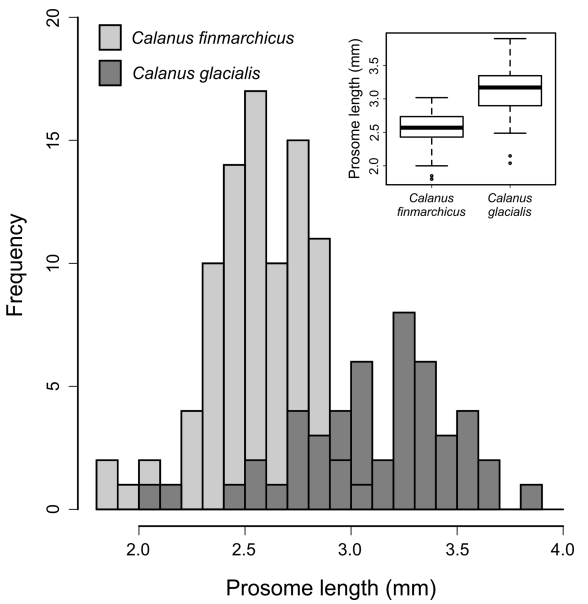

FIGURE 2 | Prosome length distribution of adult females of *Calanus finmarchicus* and *Calanus glacialis* from the Fram Strait. Inset boxplot shows the median prosome lengths (thick horizonal lines), their interquartile ranges (top and bottom of boxes = third and first quartile, respectively) and 1.5 times the interquartile ranges (whiskers).

Using Morphological Characteristics to Distinguish Between *C. finmarchicus* and *C. glacialis*

Antennae redness was consistently the best at discriminating between *C. finmarchicus* and *C. glacialis* across all of the metrics used, and whole animal redness was consistently the worst (**Table 1A**). The performance of the explanatory variables followed the following rank order: antennae redness > genital somite redness > prosome length > whole animal redness, with the latter explaining only 5% of the observed deviance. Interestingly, the overall accuracy of using antennae redness and genital somite redness to discriminate between the two species were the same (**Table 1A**), yet each of these explanatory variables had different specificity (**Table 1B**); *C. finmarchicus* were more accurately identified using genital somite redness (accuracy = 98.9%), whereas *C. glacialis* were more accurately identified using antennae redness (accuracy = 85.7%). Prosome length, the third best of the examined response variables, correctly identified *C. finmarchicus* and *C. glacialis* in 97% and 76% of the cases, respectively. The high level of accuracy for identifying *C. finmarchicus* relative to *C. glacialis* reflects the less variable size range of prosome length in the former (see inset boxplot in **Figure 3**).

DISCUSSION

This study assessed the reliability of identifying *C. finmarchicus* and *C. glacialis* in the field using morphological characteristics

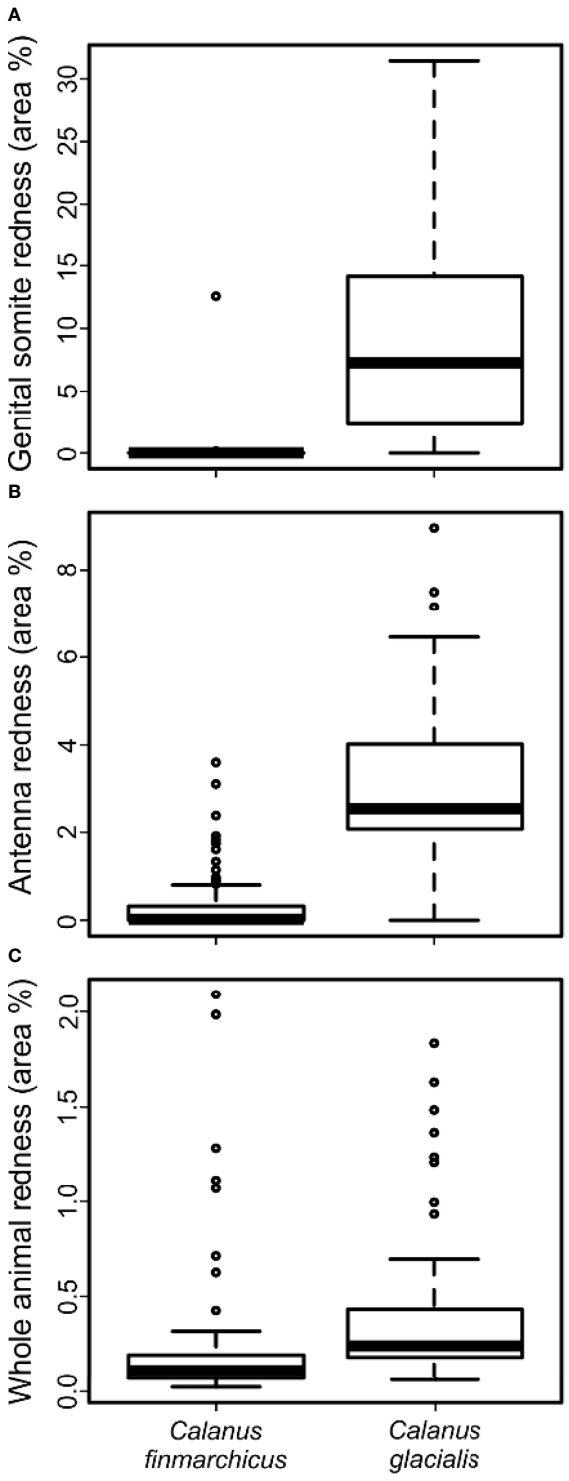

FIGURE 3 | Percentage of red pixels (area %) within selected regions of *Calanus finmarchicus* and *Calanus glacialis*: Redness of the genital somite **(A)**, antennae **(B)** and whole animal **(C)**. Boxplots illustrate the median (thick horizonal lines), interquartile ranges (top and bottom of boxes = third and first quartile, respectively) and 1.5 times the interquartile ranges (whiskers).

that are visible by eye. Molecular analysis of a 16S rDNA barcode confirmed that the best overall morphological features for distinguishing between adult females of these two species were the redness of the antennae and the genital somites; both of these criteria generally outperformed prosome length as a distinguishing characteristic, although even prosome length showed good, overall performance. Interestingly, the accuracy of using redness of the antennae and genital somites to discriminate between the two species were the same, yet each of these explanatory variables had different specificity. *C. finmarchicus* were more accurately identified by the absence of redness in the genital somites (**Table 1**); of the 90 imaged females, 89 (98.9%) were correctly identified by the level of red pigmentation in this area, whereas only 83 (92.2%) were correctly identified on the basis of their antennae redness. By contrast, *C. glacialis* were more accurately identified using antennae redness; of the 49 imaged females, 42 (85.7%) were correctly identified by the red pigmentation in the antennae, whereas only 36 (73.5%) could be identified on the basis of the redness of their genital somites.

Previous studies have indicated that live females of *C. finmarchicus* and *C. glacialis* can be separated by the red pigmentation of the antennae and genital somites from both East and West Greenlandic waters (Nielsen et al., 2014). Our redness values for the antennae and genital somites for *C. glacialis* were ~50% lower than those in Nielsen et al. (2014), yet they were sufficient to clearly distinguish between the two species. Choquet et al. (2018) also investigated the reliability of using pigmentation of the antennae and genital somites to identify *C. finmarchicus* and *C. glacialis* from the White Sea, Svalbard and three Norwegian fjords. In their study, they found that red pigmentation was variable, with significant differences between the two species, among developmental stages and locations sampled. Nevertheless, the majority of *C. finmarchicus* tended to have pale antennae, whereas the majority of *C. glacialis* tended to have red ones. Exceptions to this appear to have been from different developmental stages where males of both species were pale without exception, and *C. glacialis* CIV were generally less pigmented than *C. glacialis* CV and CVI. When they compared redness of antennae versus redness of genital somites for both species from distinct locations, in all but two cases, animals with pale antennae and genital somites were *C. finmarchicus*. All individuals with red antennae and red genital somites were *C. glacialis*, although some *C. finmarchicus* from Svalbard and Saltenfjord did display redness in the antennae. Our findings corroborate this: *C. finmarchicus* were most reliably identified by the absence of redness in the genital somite areas, whereas *C. glacialis* were most accurately identified by possessing redness in the antennae.

Trudnowska et al. (2020) suggested that the pigmentation of antennae and genital somites in both *C. finmarchicus* and *C. glacialis* were highest in copepods inhabiting their preferred water mass, or their 'comfort zone'. *C. finmarchicus* CV had high red pigmentation in the antennae when sampled from the Atlantic domain of the Polar Front and *C. glacialis* CV had high red pigmentation in the antennae and genital somites when sampled

TABLE 1 | Performance metrics **(A)** and confusion matrices **(B)** for each of the explanatory variables to discriminate between *C. finmarchicus* (n = 90) and *C. glacialis* (n = 49).

A	Assessment criterion	Explanatory variable			
		Prosome length	Genital somite redness	Antennae redness	Whole animal redness
	Explained deviance (d^2)	0.441	0.503	0.595	0.053
	AIC	104.9	93.7	77.0	174.9
	Area under ROC curve	0.898	0.930	0.941	0.807
	Overall accuracy	0.892	0.899	0.899	0.669
B	Correctly identified *C. finmarchicus*	87 (0.966)	89 (0.989)	83 (0.922)	85 (0.944)
	Incorrectly identified *C. finmarchicus*	3 (0.033)	1 (0.011)	7 (0.078)	5 (0.056)
	Correctly identified *C. glacialis*	37 (0.755)	36 (0.735)	42 (0.857)	8 (0.163)
	Incorrectly identified *C. glacialis*	12 (0.245)	13 (0.265)	7 (0.143)	41 (0.837)

*Values in **(B)** represent the absolute number of individuals identified (with proportions in parentheses). AIC, Akaike Information Criteria; ROC, receiver operating characteristic. Shaded boxes indicate the best performing variables.*

from the Arctic domain of the Polar Front. For both species, the amount of redness progressively decreased from the open water stations to the inner fjord and glacial bay stations. If their hypothesis is correct, our observations of poorly pigmented adult female *C. finmarchicus* in the Fram Strait may indicate that these animals are far less suited to the local conditions than *C. glacialis*, which were more pigmented. Depth of occurrence may also influence the intensity of red pigmentation in *Calanus* spp., with deeper-dwelling animals having reduced need for carotenoid pigments (redness) that protect against the harmful effects of ultraviolet irradiance on stored lipids (Trudnowska et al., 2020). Unfortunately, the animals in our study were collected using vertical hauls from 200 m to the surface, and hence variation in red pigmentation with depth could not be assessed.

Previously molecular identification has highlighted how using prosome length as a species discriminator for *Calanus* has led to erroneous results (Breur, 2003; Lindeque et al., 2004; Parent et al., 2011; Gabrielsen et al., 2012; Nielsen et al., 2014; Choquet et al., 2018). For example, Gabrielsen et al. (2012) found that prosome length systematically overestimated the abundance of *C. finmarchicus* at the expense of *C. glacialis* from three Arctic fjords. There is often an overlap between the prosome length of the two species, with the extent of this overlap depending on the location where the species co-occur, the temperature at which development occurred and the likelihood of advection and hence mixing of the species or populations of the same species. In our study, the use of prosome length to distinguish between *C. finmarchicus* and *C. glacialis* achieved not dissimilar overall accuracy compared to using the redness of genital somites and antennae (**Table 1**). Nevertheless, it underperformed relative to the use of genital somites redness when identifying *C. finmarchicus*, and underperformed relative to the use of antennae redness when identifying *C. glacialis*. Prosome length correctly identified 87 of the 90 (96.6%) imaged *C. finmarchicus*, but only 37 of the 49 (75.5%) *C. glacialis*. The increased accuracy when identifying *C. finmarchicus* reflects the less variable size range of the individuals of this species in our study, relative to *C. glacialis* (see inset boxplot in **Figure 2**). Exactly why the size range of individual *C. finmarchicus* was less variable than *C. glacialis* in our study is unknown. It may relate to a more variable temperature range experienced by the copepodites of the latter species during their development as in Parent et al., (2011). Alternatively, it may suggest that the sampled *C. glacialis* were not from a single generation or even population, and instead, consisted of a local population mixed with at least some larger individuals that have been carried south with the East Greenland Current which carries cold polar water towards the South West of the Fram Strait (de Steur et al., 2009). This interpretation has previously been used to explain why single-species populations of *Calanus* in the Arctic and Atlantic Oceans have bimodal size distributions (Lindeque et al., 2006; Trudnowska et al., 2020). Regardless, it is clear that the prosome lengths of *C. finmarchicus* and *C. glacialis* overlap and cannot be considered as a strong criterion for separating the two species, particularly if the species are being used as representatives of Atlantic versus Arctic water masses and thus climatic indicators.

Our study does, however, suggest, that antennae redness, somite redness and prosome length all achieve a sufficient level of accuracy for identifying adult female *Calanus* spp. in the Fram Strait for use in experiments. For the purposes of this study, adult female *Calanus* spp. were picked at random, making no judgement as to whether they were *C. finmarchicus*, *C. glacialis* or indistinguishable. However, when picking live animals for use in experiments, we suggest that increased accuracy of species identification may be achieved if only those animals that unambiguously fulfil the pigmentation and/or size criteria were chosen. For example, picking only smaller individuals that showed no redness in the antennae and/or genital somites would likely yield a cohort of individuals dominated by *C. finmarchicus*, whereas selecting larger animals with increased levels of pigmentation in the antennae and/or genital somites would likely produce a sample dominated by *C. glacialis*. Accurately determining species identification of live animals in the field using morphological characteristics that are visible by eye greatly facilitates the implementation of experiments to determine how individual species will respond and adapt to climate change in the Arctic. Nevertheless, it is clear that no single morphological characteristic can determine the species of live *Calanus* with 100% accuracy and we suggest that it is prudent to integrate the use of molecular identification techniques into all future studies working on *Calanus* spp. in the Arctic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Samples were collected, sorted and photographed by DM. AL and IH carried out image analysis to determine antenna and genital somite redness, and prosome length. Molecular analyses were performed by HP and IH. DM undertook data analysis. The manuscript was written by PL, DM and IH with further input from all authors. All authors contributed to the article and approved the submitted version.

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