SHORT COMMUNICATION

Lipid sac area as a proxy for individual lipid content of arctic calanoid copepods

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We present an accurate, fast, simple and non-destructive photographic method to estimate wax ester and lipid content in single individuals of the calanoid copepod genus *Calanus* and test this method against gas-chromatographic lipid measurements.

KEYWORDS: *Calanus*; lipid sac; energy; image analysis; wax ester; lipid content; gas chromatography

In the Arctic, the three co-occurring calanoid copepods *Calanus glacialis*, *C. finmarchicus* and *C. hyperboreus* are the key trophic links between primary producers and higher trophic levels (Lee *et al.*, 2006). *Calanus* spp. convert low-energy carbohydrates and proteins, but also dietary fatty acids from their micro algal diet into high-energy wax esters, making up to 70% lipids of their dry mass (Falk-Petersen *et al.*, 2009). Lipids are mainly stored in a lipid sac which can fill more than 80% of the body cavity in older copepodite stages (Miller *et al.*, 1998; Sargent and Falk-Petersen, 1988; Miller *et al.*, 2000; Lee *et al.*, 2006). Due to their abundance and extensive energy storage, *Calanus* spp. form an important source of food for many predators, e.g. herring (*Clupea harengus*) (Varpe *et al.*, 2005), basking sharks (*Cetorhinus maximus*) (Sims *et al.*, 2003) and little auks (*Alle alle*) (Steen *et al.*, 2007).

The lipid sac content of a single specimen is closely related to the total energy reserves of that individual, and there is high intra-specific variability in lipid sac size depending on developmental stage and time of the year (Arts and Evans, 1991; Søreide *et al.*, 2008). Importantly, behavioral and life-history strategies of many copepods are state-dependent with individual energy reserves being a key state (Fiksen and Carlotti, 1998; Hays *et al.*, 2001; Varpe *et al.*, 2009; Søreide *et al.*, 2010). Consequently, an accurate, easy, cheap and fast method to determine the lipid content of copepods is of great interest.

There has been a range of attempts to measure total lipid contents in copepods using digital imaging (video, still image), chemical analysis and visually determined indices, with pioneering work such as that of Sushkina (Sushkina, 1961) and Petipa (Petipa, 1964). A review of these methods can be found in Lee *et al.* (Lee *et al.*, 2006). Here we have measured both the lipid sac area as well as the wax ester and total lipid content of individual copepods by digital image analyses and gas chromatography, respectively. This allows for a correlation between area and lipids to be estimated. Our

work adds to previous analyses by Miller *et al.* (Miller *et al.*, 1998) where lipid sac size (but not area as in our work) from optical measurements was related to lipid levels as measured by gas chromatography. Their measurements were from *Calanus finmarchicus* copepodite stage V. We used a broader range of species and developmental stages, namely the calanoid copepods *C. finmarchicus, C. glacialis* and *C. hyperboreus, stages* CIV, CV and adult females (in non-reproductive state). These three species were sampled around Svalbard, Norway at different locations and different times of the year (January, July and September). We assumed that the variation in wax ester content was dependent on the lipid sac size and thus independent of species and developmental stages. The *Calanus* specimens were individually picked out of the total sample using a pipette. As copepods soon after their death loose most of their transparency, only living, intact individuals without any visual damage to the lipid sac were chosen. To reduce their mobility, the specimens were placed in a single droplet of water in a Petri dish and photographed from a lateral view. For the photographs, we used a Leica stereomicroscope with a Sony HDR-HC7 video camera in photo mode. The camera was mounted on one of the microscope tubes with a video camera mount. For every magnification and camera zoom level used, a picture of a calibration slide was taken. The individuals were then singly placed into Eppendorf or cryo vials and quickly frozen in liquid nitrogen. Samples were stored in biofreezers at $-80°C$ until further lipid analyses.

For the image analysis, the free, multi-platform image analysis software "ImageJ" was used (Rasband, 1997–2009). The pixel-to-μm ratio was calibrated using the image of the appropriate calibration slide. The differences in contrast between lipid sac and the rest of the animal were not sufficient to define thresholds for automated measuring functions of ImageJ. To allow measurements, the perimeter of the lipid sac (Fig. 1A) and the length of the prosome were outlined manually. The ImageJ output is an Excel or text file with measures of lipid sac area and prosome length. Area is derived from a count of pixels inside the perimeter outline, transformed to square micrometer using the calibration as described above.

Tessier and Goulden (Tessier and Goulden, 1982) have previously pointed out that all optical methods are biased when they are applied by different scorers. To check reproducibility of the image measurements, prosome length and lipid sac area of 215 images were measured twice, once by one of the authors and the second time by a student after a short introduction to the method. Measurements were highly correlated ($n = 215$, r^2 between 0.92 and 0.99), suggesting high repeatability of the proposed method.
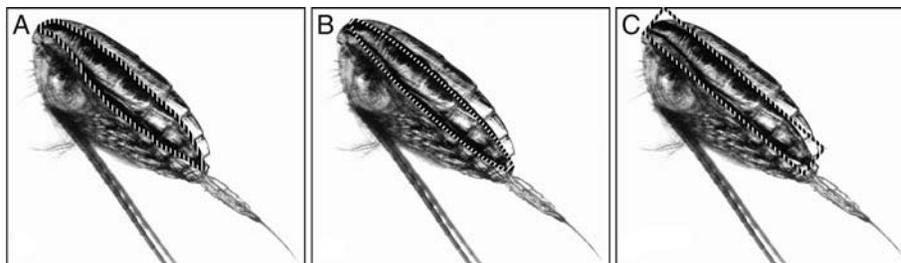

Fig. 1. Lipid sac outlines according to three different measuring methods: (**A**) perimeter, (**B**) oblate spheroid, (**C**) length × width.
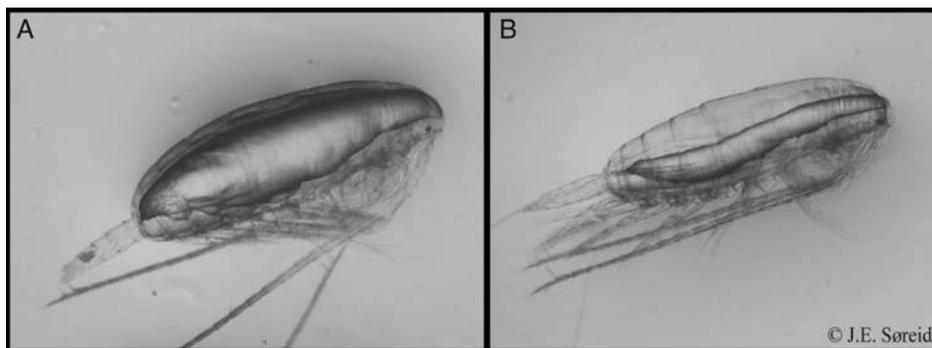

Fig. 2. (**A**) A well filled lipid sac, and (**B**) a thin and elongated lipid sac.

Of the 215 specimens photographed and measured, 52 specimens with variable lipid sac sizes ranging from small to large (Fig. 2) were selected for gas chromatographic lipid analyses. Single specimens were dried in a freeze dryer for 12 h and transferred into pre-weight glass vials for dry weight (DW) determination on a Sartorius R200D balance (accuracy $\pm$ 0.02 mg).

After freeze-drying, lipids were extracted from single specimens using dichloromethane:methanol (2:1 by vol.) (Folch *et al.*, 1957). The fatty acid and alcohol compositions were analysed by gas-liquid chromatography (GC) according to Kattner and Fricke (Kattner and Fricke, 1986). Lipids were converted to their methyl esters and free fatty alcohols by trans-esterfication in methanol containing 3% concentrated sulphuric acid at 80°C for 4 h. After extraction with hexane, fatty acid methyl esters and alcohols were analysed with a Hewlett-Packard 6890 Series gas chromatograph with a DB-FFAP fused silica capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness) using temperature programming (160–240°C at 4°C min^{-1}, hold 15 min). For recording and integration, Class-VP software (4.3) (Shimadzu, Germany) was used. Fatty acids and fatty alcohols were identified with known standard mixtures, and if necessary additional confirmation was obtained by gas chromatography-mass spectrometry (Kattner *et al.*, 1998). The amount of total lipid was calculated as the sum of fatty acid methyl esters and alcohols. Wax ester (WE) proportion was calculated from fatty acid and alcohol content. For 8 of the 52 samples, no total lipid content was obtained due to contamination, whereas for 5 of the 52 samples no dry weight data were obtained (online Supplementary Table SI).

All statistical analyses were done in R (R Development Core Team, 2007) and the significance level was set to $P \leq 0.05$.

The most direct and simple measure of the lipid sac in a two-dimensional image is the lipid sac area. There are several different ways to obtain an approximation of the area of an object. A rough approximation can be achieved by multiplying length and width, measured at the largest respective distances as it was done in several earlier studies (Table I). A more accurate method is the perimeter line, which results in an area based on all pixels included within the perimeter. By using lipid sac area directly, we also avoid the problems that occur in transforming area measurements to volumetric units, and transforming those again to weight.

Table I: Lipid sac measurement methods with description of which geometrical shape the lipid sac measurements are based on

Standardized equation	Species (reference)	r^2	Geometrical shape
Equation 1: $$WE = 0.167A^{1.42}$$	*C. finmarchicus, C. glacialis, C. hyperboreus* (this study)	0.95	Area *A* based on perimeter (Fig. 1A)
Equation 2: $$V = \frac{4}{3}\pi\frac{L}{2}\left(\frac{W}{2}\right)^2$$	*Calanus helgolandicus* (q[1]), *C. finmarchicus* (b, d, j[1]), *C. hyperboreus* (j[1]), *C. euxinus* (l, n), *Calanoides carinatus* (a), *Diaptomus sicilis* (e), *Oithona similis* (k)	0.90	Sphere, ellipsoid, prolate spheroid (Fig. 1B)
Equation 3: $$V = \frac{1}{4}\pi\frac{1}{L}A^2$$	*Calanus finmarchicus* (p, g), *Pareuchaeta norvegica* (o)	0.76	Cylindrical tube (Fig. 1C)
Equation 4: $$V = \frac{1}{4}\pi LW^2$$	*Pseudocalanus* sp. (i)	0.90	Cylindrical tube (Fig. 1C)
No equation, volume calculation by disc-integration	*C. finmarchicus* (h)	–	–

Equations are expressed in different notations in the literature (see references). To simplify comparison, they have been transformed to standardized equations. The fit of the regression model, i.e. the proportion of total variability explained (r^2) is shown for each equation [equation (4) referring to untransformed data].
Abbreviations in standardized equations (different in original equation): *L*, length; *A*, area; *W*, width.
References: (a) Arashkevich *et al.* (1996); (b) Arashkevich *et al.* (2004); (d) Plourde & Runge (1993); (e) Arts and Evans (1991); (g) Miller *et al.* (1998); (h) Miller *et al.* (2000); (i) Reiss *et al.* (1999); (j) Pasternak *et al.* (2001); (k) Narcy *et al.* (2009); (l) Svetlichny *et al.* (2009); (m) Svetlichny *et al.* (2006); (n) Svetlichny *et al.* (1998); (o) Vestheim *et al.* (2005); (p) Hassett (2006); (q) Petipa (1964).
[1]The authors do not give any equation, but according to the parameters measured, it can be assumed that equation (1) was used.
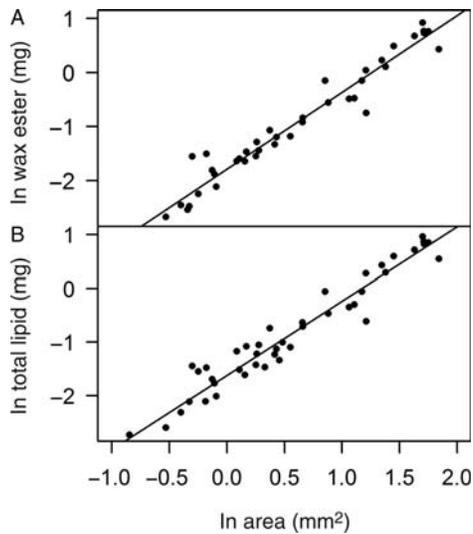
Fig. 3. Wax ester (WE, **A**) and total lipid (TL, **B**) obtained by gas chromatography plotted as a function of lipid sac area (A). WE (mg), TL (mg) and A (mm^2) are ln transformed. (**A**) $n = 44$, $r^2 = 0.95$, $P < 0.001$, linear regression: $\ln WE = -1.79 + \ln A^{1.42}$. (**B**) $n = 44$, $r^2 = 0.94$, $P < 0.001$, linear regression: $\ln TL = -1.62 + \ln A^{1.38}$.

Wax ester, total lipid and lipid sac area data were ln transformed, a standard way of presenting allometric relationships (Burton, 1998). The subsequently presented equations are derived from the ln equations, following the general power law relation

$$Y = aX^b \quad (5)$$

and the logarithmic equivalent:

$$\ln Y = \ln a + b \ln X \quad (6)$$

Scaling coefficients and exponents for wax ester (WE) in mg predicted by area (A) in mm^2 in Fig. 3A are:

$$WE = 0.167A^{1.42} \quad (1)$$

and similarly for total lipid (TL) in mg predicted by area (Fig. 3B):

$$TL = 0.197A^{1.38} \quad (7)$$

Total wax ester content ranged from 0.041 mg for the smallest to 2.506 mg for the specimens with largest lipid sacs, and a high correlation ($r^2 = 0.95$ for ln transformed, $r^2 = 0.90$ for untransformed data) between the lipid sac area and the wax ester content was found (Fig. 3A). Due to the high percentage of wax ester (44–97% of total lipid, mean $84 \pm 13\%$, see online Supplementary Table SI), the correlation of lipid sac area to total lipid (Fig. 3B) was almost identical (range: 0.048–2.600 mg, $r^2 = 0.94$ ln transformed, $r^2 = 0.92$ untransformed data).
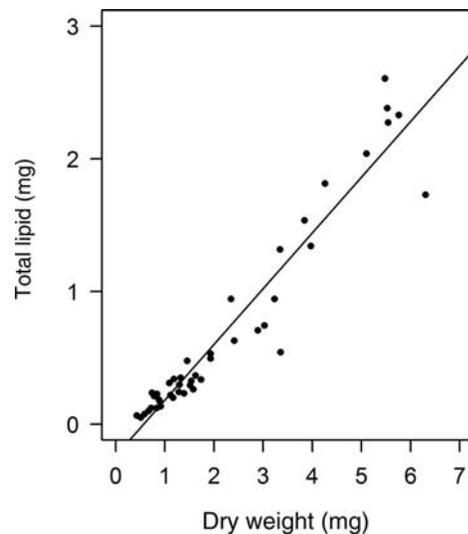

Fig. 4. Correlation between total lipid from gas chromatography and dry weight (entire specimens). $n = 39$, $r^2 = 0.91$, $P < 0.001$. Linear regression: $TL = -0.221 + 0.419DW$.

A classic method to gain information on energy content of an animal is to measure the dry weight. Linear regression of individual dry weights versus total lipid results from GC revealed highly significant ($r^2 = 0.91$) relationships with high precision (Fig. 4). Mean total lipid content was 51% of total dry weight ($n = 40$, min. 25%, max. 85%, SD: 13%). The linear regression for this relationship is

$$TL = 0.037 + 0.453DW \quad (8)$$

where TL (mg) is total lipid and DW (mg) is dry weight.

The estimated correlation between lipid sac area and dry weight (Fig. 4) indicates that dry weight is highly correlated with the lipid sac content within the size range of lipid sacs analyzed. Dry weight of frozen individuals can therefore be used to estimate total lipid if there are no possibilities to take pictures of live specimens.

Two different geometrical approximations to the actual lipid sac shape have been suggested earlier (see Table I), an ellipsoid (or spheroid) and a cylindrical tube (see Fig. 1B and C for actual application of the shapes). In addition, Miller et al. (Miller et al., 2000) used a more complex method, dividing the lipid sac in small disc-shaped units and summing up the volume of these. As is apparent from Fig. 1, there is an overestimation of lipid sac size for lipid sacs that do not follow the geometrical shapes, since the shapes are based on maximum extension measurements. We calculated the theoretical lipid sac content based on the published equations (Table I) using length/width/area measurements from
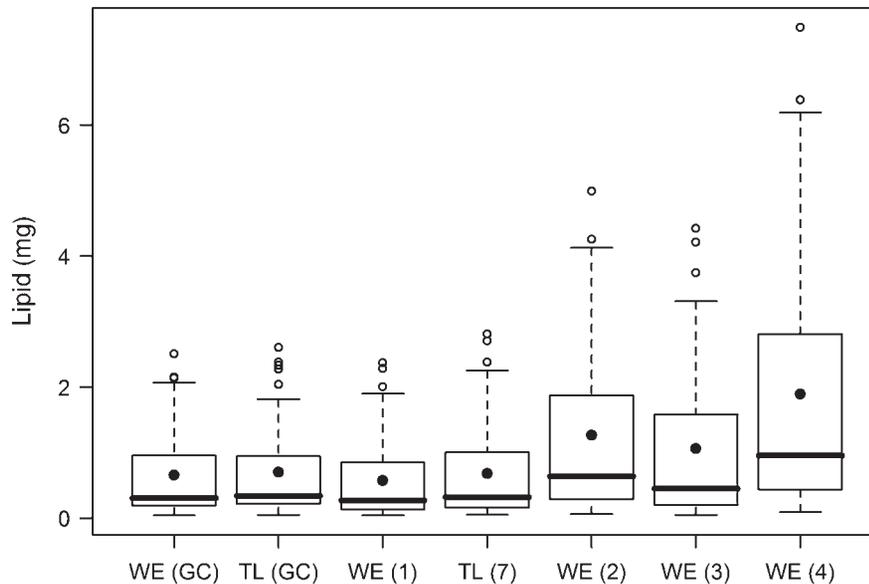
Fig. 5. Wax ester (WE) and total lipid (TL) measured by gas chromatography and calculated WE and TL according to the regression from this publication [equations (1) and (7)] and according to the other volume equations from Table I, assuming a density of 0.9 g mL^{-1} (Miller *et al.*, 1998) when calculating lipid weight from lipid sac volume. Whiskers extending to 1.5 × interquartile range or maximum data point.

our samples (see Supplementary Table SI for details) and plotted the expected results against the actual measurements (Fig. 5). Based on these measurements, equations (2) and (3) overestimated the lipid content by a factor of approximately 2, whereas equation (4) had a corresponding overestimation by a factor of approx. 3 [mean WE (GC): 0.66 mg, mean WE equation (2): 1.26 mg, mean WE equation (4): 1.90 mg]. Some part of this mismatch can be explained by the overestimation of the lipid sac size by geometrical shapes that cover a larger area than the actual lipid sac. For all samples analyzed here, area calculated by length × width is up to 75% larger than by perimeter line [Online Supplementary Table SI: LSA% (LSL × LSW)]. Miller *et al.* (Miller *et al.*, 1998) found the same problem in their image-to-lipid calculations, where video estimates were about 2-fold of the Iatroscan results, and suggested problems with an internal standard for the Iatroscan as a source for the discrepancy. In our study, the total lipid weight ranges from 25 to 85% of the dry weight, which is well in the range of published data (Falk-Petersen *et al.*, 2009), supporting that the GC estimates are also in reasonable range and that the overestimation is caused mainly by volume equations not reflecting the real shape of a lipid sac accurately enough.

Although we only have data on the three major Arctic *Calanus* species, the method described may be applicable to other similar species, e.g. the Antarctic *Calanoides acutus*, which shows a life-history similar to the Arctic *Calanus* (Atkinson, 1998; Varpe *et al.*, 2007) and which also stores large amounts of wax ester in a distinct lipid

sac (Kattner and Hagen, 1995; Albers *et al.*, 1996). In the Pacific, *Calanus pacificus, C. marshallae, Neocalanus plumchrus* and *N. cristatus* also store lipid in a single large lipid sac (Miller *et al.*, 1984; Conover, 1988) which potentially could be measured with the method described here.

With the method and the correlations described, it is possible to give a good estimate of the wax ester and total lipid content of copepods which have a lipid sac. Large numbers of specimens can be processed in a short time at low cost, and data can be gathered easily in the field. Furthermore, because of the non-destructive nature of the method, repeated measures can be done on the same individual, which can be useful during for instance feeding experiments. Typical applications for this method could be predator–prey studies as in Steen *et al.* (Steen *et al.*, 2007), where the energy content of the prey items is of importance, or as a method in tests of the proximate and ultimate function of lipids in *Calanus* species (Campbell, 2004; Fiksen *et al.*, 2004; Irigoien, 2004).

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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