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Authors(s):	Inês Leal (Corresponding Author), Katrin Bohn (Co-author), Stephen John Hawkins (Co-author), Stuart Jenkins (Co-author), Augusto Flores (Co-author), Réjean Tremblay (Co-author)
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Lipid allocation in late-stage barnacle larvae from subtropical and temperate waters

Inês Leal[1,*], Katrin Bohn[2,3], Stephen J. Hawkins[3,4], Stuart R. Jenkins[5], Augusto A. V. Flores[6] & Réjean Tremblay[1]

[1] Institut des sciences de la mer, Université du Québec à Rimouski, 310 Allée des Ursulines, Rimouski, QC G5L 2Z9, Canada
[2] Natural England, Apex Court, City Link, Nottingham, NG24LA, UK
[3] Ocean and Earth Science, National Oceanography Centre Southampton, University of Southampton, European Way, Southampton SO14 3ZH, UK
[4] Marine Biological Association of the UK, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK
[5] School of Ocean Sciences, Bangor University, Anglesey LL59 5AB, UK
[6] Centro de Biologia Marinha, Universidade de São Paulo, Rodovia Manoel Hypólito do Rego, Km 131,50, S/N - Praia do Cabelo Gordo, São Sebastião - SP, 11600-000, Brazil

* Corresponding author: ines.agravasconcelosleal@uqar.ca

RUNNING PAGE HEAD: Larval physiology of two chthamalids

ABSTRACT

The transition of planktonic late-stage barnacle larvae to a benthic life requires enough energy to power settlement and metamorphosis, and may be compromised by food limitation during early ontogeny. We carried out a comparative study to better understand the larval physiology of space-monopolizing barnacles exposed to contrasting regimes of primary productivity: *Chthamalus bisinuatus* under a meso-oligotrophic regime on the southeastern coast of Brazil, and *Chthamalus montagui* under a highly-productive regime on the south coast of the British Isles. We used an index based on lipid composition, the triacylglycerol (TAG) to phospholipid (PL) ratio, to characterize lipid allocation (energy/structure) in the tissues of cyprid larvae and anticipated depleted TAG reserves in cyprids from less productive waters. Despite the considerably different levels of primary productivity between subtropical (1.31 ± 0.4 mg Chl-a/m^3) and temperate waters (3.09 ± 1.2 mg Chl-a/m^3), TAG/PL and settlement success were comparable for *C. bisinuatus* and *C. montagui*. Lipid allocation of daily cohorts was also comparable for both chthamalids, with cyprids equally storing TAG reserves (≥ 50 % of total lipid content). This points to an energetic threshold below which nauplii cannot develop to a cyprid and/or selection for lipid accumulation under poor trophic conditions. We highlight the challenges of directly relating estimates of primary productivity with food supply and larval physiological status, as lower Chl-a concentrations do not necessarily indicate food limitation for barnacle nauplii. We propose a conceptual model to clarify the process of lipid allocation (energetic to structural lipids) in the tissues of cyprid larvae.

KEYWORDS: Supply-side Ecology, Settlement dynamics, Larval physiology, Lipids, Energetics

1. INTRODUCTION

The recruitment dynamics of marine benthic organisms have been extensively studied for over seven decades (e.g. Barnes 1956, Connell 1961, 1985, Crisp 1976, Hawkins & Hartnoll 1982, Roughgarden et al. 1985, Rodriguez et al. 1993, Jenkins et al. 1999, Barbosa et al. 2016, Giménez et al. 2017). Variations in these dynamics have been related to spatial (e.g. Burrows et al. 2010) and temporal (e.g. Jenkins et al. 2008, Kasten et al. 2019a) patterns of pelagic food supply as indicated by chlorophyll-*a* concentration in coastal waters (Hawkins & Hartnoll 1982, Menge 1997, Leslie et al. 2005, Mazzuco et al. 2015). For instance, Burrows et al. (2010) reported that barnacles reached larger sizes in areas of high chlorophyll-*a* concentration, suggesting that food-limited growth, rather than differential survival caused this trend. High phytoplankton concentrations have also been suggested to increase recruitment by increasing the survival of larvae and recruits, by producing better fed, larger individuals (Menge et al. 1997). Moreover, more evidence has emerged that plasticity in larval *condition* can regulate post-settlement events, independent of larval *abundance* (Jenkins 2005, Tremblay et al. 2007, Allen & Marshall 2010, Giménez 2010). Across a variety of taxa, juvenile performance can be compromised by poor physiological condition of preceding larval stages (e.g. in the gastropod *Nucella ostrina*, Moran & Emlet 2001; barnacle *Semibalanus balanoides*, Jarrett 2003; bryozoan *Watersipora subtorquata*, Marshall & Keough 2004; tubeworm *Hydroides diramphus*, Allen & Marshall 2010; crab *Carcinus maenas*, Giménez 2010).

Many factors can affect larval condition, including: (1) quality and quantity of pelagic food sources (Burrows et al. 2010, Toupoint et al. 2012), (2) pelagic larval duration (Pechenik et al. 1993) and/or (3) maternal investment (Freuchet et al. 2015, Kasten et al. 2019b). Such effects appear to vary strongly under different environmental conditions (Marshall & Keough 2004).

Temporal changes in food availability are generally considered to be greater at higher latitudes as a result of seasonal changes in physical variables affecting primary production (Lawrence 1976). Low-latitude systems lack the large phytoplanktonic blooms found at higher latitudes (Ciotti et al. 2010). They are typically oligotrophic year-round and depleted of high-quality dietary fatty acids (Colombo et al. 2017, Leal et al. 2018, 2019), shown to be critical for the development of invertebrate larvae (Pernet et al. 2004, Tremblay et al. 2007). Latitudinal changes in egg size (i.e. an increase in egg size with a decrease of temperature) were addressed by classic works by Thorson (1950), who attributed developmental trends to different food availability in different latitudes, and by Rass (1941), who attributed egg size trends to temperature-dependent changes in yolk accumulation patterns (see Laptikhovsky 2006, for a new look at Thorson's and Rass's rules). Further, a decreasing latitudinal trend in lipid storage has also been reported for zooplankton groups, with those found in polar systems accumulating the largest lipid reserves (e.g. planktonic crustaceans, such as the amphipods *Themisto libellula* and *T. abyssorum*, the Antarctic krill *Euphausia superba* and the copepod *Calanus propinquus*; Lee et al. 2006). In coastal waters, such generalizations are difficult as phytoplankton blooms (that lead to a build-up of storage lipids in planktonic larvae) may result not only from upwelling of nutrient-rich waters, but also from local wind forcing and coastal circulation (e.g. scattered events of high productivity in subtropical shores that raise baseline oligotrophic conditions to eutrophic ones during short periods, 1 to 2 days; Kasten & Flores 2013) plus nutrient-rich estuarine plumes (Gorman et al. 2019).

On intertidal shores, enhanced quality of settling larval stages of benthic species may be critical to allow for a rapid metamorphosis and early juvenile survival (e.g. subtropical chthamalid barnacles with larger lipid reserves grow faster for the first days in the benthos; Leal et al. 2020). Triacylglycerol (TAG) is often the major storage lipid and the primary energetic source of lecithotrophic meroplanktonic larvae (e.g. acorn barnacle *Semibalanus balanoides*, Holland & Walker 1975; king crab *Lithodes santolla*, Kattner et al. 2003). Besides its energetic supply role, TAG functions as a source of essential fatty acids for membrane integrity, regulating the physiological metabolism of many crustacean species, especially the synthetic process of moulting hormones (O'Connor & Gilbert 1968). In non-feeding larvae, depleting TAG reserves can affect pre-attachment exploratory behaviour, so that larvae become less discriminating in their 'choice' of settlement substrate (Miron et al. 2000, Marshall & Keough 2003, Tremblay et al. 2007), and may ultimately lead to 'desperate' settlement, that is, metamorphosis occurs in response to a nonspecific cue or even spontaneously (Knight-Jones 1953, Crisp 1955, Lucas et al. 1979). Further, competent larvae with higher energy reserves have been shown to settle in better quality microhabitats (Marshall & Keough 2003), grow faster for the first days in the benthos (Thiyagarajan et al. 2003, Leal et al. 2020), and have greater chances of post-settlement survival (Thiyagarajan et al. 2005). A deeper understanding of supply-driven population dynamics (e.g. Underwood & Fairweather 1989, Minchinton & Scheibling 1991, Bertness et al. 1992, Delany et al. 2003) in the intertidal realm would therefore benefit from studies exploring larval lipidomics of foundation benthic species.

In the present study, we compared the larval physiology, supply, and settlement of two closely related space-monopolizing barnacles of the genus *Chthamalus*: the subtropical *C. bisinuatus* and temperate *C. montagui*, inhabiting shores exposed to relatively low and high coastal primary productivity, respectively. We specifically focused on lipid allocation in late-stage barnacle larvae, the settlement stage. During their planktonic phase, barnacles pass through 6 naupliar stages before moulting into a cyprid. The nauplius stage I is non-feeding, surviving for a few days on glycoprotein and lipid yolk reserves before moulting to stage II (Rainbow & Walker, 1977). Stages II to VI are planktotrophic, feeding on phytoplankton, with nauplii increasing in size at each moult, and building up lipid reserves needed to support the non-feeding cyprid, specialized for substrate exploration and settlement. We assessed lipid allocation in cyprid larvae by using a ratio of energetic to structural lipids (triacylglycerol to phospholipid; TAG/PL ratio), while also measuring other larval traits, including size, lipid droplet cover, and total lipid content. Given the generally poorer primary productivity at subtropical shores, we anticipated lower TAG/PL and settlement success of *C. bisinuatus* compared to *C. montagui* cyprids, which (naupliar) development occurs in more productive waters. A conceptual model clarifying the process of lipid allocation and energy storage in the tissues of cyprid larvae has been developed to place our work into a wider context.

2. MATERIAL AND METHODS

2.1. Study sites and species

Late-stage (cyprid) larvae of two chthamalid barnacle species were sampled from two different latitudes (subtropical and temperate) in the Atlantic: *Chthamalus bisinuatus,* on the southeastern coast of Brazil in the São Sebastião Channel, and *Chthamalus montagui,* on the south coast of the British Isles, in Plymouth Sound in the English Channel (see Figure 1). These species are considered ecological equivalents as they occupy the same ecological niche (the upper midlittoral zone) forming a conspicuous *Chthamalus* zone. Chthamalid cover was mostly close to saturation at all sampled sites (80–100%). Comparable shores were sampled at two sites per region (separated by 3–6 km), during March 2015 in São Sebastião and August 2015 in Plymouth Sound. These dates correspond to late summer/early autumn months in either region.

Samples were first processed at the Center for Marine Biology of the University of São Paulo (CEBIMar/USP, São Paulo, Brazil) and at the Marine Biological Association of the United Kingdom (MBA, Plymouth, British Isles), and later analysed at the Institute of Ocean Sciences of Rimouski (ISMER/UQAR; Rimouski, Canada).

In Brazil, the rocky shores of Baleeiro Head (23°49'41"S, 45°25'22"W) and Feiticeira Beach (23°50'38"S, 45°24'28"W) were sampled. Facing the São Sebastião Channel (SSC, northern coast of São Paulo State), these shores are characterized by a steep rocky habitat with a nearly saturated (close to 100% cover) *Chthamalus* zone in the mid-high littoral (e.g. Kasten and Flores 2013; Barbosa et al. 2016). Natural populations of *C. bisinuatus* along the São Paulo coastline may exhibit a very clear fortnightly rhythm of larval release (Bueno et al. 2010). The SSC is principally a meso-oligotrophic system, with a seasonal chlorophyll-*a* pattern reaching maximum concentrations in winter (0.36 ± 0.12 mg/m^3; Ciotti et al. 2010). During late summer, organic detritus dominate nearshore suspended matter composition over the SSC (Leal et al. 2018).

In the English Channel, the rocky shores of Mount Batten (50°21'28"N, 4°7'41"W) and Plymouth Hoe (50°21'47"N, 4°8'17"W) were sampled. These shores are located in Plymouth Sound (PS), an area characterized by dense populations of chthamalid barnacles (e.g. Southward 1991, Jenkins 2005). Of these, *C. montagui* is more abundant in embayed areas where there are more particles suspended in the water column (Burrows et al. 1999). Here *C. montagui* breeds between the beginning of May and the end of September, with asynchronous multiple broods within a defined breeding season (Burrows et al. 1992). Settlement patterns of chthamalids over PS are largely dictated by larval choice rather than late-stage larval supply (Jenkins 2005). The English Channel, as is typical of temperate systems, has two yearly productivity peaks (spring bloom of diatoms followed by summer dominance of flagellates; Rodriguez et al. 2000). In contrast with other cold-temperate acorn barnacle species such as *Semibalanus balanoides*, in which release of single larval brood is synchronized to match the predictable spring phytoplankton bloom of diatoms (e.g. Barnes 1956, Crisp & Spencer 1958, Hawkins & Hartnoll 1982), flagellates appear to be the type of phytoplankton which best promotes successful development of the planktonic larvae of *Chthamalus* (Moyse 1963; Burrows et al. 1992). Chlorophyll-*a* concentration in the study area (measured at the Western Channel Observatory Station L4; https://www.westernchannelobservatory.org.uk/) is at its lowest during winter, and higher during the characteristic spring and autumn phytoplankton blooms (reaching nearly 3 mg/m^3 from August through September; Skákala et al. 2018).

2.2. Field sampling

Sampling was conducted consecutively over a 4-day period in Brazil (03-March to 06-March-2015) and in the British Isles (19-Aug to 22-Aug-2015). Two replicate plankton tows (10 m apart; mesh 160 μm; 50-100 m tow length at sub-surface) were collected daily at each of the 2 sites in the São Sebastião Channel and Plymouth Sound, 100-200 metres offshore, to estimate larval supply (cyprids/m^3) and larval morpho-physiological traits. A minimum of 20 cyprids per tow sample were collected for morphometric measures (see below 2.4) and lipid analysis (see below 2.5). Whilst *C. bisinuatus* was the single chthamalid barnacle species occurring on the subtropical shores sampled, the temperate *C. montagui* co-occurred with a congener, *C. stellatus*. Morphological differences between the larvae of *C. stellatus* and *C. montagui* have been previously described, with *C. montagui* being consistently smaller than its congener (over 1500 chthamalid cyprids sampled in the PS area; Jenkins 2005). The cyprid carapace length was therefore used to distinguish between both temperate *Chthamalus* spp. (Power et al. 1999). All temperate cyprids analysed were well below 540 μm (see results), the reported length threshold for *C. montagui* (Powers et al. 1999, Jenkins 2005). Daily settlement rates (individuals/plate/day) were determined by random deployment of 10 settlement plates (10cm × 10cm; covered with rough tape 3M™ Safety-Walk) over the 4-day sampling period at each site. Plates were replaced every 24 hours and surroundings were cleared for potential predators. Settlement success was estimated by calculating settlement/supply ratios, and the size of the recently metamorphosed juveniles (rostro-carinal diameter) was measured (see below 2.4).

2.3. Remote estimation of primary productivity

The trophic conditions at each sampling region were characterized using remote estimates of chlorophyll-*a* concentration (Chl-*a*; mg/m^3), a commonly used proxy of food supply (similarly to Mazzuco et al. 2015, Kasten et al. 2019a). Remote sensing data for surface Chl-*a* was acquired from the MODIS-Aqua sensor through the OceanColor database (https://oceancolor.gsfc.nasa.gov/l3/). Level-3 mapped images of 4 km resolution were processed using the software SeaDAS (Version 7.5.3). Local estimates were averaged for one specific cell (4 km × 4 km) for each region, as sites within regions were not distant enough to warrant site-specific cells. Daily averages were extracted for 5 dates where remote sensing data was available for the São Sebastião Channel (23°56'15" S, 45°26'15" W; dates: 23-02-15, 25-02-15, 27-02-15, 03-03-15, 04-03-15) and Plymouth Sound (50°16'15" N, 4°11'15" W; dates: 06-08-15, 08-08-15, 17-08-15, 18-08-15, 23-08-15). The extracted dates spanned the pelagic larval development (from nauplius to cyprid) of both species (12 days for *C. bisinuatus*, Barbosa et al. 2016; 14-17 days for *C. montagui,* Burrows et al. 1999).

2.4. Morphometric analysis

We focused on cyprid traits that are known to affect post-settlement performance (Thiyagarajan et al. 2003, Jarret 2003, Tremblay et al. 2007, Leal et al. 2020): (1) size (carapace length; µm) and (2) lipid droplet cover (droplets area/body area; %). The transparent carapace of cyprid larvae makes it easy to visualize the conspicuous lipid droplets stored anteriorly, around the midgut and at the bases of the thoracic limbs (Figure 2), that constitute the cyprid's main energetic reserves (Rainbow & Walker 1977, Tremblay et al. 2007). Recent evidence using *C. bisinuatus* cyprids shows that the visual estimation of lipid droplet cover can be used as a proxy of TAG content, given the strong correlation between visual lipid area and nile-red stained lipid area under a fluorescence microscope (Guerra et al. *unpublished*). Based on this, size and lipid droplet cover were measured for 170 *C. bisinuatus* cyprids, and 153 *C. montagui* cyprids. Cyprids were photographed alive under a stereomicroscope (100 ×) in lateral view for later measurements through image analysis. Similarly, juveniles settled on the artificial plates (1-day old) were photographed daily under a stereomicroscope to obtain measurements of rostro-carinal diameters (148 *C. bisinuatus* juveniles, and 442 *C. montagui* juveniles). All morphometric analyses were done using the software Fiji, an open-source platform for scientific image analysis (Schindelin et al. 2012).

2.5. Lipid analysis

To quantify the larval total lipid content (µg/larva) and lipid fractions (classes), cyprids collected from nearshore waters were preserved at -80°C in dichloromethane-methanol vials (2:1, v/v) for lipid analysis (2 samples of 20 cyprids per day, per site, comprising a total of 640 cyprids). Lipid extraction was done according to Folch et al. (1957) and classes determined using an Iatroscan Mark-VI analyzer (Iatron Laboratories Inc., Tokyo, Japan) developed in a four-solvent system (Parrish 1999). Three main lipid classes were identified: Triacylglycerols (TAG), phospholipids (PL) and acetone mobile polar lipids (AMPL). TAG are storage lipids that make up most of the energetic reserves, PL are the main components of cell membranes, and AMPL are a group constituted mainly from glycolipids, pigments, and degradation products of PL (Parrish 1999). Storage lipids are generally associated with energy supply, while structural lipids are important for the cell membrane stability (Fraser 1989). Most of the variability in individual lipid content reflects changes in TAG levels because this class of lipids most closely reflects changes in factors such as food supply and temperature, which are known to influence the deposition or loss of lipids (Arts et al. 1993).

2.5.1. The TAG/PL ratio

Given the size-dependency associated with TAG (Fraser 1989), the ratio of triacylglycerol to phospholipid (TAG/PL ratio) was used to characterize lipid allocation in larval tissues, that is, standardized TAG measures by a lipid class related to body size, PL. The ratio of storage to membrane lipids has been found to be a good indicator of physiological condition in a number of fish, bivalve and crustacean larvae (Fraser, 1989, Lochmann et al. 1995, Harding & Fraser 1999). Håkanson (1993) defined fish larvae in poor condition to be those with a ratio less than 0.2. For lobster larvae, Harding & Fraser (1999) considered a condition index less than 0.1 the 'point-of-no-return'. For chthamalid barnacles, the threshold of TAG below which nauplii and subsequent cyprid stages do not survive in the wild is yet to be estimated. However, through a laboratory experiment, Freuchet et al. (2015) have shown that the ratio of storage to membrane lipids in *C. bisinuatus* nauplii (larval feeding stages) exposed to low food supply corresponded to 0.7, as opposed to a ratio of 1.9 in nauplii fed high food supply. As TAG reserves in (non-feeding) cyprid larvae are largely linked to those accumulated during the precedent naupliar stages, we argue that the TAG/PL ratio can be used to distinguish cyprids with high endogenous TAG reserves (ratio far greater than 1) that fuel larval metabolic activity, such as escaping from predators and swimming (pertinent for substrate exploration behaviour; Miron et al. 2000, Tremblay et al. 2007), from energy-depleted cyprids (ratio far below 1), maintaining basal metabolism.

2.6. Statistical analysis

A nested Analysis of Variance (ANOVA) was used to determine if statistically significant differences in settlement/supply ratio (dependent variable) existed between Species/Regions (*C. bisinuatus* in the São Sebastião Channel *vs*. *C. montagui* in the English Channel). A balanced design with Sites (Site 1, Site 2) nested within Region was used. With this approach, we tested the hypothesis that larger settlement/supply ratios would be expected in areas with greater productivity (Plymouth Sound in the English Channel, see results) with regional differences overwhelming any small-scale variation. Using the same design, differences in larval lipid allocation (TAG/PL ratio) between *C. bisinuatus* and *C. montagui* were tested by running a nested ANOVA. Differences in frequency distributions of cyprid size, lipid droplet cover, and juvenile size, between species were tested using two-sample independent Kolmogorov-Smirnov tests, to understand whether selection pressures on morphological larval traits differed among subtropical and temperate environments. To investigate the relationship between morpho-physiological larval traits, a Pearson correlation matrix was computed for the variables cyprid size, lipid droplet cover, total lipid content, and TAG/PL ratio. The R software (version 3.6.1; R

Core Team 2019) and packages ggplot2 (Wickham 2016) and ggpubr (Kassambara 2019) were used to conduct statistical analysis and as a graphical tool.

3. RESULTS

3.1. Local conditions: trophic regime, supply, and settlement

Monthly estimates of pelagic primary production (Chl-*a*) for 2015 (Fig. 1) revealed that sampling was carried out during a peak of Chl-*a* for both the São Sebastião Channel (SSC) (March, 2.85 mg/m^3) and Plymouth Sound (PS) (August, 5.17 mg/m^3). Throughout the sampling period, daily estimates averaged 1.31 ± 0.4 mg/m^3 for the SSC, contrasting with the higher Chl-*a* concentration of PS, 3.09 ± 1.2 mg/m^3 (Fig. 3A). Larval supply (Fig. 3B) was around three times higher at the temperate shores (158 ± 46 ind/m^3) compared to the subtropical ones (46 ± 14 ind/m^3). The same pattern held true for daily settlement rates (Fig. 3C), with *Chthamalus montagui* settling at higher rates (~11 settlers/plate/day) compared to its subtropical congener *Chthamalus bisinuatus* (~ 4 settlers/plate/day). However, settlement/supply ratios (Fig. 3D) were similar, with no differences being found between regions, nor between sites within regions ($p >$ 0.05; Table 1). This indicates comparable rates of larval settlement per number of larvae in the water column, that is, *C. bisinuatus* cyprids settled in the same proportion as those of *C. montagui* despite developing in less productive waters. Within-region variability (coefficient of variation; % CV) was, however, high for supply (SSC: 68 % ; PS: 72 %) and settlement (SSC: 73 %; PS: 56 %) patterns, suggesting that the generality of these results must be interpreted with caution, in light of the variability observed.

3.2. Morpho-physiological larval traits

The size-frequency distribution curves differed between species for the cyprid stage (Kolmogorov-Smirnov $K_{d\ 170,\ 153} = 7.00$, $p < 0.0001$; Fig. 4A) and juvenile stage ($K_{d\ 148,\ 442} = 7.50$, $p < 0.0001$; Fig. 4C), with *C. montagui* being consistently larger than its subtropical congener. Lipid droplet cover distributions also differed ($K_{d\ 170,\ 153} = 5.04$, $p < 0.0001$; Fig. 4B), with *C. bisinuatus* having greater covers than the temperate *C. montagui*. *C. bisinuatus* averaged 224 ± 28 μm in cyprids' carapace length, 24 ± 8 % in lipid droplet cover, and 226 ± 23 μm in juvenile size (Mean ± SD). *C. montagui* averaged 290 ± 70 μm in cyprids' carapace length, 14 ± 7 % in lipid droplet cover, and 283 ± 41 μm in juvenile size.

A detailed description of the lipid classes found in the larval tissue of both chthamalids is given in Table 2. Total lipid content averaged 50 μg/cyprid for *C. bisinuatus*, and 48.5 μg/cyprid for *C. montagui*. Triacylglycerols (TAG) and phospholipids (PL) were the major lipid classes of both species, constituting more than 90% of the total larval lipid content. The TAG/PL ratio was similar for both species (1.2 for *C. bisinuatus*, and 1.3 for *C. montagui*; $p > 0.05$, Table 1), indicating that larvae similarly allocated lipids to energy storage and cellular structure, and that larvae had previously accumulated TAG reserves during their naupliar development. There was, however, considerable intra-specific variation (% CV) in this condition index (30 % in *C. bisinuatus* and 32 % in *C. montagui*), which might explain the nearly significant effect of site in the ANOVA comparisons ($p = 0.05$; Table 1).

The relationship between the TAG/PL ratio and other larval traits shows that cyprids with similar physiological status may vary in size (Fig. 5A), lipid droplet cover (Fig. 5B), and total lipid content (Fig. 5C); yet, some patterns emerged. The highest ratios (TAG/PL ≥ 1.5) found in *C. montagui* cyprids comprised small-sized cohorts (< 300 μm) with lipid droplets covering a greater area of the body (≥ 20 % cover). This was confirmed by the positive relationship between

TAG/PL and lipid droplet cover (p = 0.01, r = 0.90; Table 3). The lipidic costs associated with larger sizes can be further evidenced by the (nearly significant) negative relationship between size and total lipid content (p = 0.05, r = -0.74; Table 3). No significant relationships were detected among *C. bisinuatus* larval traits (Table 3). *C. bisinuatus* cyprids had a similar size-range (200 – 245 μm) regardless of TAG/PL ratio, but similar to their temperate counterparts, the highest ratios were found in cyprids that had a greater lipid droplet cover (≥ 20 % cover).

4. DISCUSSION

In this study, we show that similar lipid allocation in late-stage barnacle larvae can occur in *Chthamalus* species exposed to contrasting trophic regimes, contrary to the expected depletion of TAG reserves in cyprid larvae from less productive waters. This points to an energetic threshold below which nauplii cannot develop to a cyprid and/or selection for lipid accumulation under poor trophic conditions. The equivalence in physiological status between larvae given by the TAG/PL ratio was reflected in our finding of similar supply/settlement ratios in *Chthamalus bisinuatus* and *Chthamalus montagui*. We highlight the challenges of directly relating estimates of primary productivity with food supply and larval physiology, as lower Chl-*a* concentrations do not necessarily indicate food limitation for barnacle nauplii. We also propose a conceptual model for the integration of morpho-physiological traits when assessing lipid allocation (energetic to structural lipids) in the tissues of cyprid larvae.

4.1. The ratio of storage to membrane lipids: TAG/PL

As reported in studies with other benthic invertebrates (Delaunay et al. 1992, Abad et al. 1995), triacylglycerols (TAG) and phospholipids (PL) were the main lipid classes found in cyprids of both *Chthamalus* species here tested. TAG alone accounted for 50 % of the total lipid content in *C. bisinuatus*, and 53 % in *C. montagui*. These values are similar to those reported by Holland and Walker (1975) in balanid cyprids, where neutral lipids comprised 66% of the total lipid fraction. The need for TAG accumulation in pre-metamorphic stages is easily understood, given the reliance on endogenous energy during metamorphosis (Lucas et al. 1979, Thiyagarajan et al. 2003). Moreover, higher TAG accumulation reduces sinking rates, which has been suggested to reduce energy costs of swimming cyprids to maintain optimal depth (Burrows et al. 1999) and allow for a more prolonged search for a suitable substrate (Lucas et al. 1979).

Total lipids and TAG are predictors of settlement success, post-metamorphic growth, and survival in other species (e.g. *Balanus amphitrite*; Miron et al., 2000; Thiyagarajan et al. 2005, Tremblay et al. 2007), and therefore an excellent reference index for larval quality. TAG adjusts quickly to changes in food supply and temperature (Lochmann et al. 1995, Freites et al. 2002), whereas PL provides structural-type functions (e.g. membrane stability) that are maintained fairly constant (Fraser 1989) and correlate with the size of larvae, as demonstrated in fish and crustaceans. We argue that the use of a lipid-based condition index (such as the TAG/PL ratio) is useful to estimate the physiological condition of barnacle cyprids, by informing how larvae allocate lipids in their tissues to energy storage and/or cellular structure (Fig. 6). We found similar ratios for both chthamalid barnacle species, with a TAG/PL ratio above 1 (1.2 *C. bisinuatus*, 1.3 *C. montagui*) indicating that larvae had accumulated energetic (TAG) reserves during their previous naupliar feeding stages and/or through maternal allocation. Estimates of the TAG/PL ratio at the point-of-no-return (i.e. of non-viable larvae in the wild) are still lacking for chthamalid cyprids and are necessary to make well-grounded inferences on larval physiological status using lipid-based condition indices. We argue that analysing several morpho-physiological traits together leads to a better understanding of larval condition (Fig. 6). For instance, focusing on size-metrics alone would render large *C. montagui* cyprids the ones with highest quality, which appears not to be the case. Subtropical *C. bisinuatus* cyprids with 200 µm carapace length had higher TAG reserves than *C. montagui* with 400 µm or more. We hypothesize that the larger the cyprid, the bigger the structural and energetical lipidic demands it has. For a balanid barnacle (*Balanus glandula*), Hentschel and Emlet (2000) found that a large nauplius with a high feeding rate and reduced time to cyprid moult might store the same amount of lipid as a smaller nauplius with a slower feeding rate and a longer naupliar duration, demonstrating the plasticity in size and age at metamorphosis, and suggested it to be an adaptation to environmental variability. As suggested for other barnacle groups (balanids, Giménez et al. 2017, Hentschel and Emlet 2000), the physiological condition of both chthamalids here described likely reflects local selection for larval phenotypes that match the requirements for juvenile survival (see 4.3. discussion below).

4.2. Effects of food supply

Our findings show that lower Chl-*a* concentrations do not necessarily indicate food limitation for barnacle nauplii (see below 4.3.2), and do not provide support for the general assumption of latitudinal gradients in larval lipid storage in this group of barnacles. Cyprid larvae of both chthamalids had similar lipid profiles and contents, despite being exposed to contrasting trophic regimes during their development. This might have been caused by differing (1) competition for phytoplankton, (2) nutritional quality of food resources, and/or (3) physiology linked to differential lipid retention (see below 4.3.1). Starvation appears to be the biggest threat for pelagic crustacean larvae, being driven by high metabolic demands (Morgan 1995) and food limitation (Bashevkin & Morgan 2020). In nearshore waters, herbivorous zooplankton are, however, rarely limited by food (Strathmann 1996), and barnacle nauplii are unlikely to be strongly affected by competition (Bashevkin & Morgan 2020). To the authors knowledge, limited information is available either on particle size ingested by barnacle nauplii, or on selective feeding behaviour, and for chthamalids, the only evidence was published by Stone (1989), under laboratory conditions. This gap in the scientific literature was also recently pointed by Bemal and Anil (2019). The authors found that picoplankton (< 2.0 µm), particularly the picocyanobacteria *Synechococcus*, occupy a key position in the naupliar diet of *Amphibalanus amphitrite*. Unlike larger phytoplankton (nano and micro), picocyanobacteria and other picophytoplankton are better adapted to low-nutrient conditions, contributing significantly to the bulk of primary production in oligotrophic waters (Bemal & Anil 2019). Even species adapted to more productive regimes, such as the nauplii of *Chthamalus montagui* and *Balanus perforatus*, have been grown successfully when fed on small cells (< 5 µm) of *Micromonas pusilla* and *Isochrysis galbana* (Stone 1989). Vargas et al. (2006) also reports that nauplii of two barnacle species, *Jehlius cirratus* and *Notobalanus flosculus* from central Chile, primarily graze on picophytoplankton and small nanophytoplankton (< 5 µm) when incubated in natural sea water. While low amounts of phytoplanktonic food resources are present over the SSC, detrital pathways (e.g. phytodetritus, bacteria) prevail in these subtropical waters (Leal et al. 2018). It is possible therefore that naupliar stages of barnacles over the SSC are adapted to explore other sources of food in their diet to build their energetic (TAG) reserves. Recent studies on the dietary fatty acid composition of cyprid larvae inhabiting different latitudes (*Chthamalus* spp. and *Semibalanus* sp.) show that lipid reserves can be built from different food sources, not simply microalgae (Leal et al. *in prep*). In *C. bisinuatus* for instance, a large proportion of energetic reserves contain trophic tracers for terrestrial detritus and macrophytes (Leal et al. *in prep*).

Triacylglycerols, the main constituent of larval reserves, are tri-esters consisting of a glycerol bound to three fatty acids, so that there are many different types of TAG, with the main division existing between saturated (e.g. 16:0 palmitic acid, 18:0 stearic acid) and unsaturated types (e.g. ω-3 and ω-6 essential fatty acids). As the fatty acid composition of lipid classes was not characterized in this study, it is not possible to ascertain what type of fatty acids were the building blocks of the TAG reserves for each species. It is possible that in temperate waters, microalgae-derived fatty acids (unsaturated) make up most of the food available for *C. montagui* nauplii, whereas in subtropical waters, detrital matter (saturated) makes up most of the diet of *C. bisinuatus* nauplii. That would mean that, although both species have similar TAG contents, their fatty acid composition is probably different. We therefore urge further research to reconcile the trophic ecology (e.g. feeding behaviour, feeding rates, dietary fatty acid composition) of chthamalid barnacles.

Shifts in food during the ultimate naupliar instar (VI) particularly affect the cyprid's lipid accumulation, stored in (neutral) lipid droplets (Hentschel & Emlet 2000). Considering the duration of pelagic larval development from nauplius to cyprid of both *Chthamalus* species – 12 days for *C. bisinuatus* (Barbosa et al. 2016), and 14-17 days for *C. montagui* (Burrows et al. 1999) – cyprid cohorts sampled might have been exposed to different food supply that reflect a lag in their TAG/PL ratio. For example, scattered events of high primary productivity during short periods (1 to 2 days) have been reported for the SSC (Kasten & Flores 2013). Such variability likely leads to differential condition of cyprid cohorts, as recently reported for *C. bisinuatus* cyprids (reaching values as high as 52%; Leal et al. 2020). This suggests that selection for cohorts with high TAG reserves may occur in subtropical shores. The physiological condition of cyprids is also largely determined by maternal allocation (Kasten et al., 2019b). In subtropical

C. bisinuatus, carry-over effects of maternal allocation seem to have the same effects as pelagic inputs to larvae in the determination of cyprid size (c.a. 13% to 14% size increase; Kasten et al. 2019b), which may compensate for the low food supply in these subtropical shores.

4.3. Implications

4.3.1. Physiological thresholds in cyprid larvae

We suggest that there may be a threshold above which greater sizes are achieved in detriment of lipid reserves, a cost that is likely too high for species that are exposed to low food supply. Lipid accumulation has been suggested to be an adaptive strategy for species inhabiting habitat-limited shores (e.g. maternal provisioning essential at crowded shores, Kasten et al. 2019b). It has also been suggested for *Semibalanus balanoides* larvae along the coast of West Scotland by Giménez et al. (2017), who observed a counter-gradient pattern in larval reserves and Chl-*a* and suggested an adaptive role of increased reserves in buffering juveniles from low food availability. They argued that ultimately, local adaptation will drive larval phenotypes to match the requirements for juvenile survival. In the case of *C. bisinuatus*, it is possible that TAG reserves (stored as lipid droplets) for settlement and metamorphosis may offset the detrimental effects of food scarcity during the critical first days in the benthos, where juveniles must grow as fast as possible (*C. bisinuatus* cyprids with more stored reserves lead to juveniles that grow faster; Leal et al., 2020). In cold-temperate shores, peaks of *C. montagui* settlement follow major phytoplanktonic blooms. Thus, food is likely not limiting at the onset of juvenile feeding, and larvae may invest on size to counterbalance the slower growth rates imposed by low temperatures. The impact of the observed physiological traits on metamorphic performance will also depend on the post-metamorphic environment. Post-settlement processes (e.g. predation, desiccation, microhabitat availability) can accentuate the patterns set pre-settlement or during settlement (Bohn et al. 2013a, b), making a comprehensive understanding of larval physiology in supply-side ecology critical.

A caveat must be given to comparing one species each in two locations, albeit with local replication using a nested design, if wanting to draw wider conclusions about latitudinal patterns. Ideally, more than one species should be used in each locality, with several localities in both sides of the ocean. Unfortunately, there is only one chthamalid barnacle species in southeast Brazil. In Europe, *C. stellatus* has a more offshore larval distribution and morphology as well as longer larval life with greater dispersal (Burrows et al. 1999, Pannacciulli et al. 1997) and is more prevalent on exposed headlands (Crisp, Southward & Southward 1981, Jenkins 2005). The field-based results from our comparative study, however, plus the final proposed conceptual model, contribute to a better understanding of the physiology of settling barnacle larvae of closely-related *Chthamalus* species, and motivate further research on the effects of larval energetics in shaping settlement of this important group of habitat-forming species.

4.3.2. The challenge of remotely estimating food supply

We highlight the challenge of directly relating estimates of primary productivity (Chl-*a*) with food supply and larval physiological status. We observed similar TAG/PL for barnacle cyprids exposed to considerably different levels of Chl-*a* (1.31 ± 0.4 mg/m^3 in the SSC, southeast Brazil, *vs.* 3.09 ± 1.2 mg/m^3 in PS, southwest British Isles), for a period spanning 2-weeks prior sampling. Other studies have not found correlations between Chl-*a* and larval traits (e.g. Giménez et al. 2017, Ewers-Saucedo & Pappalardo 2019). As suggested by Ciotti et al. (2010), the general spatial patterns for Chl-*a* exist in the surface ocean (see Appendix Fig. 1), resulting from differences in nutrient and light availability for phytoplankton growth set by regional and global physical processes, as well as other important but less understood processes such as grazing, sedimentation and advection rates for phytoplankton cells. However, they neither translate directly into primary production nor exportation rates for phytoplankton carbon. Mechanisms related specifically to food quality (e.g. phytoplankton species, organic matter composition), cannot be directly associated to Chl-*a* patterns. Further, nearshore trophic subsidies to rocky shore communities, e.g. surf-zone hydrodynamics as subsidies of phytoplankton (*sensu* Morgan et al. 2018) by increasing the nutrient uptake, light exposure, and suspension of surf diatoms from the sediment into the water column, are often overlooked. The high concentrations of phytoplankton (far higher concentrations of phytoplankton in surf-zones than offshore; Shanks et al. 2016, 2017), particulate organic matter, and detritus, can sustain short and highly productive food webs. Most importantly, together with land-derived organic subsidies (e.g. rainfall-driven inputs in shallow subtropical waters; Gorman et al. 2019), such nutrient apport to nearshore marine communities fails to be detected by remote estimates of primary productivity, compromising assumptions made on food supply for suspension feeders, especially in areas with strong detrital-based nearshore food webs, typical of low-latitudes (e.g. Leal et al. 2018, 2019).

5. CONCLUSION

Quantifying the lipid class fractions (TAG, PL) that are present in the total lipid content of larvae gives important information regarding the way lipid compounds are allocated (energy storage/cellular structure). The similar TAG/PL ratio observed for two closely-related barnacle species exposed to different levels of surface Chl-*a* highlights the challenges of directly relating estimates of primary productivity with larval physiological status, and the need to better describe trophic resources available for pelagic naupliar stages. We hope to motivate further research, ideally with more replicate species, to unveil latitudinal patterns in lipid allocation in this important group of barnacles.

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TABLES

Table 1. Nested analyses of variance (ANOVA) summary table for regional/species differences (São Sebastião Channel/*C. bisinuatus versus* Plymouth Sound/*C. montagui*) in supply to settlement patterns and in lipid allocation in cyprid tissues given by the TAG/PL ratio.

	Settlement/Supply ratio					**TAG/PL ratio**				
Source	*df*	*SS*	*MS*	*F*	*p*	*df*	*SS*	*MS*	*F*	*p*
Region	1	0.01	0.01	0.40	0.59	1	0.36	0.36	2.80	0.11
Site(Region)	2	0.05	0.03	0.37	0.71	2	0.91	0.47	3.57	0.05
Residuals	4	0.28	0.07			20	2.55	0.13		

Table 2. Lipid class composition of the cyprids collected from the subtropical (*C. bisinuatus*) and temperate (*C. montagui*) nearshore waters. Lipid classes (% ± SE), divided in TAG (triacylglycerol), PL (phospholipids) and AMPL (acetone mobile polar lipids), constituted the larval total lipid content (µg/larva ± SE). The TAG/PL Ratio, i.e. the ratio of storage to membrane lipids, is provided for both species.

Lipid class (%)	***Chthamalus bisinuatus***	***Chthamalus montagui***
TAG	49.8 ± 1.8	52.8 ± 2.1
PL	41.3 ± 1.4	41.6 ± 1.9
AMPL	8.8 ± 0.9	5.6 ± 0.4
∑ Lipid content (µg/larva)	50.0 ± 1.9	48.5 ± 2.3
TAG/PL Ratio	**1.21**	**1.27**

Table 3. Pearson correlation coefficients for cyprid morpho-physiological traits. Each sample (*n*) corresponds to the daily average of two replicate plankton tows, representing daily cohorts. Significance denoted as '***' 0.001; '**' 0.01; '*' 0.05; '**.**' 0.1.

		Chthamalus bisinuatus			***Chthamalus montagui***		
Variable		Size	Droplet cover	Total lipid	Size	Droplet cover	Total lipid
TAG/PL	*r*	-0.10	0.60	0.00	-0.55	0.90	0.82
	p-value	0.81	0.11	0.99	0.20	0.01 **	0.01 **
	n	8	8	8	7	7	8
Size	*r*		0.08	-0.43		-0.64	-0.741
	p-value		0.85	0.29		0.12	0.05 **.**
	n		8	8		7	7
Droplet cover	*r*			0.04			0.84
	p-value			0.93			0.02 *
	n			8			7

FIGURES

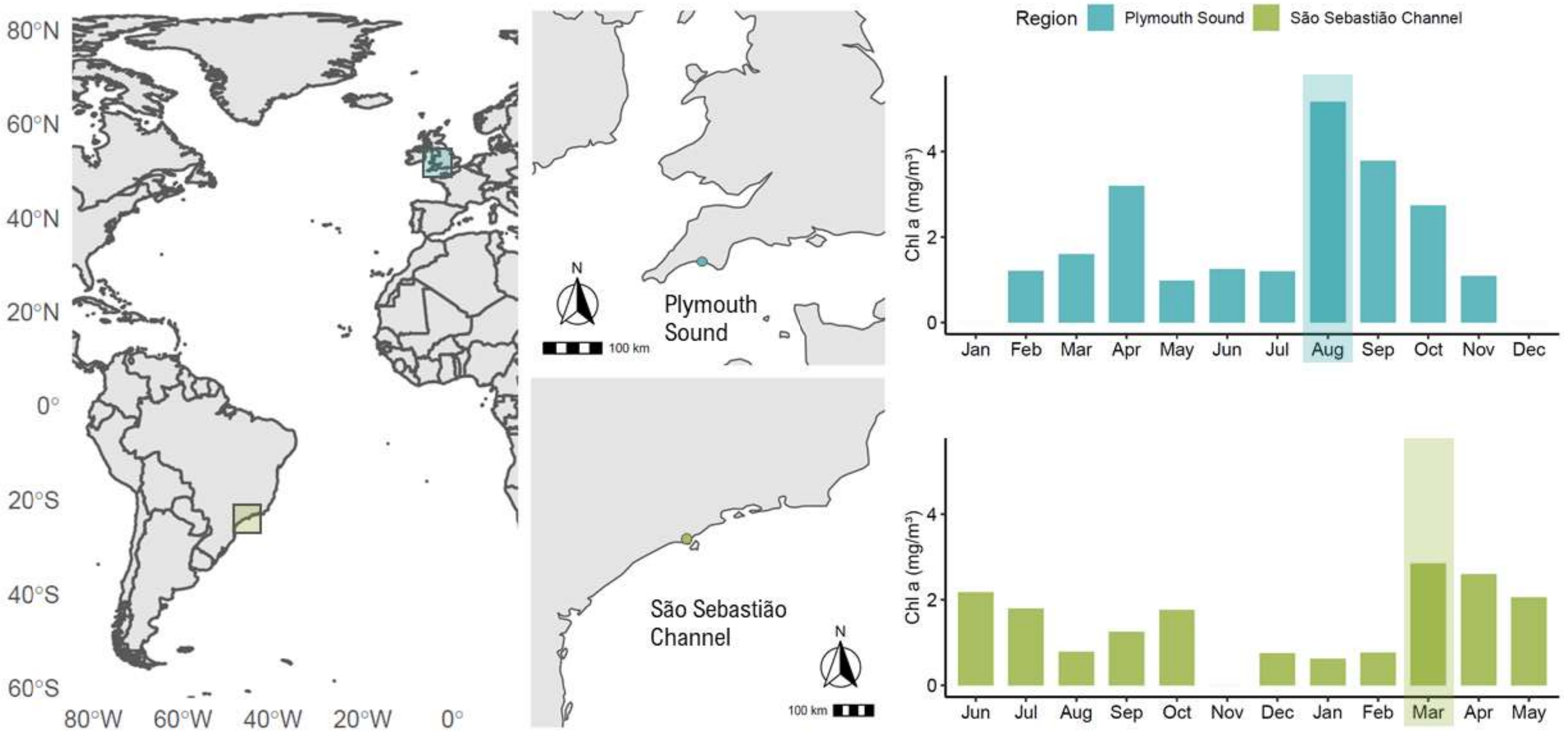

Figure 1 – Map displaying sampled locations: southwest of the British Isles (Plymouth Sound) and southeast Brazil (São Sebastião Channel). Monthly estimates of surface chlorophyll-*a* concentration (Chl a; mg/m^3), acquired from MODIS-Aqua NASA Ocean Color database using Level-3 mapped images of 4 km resolution, indicate the year-round (winter solstice to summer solstice) pelagic primary productivity found at each location during 2015. Shaded months represent the sampling period.

Figure 2 – Live *Chthamalus bisinuatus* cyprid larvae in lateral view. Conspicuous lipid droplets are visible in the anterior part of the carapace, the main larval energy storage.

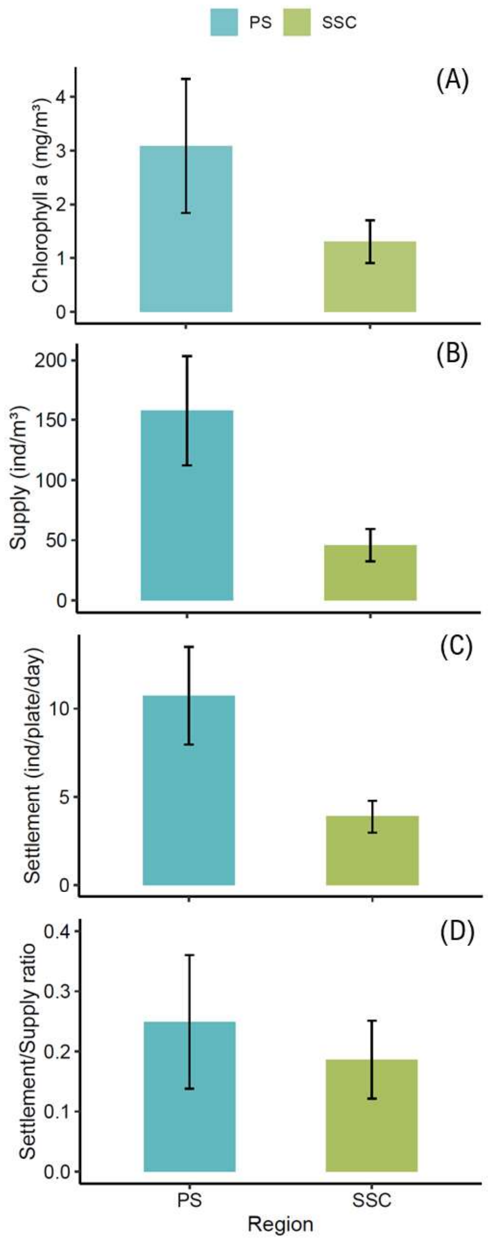

Figure 3 – Daily parameters found at each sampling location, (A) Chlorophyll-*a* concentration, i.e. estimate of primary productivity, (B) Supply, i.e. cyprid abundance, (C) Settlement rate, and (D) Settlement/Supply ratio, i.e. a measure of settlement success. Error bars correspond to mean ± SE. SSC stands for São Sebastião Channel (*C. bisinuatus*) and PS for Plymouth Sound in the English Channel (*C. montagui*).

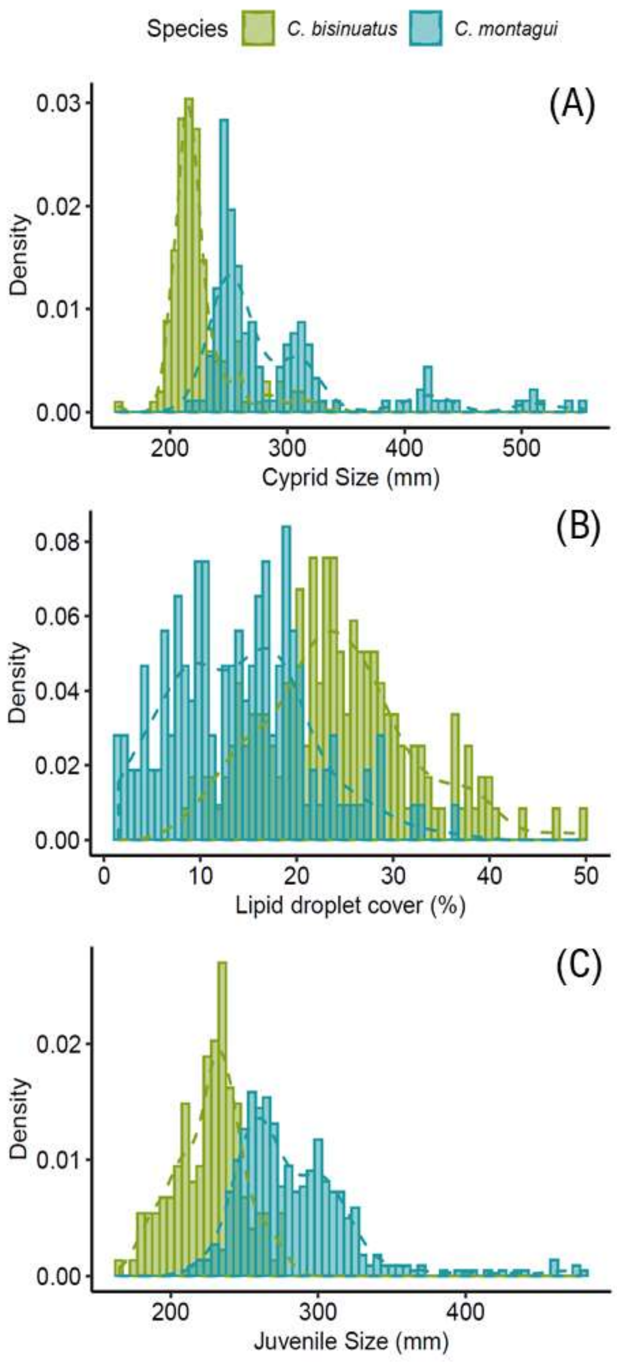

Figure 4 – Histograms showing the distributions with density curves for *C. bisinuatus* (subtropical) and *C. montagui* (temperate): cyprid larvae (A) carapace length and (B) lipid droplet cover, and early (1-day old) juveniles (C) rostro-carinal diameter.

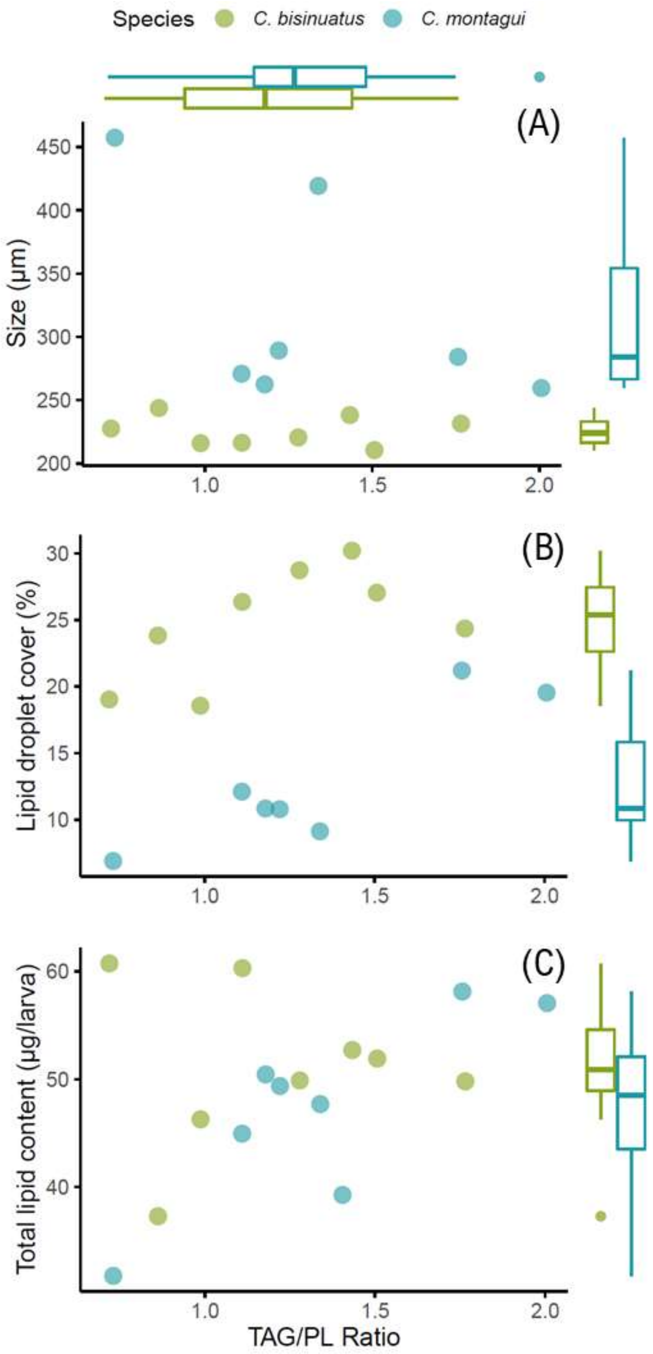

Figure 5 – Series of scatterplots with marginal boxplots showing the TAG/PL of daily cyprid cohorts (daily average of two replicate plankton tows per site) plotted against other morpho-physiological traits: (A) size, (B) lipid droplet cover, and (C) total lipid content.

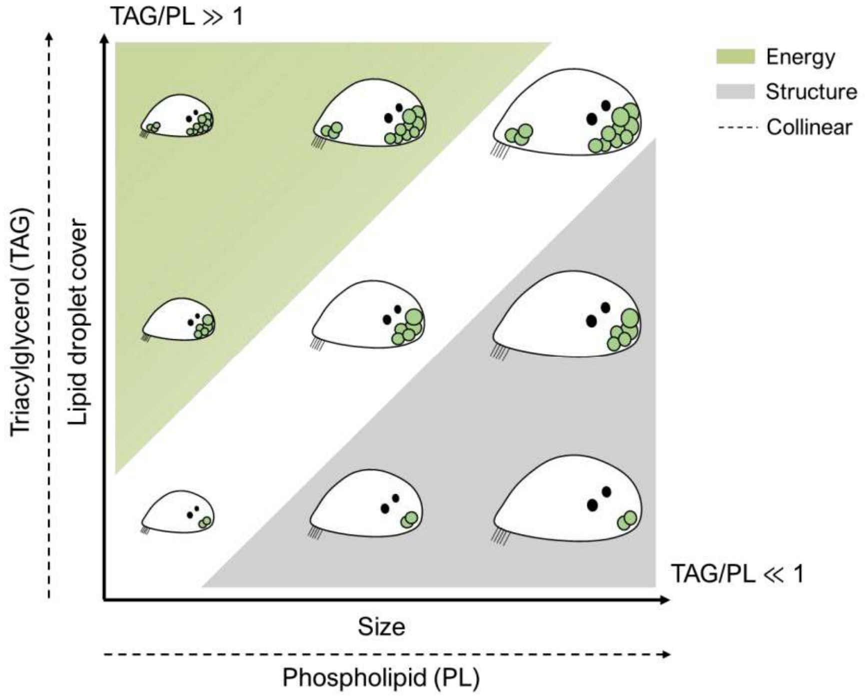

Figure 6. Conceptual model for the integration of morpho-physiological traits when assessing lipid allocation (energy, structure) in late-stage barnacle larvae. The triacylglycerol (TAG) to phospholipid (PL) ratio can be used as an index of physiological condition. For instance, larvae with a TAG/PL = 1 (non-shaded area of the scheme) resemble in physiological condition, although having different sizes and lipid reserves, illustrating that larger sizes incur higher metabolic costs. Larvae with TAG/PL ≫ 1 accumulate more energetic (TAG) reserves as opposed to larvae showing TAG/PL ≪ 1. This model clarifies how cyprid physiological condition can be estimated with visual traits, when size and lipid droplet cover are integrated together.

APPENDIX

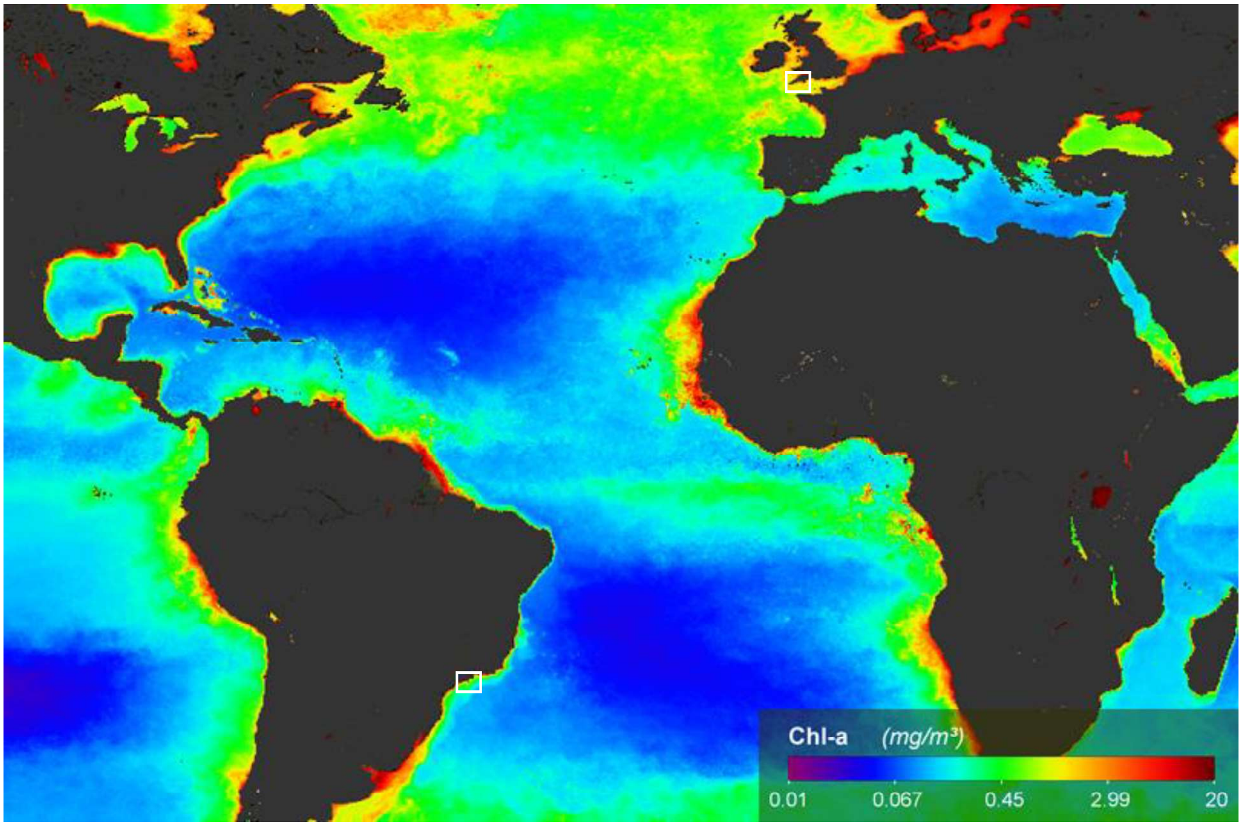

Figure 1 – Surface chlorophyll-a concentration (Chl-a; mg/m^3) map, produced using data from MODIS-Aqua NASA Ocean Color database using Level-3 mapped images of 4 km resolution, showing the latitudinal patterns of pelagic Chl-a during 2015. Sampled coasts indicated (São Sebastião Channel, Brazil, and the English Channel, British Isles).