Growth of juvenile *Pinna nobilis* in captivity conditions: dietary and pathological constraints

Patricia Prado[1] *, Pep Cabanes[1], Gaetano Catanese[2,3], Francesca Carella[4], Noelia Carrasco[1], Amalia Grau[2,3], Sebastián Hernandis[5], Jose Rafael García-March[5], José Tena[5], Nuno Caiola[1], Karl B. Andree[1]

[*1]IRTA-Sant Carles de la Ràpita. Ctra. Poble Nou Km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2]Laboratori d'Investigacions Marines i Aqüicultura, (LIMIA) - Govern de les Illes Balears, Av. Gabriel Roca 69, 07158 Port d'Andratx - Mallorca (Spain).

[3]Instituto de Investigaciones Agroambientales y de Economía del Agua, (INAGEA) (INIA-CAIBUIB). Ctra. Valldemossa km. 7,5 Ed. Edifici Guillem Colom Casasnoves, 07122 Palma de Mallorca - Illes Balears

[4]University of Naples Federico II, Department of Biology, Corso Umberto I 40, 80138 Naples, Italy

[5]Institute of Environment and Marine Science Research (IMEDMAR). Universidad Católica de Valencia SVM, Avda. del Puerto s/n, 03710 Calpe, Alicante, Spain

Abstract

The fan mussel, *Pinna nobilis*, is an endemic Mediterranean species, whose populations have been seriously affected by infectious diseases. The effect of diet composition on growth and survival rates, and on nutritional metrics including ingestion and absorption rates, together with food preferences were investigated in 48 juveniles. Individuals were initially acclimated to conditions of captivity with a mixed diet of three species of microalgae cultured *in situ* and riverine sediment. Then, they were changed to different diets based on combinations of commercial phytoplankton gels and riverine sediments except for a control group that was maintained under the acclimation conditions. Diet A consisted of *Tetraselmis chuii*; diet B on a 2 species mix of *T. chuii* and *Isochrysis* aff. *galbana* (T-ISO), diet C on a 3 species mix *T. chuii*, T-ISO and *Phaeodactylum tricornatum*, diet D on riverine sediment without microalgae, and diet E on *T. chuii* and riverine sediment. Individuals under experimental diets were fed *ad libitum* once per day and the water with food excess replaced before the next ration. The control diet showed the highest survival and growth (50% vs. 2.5% survival and ca. 6 mm vs. <1 mm shell growth· month^{-1}), but rates were much lower than those of field animals (by ca. 30-40%). Mortality was ultimately associated to presence of *Vibrio mediterranei*, but our results suggest that diet quality is an important factor mediating host condition and disease resistance. Individuals fed sediments showed the lowest levels of nutritional performance, with higher ingestion (up to 5.5 times higher) and lower absorption rates (by approx. 60%), suggesting a poor adaptation to feed on detrital material. Additionally, individuals showed a significantly higher consumption of less voluminous microalgae (T-ISO and *P.tricornatum*) during food preference assays. The experimentation suggests that the development of new diets nutritionally similar to those available in the field and the finding of new food supplements promoting disease resistance is a research priority for the optimal maintenance of the species under long-term captivity conditions.

Keywords: Microalgae preferences; Ingestion rate; Absorption efficiency; *Vibrio mediterranei*

1 Introduction

The fan mussel, *Pinna nobilis* is a bivalve mollusk endemic to the Mediterranean Sea, and also the largest and one of the longest living, since it can reach more than one meter in length and live over fifty years (Butler et al., 1993; Rouanet et al., 2015). For decades, it was considered as a vulnerable species by the European Union (Habitats Directive Annex IV EEC 1992 and ASPIM Protocol Annex 2 of the Barcelona Convention) mainly due to due to culling of individuals for ornamental purposes and anthropogenic reduction of seagrass habitats. However, from the fall of 2016, populations began to be seriously affected by the spread of a parasitic disease caused by the protozoan *Haplosporidium pinnae* (Catanese et al., 2018; López-San Martín et al., 2019). Since its first appearance, the disease has spread throughout the Spanish Mediterranean coast leading to cumulative mortalities of 90-100% of the populations in Andalusia, Murcia, the Balearic Islands, and the Valencian Community (Vázquez-Luis et al., 2017). More recently, mortalities have escalated to 100% and have reached Catalonia, as well as other countries of the Western Mediterranean including France, Italy, Morocco, Tunisia, Turkey, Cyprus, Malta, and Greece. However, for reasons that are still unknown, the Fangar Bay (Ebro Delta; P Prado unpublished data) and the Mar Menor have remained unaffected by the parasite (Cabanellas-Reboredo et al., 2019).

Unlike other species of commercial bivalves susceptible to Haplosporidan parasites such as the American oyster (*Crassostrea virginica*), and the European oyster (*Ostrea edulis*) (Burreson and Ford, 2004; Arzul and Carnegie, 2015), *P. nobilis* has not yet been reproduced successfully in captivity (Trigos et al., 2018), which makes more difficult the implementation of conservation plans. Bivalve aquaculture can be a potential solution for the rehabilitation of endangered bivalve populations subjected to overexploitation (Loor et al., 2016; Lodeiros et al., 2016, 2017) but this approach requires the conditioning of healthy broodstocks. The practice usually involves a short period of gonad maturation of broodstock (from a few days to 2-3 months) using increasing temperature and excess microalgae (e.g., Martínez et al., 2000;

73 Chávez-Villalba et al., 2002). After a rearing period, the seed stock is transferred to the tidal
74 flats of estuaries and bays using culture methods that depend on the target species,
75 environmental conditions, and local tradition (FAO, 2006). For instance, the seed of *Atrina*
76 *maura*, a Pacific member of the Pinnidae family, can be grown in the benthic sands of lagoon
77 systems in densities of up to 36 ind.· m^{-2} (Gongora-Gomez et al., 2016) whereas another close
78 species, *Pinna carnea*, is reared to commercial size in suspended enclosures (Narváez et al.,
79 2000). In contrast, the severe reduction in abundance of multiple populations of *P. nobilis*
80 brings urgency to the development of protocols for the long-term maintenance and growth of
81 adult and juvenile individuals within rearing facilities with pathogen-free seawater, in order to
82 minimize possible loss of genetic diversity and guarantee the persistence of the species.
83 The proper formulation of diets is one of the most important constraints for rearing
84 bivalves in controlled systems outside their natural habitat (e.g., Pettersen et al., 2010; Ragg et
85 al., 2010; Gui et al., 2016). The size and type of food (living microalgae vs. fine suspended
86 detrital sediments) may influence filtration rates due to the ability of the gill´s ctenidial
87 structures and/ or labial palps to sort particles and reject undesired materials (Ward et al.,
88 1997; 1998; Cognie et al., 2001). According to Davenport et al., (2011) *P. nobilis* is capable of
89 significant selection for phytoplankton species and ingests large amounts of detrital material
90 (up to 95%), as well as some micro and mesozooplankton species. In addition, preferential
91 selection processes and distinctive absorption efficiencies have been indicated for different
92 species of microalgae across bivalve species such as *Crassostrea virginica* (Shumway et al.,
93 1985) and *C. gigas* (Cognie et al*.,* 2001) which might be associated to microalgae size or the
94 type of cell wall. From a nutritional perspective, food value of microalgae is related to the
95 abundance of essential amino acids (Knauer and Southgate 1999) and to carbohydrate
96 contents, which may enhance juvenile growth and regulate the metabolism of proteins and
97 lipids (Whyte et al., 1989). Equally, certain types of highly polyunsaturated fatty acids (HUFAs)
98 are generally accepted to be essential for bivalve growth (Knauer and Southgate, 1999).

99 Because a mixed algal diet increases the chances of achieving the adequate biochemical
100 composition, a microalgae mix is generally supplied without a clear knowledge of their need in
101 essential components (Muller-Feuga et al., 2003). In particular, a microalgae combination
102 including at least one Haptophyceae and one Bacillariophyceae species are often used for
103 bivalve rearing (Robert and Gérard, 1999). Elucidating a correct species-specific balanced diet
104 may also help development of a competent immune system (Delaporte et al., 2003; Hégaret et
105 al., 2004) and ease the long-term maintenance of individuals under conditions of captivity.
106 Virus and bacteria are considered the most recurrent agents of mortality within bivalve
107 hatcheries and nurseries (e.g., Segarra et al., 2010; Pernet et al., 2014) and have been
108 indicated as the most likely cause of failure in larval cultures of *P. nobilis* (Trigos et al. 2018).
109 Natural populations have been shown to be extremely vulnerable to *H. pinnae* (Catanese et al.,
110 2018) and to Mycobacteria disease (Carella et al., 2019). However, *Vibrio mediterranei* has
111 been suggested as the major agent of mortality in adults of *P. nobilis* subjected to long-term
112 captivity stress (Prado et al., 2019) and further challenge experiments have demonstrated its
113 pathogenicity in pen shell (Andree et al., submitted).
114 The Alfacs Bay has been reported to host the second largest population of *P. nobilis* in the
115 Mediterranean within over 90,000 adult individuals (Prado et al., 2014). Unfortunately, the
116 area was infected by *H. pinnae* in July 2018, although half of the inner bay remains unaffected
117 (P. Prado, unpublished data). Given the elevated risk of infection for the last remaining
118 populations on the Spanish coasts, the Spanish government authorized in November 2017 the
119 rescue of 221 adult individuals of *P. nobilis* from the coast of Catalonia, including 106
120 individuals from Alfacs Bay, which were transferred to the IRTA aquaculture facilities. Two
121 months later, in late January 2018, abundant juvenile recruitment was observed on an shallow
122 sand-bar (less than 10 cm water depth) adjacent to the area of adult collection. These
123 juveniles, which would have died in the following months due to desiccation in the sand bar,
124 constituted a perfect source of individuals for experimentation. In this context, the main aim of

this study was to investigate the effect of diet composition in the overall performance of juvenile individuals -collected from the sand bar- and maintained in tanks, in terms of growth and survival when confronted with potential diseases. In addition, we also pursued the following specific objectives: 1) to elucidate the potential causes of mortality using molecular methods for pathogen detection; 2) to assess differences in ingestion and absorption rates among diets (combinations of microalgae species and sources and/ or fine sediments); and 3) to assess possible feeding preferences for different types of microalgae.

2 Materials and methods

2.1 Collection of individuals and initial acclimation

A total of 48 juveniles of *P. nobilis* were collected from a shallow emerged area in the Alfacs Bay, at ca. 10 cm water depth in late January 2018 (a period with especially low tides locally). Individuals were the young of the year, from reproductive events during the summer period of 2017, with sizes ranging from 69 to 137 mm. Given the large threat of entrance of the parasitic disease by *Haplosporidium pinnae* at the time of collection (detected in the Alfacs Bay 5 months later), and the high probability of desiccation in the sand bar, it was considered that individuals could have a better chance of survival fully submerged within IRTA's tanks where sterilization of ambient water could eliminate exposure to many pathogens.

Juveniles were transported to the laboratory in an aerated cooler and once there transferred to six small tanks (50 L) within IRTA wet lab facilities with an open-water circuit system directly connected with seawater pumped from the Alfacs Bay. Seawater was filtered through 10, 5 and 1 µm and disinfected with UV light to ensure the absence of the haplosporidan parasite (ca. 2.8 µm size according to Darriba 2017), and then passed through an active carbon filter during the summer months in order to neutralize possible agrochemicals present in Alfacs Bay during the rice cultivation period. Experimental conditions were: salinity 36-37 ppt, Oxygen 5-8 mg/ L, pH 7-8.1, seasonal temperature (from 11.5 to 25.7 °C) and

photoperiod, and variable dissolved nutrients as present in the water source (NO_3: 81.4 ± 12 µmo L^{-1}; NO_2: 2.23 ± 0.4 µmo L^{-1}, and NH_4: 3.7 ± 0.8 µmo L^{-1}).

In order to improve the overall condition of individuals and acclimatize them to captivity, juveniles were feed twice a day with a mix of three microalgae (*Isochrysis* aff. *galbana*, T-ISO; *Tetraselmis chuii*; and *Chaetoceros calcitrans*) and sediment rich in organic material (OM) (average of 13.5%) during *circa* 3 months (late January to April). Diet rations consisted of an average of $9 \cdot 10^9$ cells of T-ISO (1200 ml), $1.2 \cdot 10^9$ (600 ml) cells of *T. chuii*, and $5 \cdot 10^9$ cells of *C. calcitrans* (600 ml) per tank with 1 g of riverine sediments <200 µm supplied twice a day. Water circulation in the tanks was closed when feeding in order to avoid food loss, allowing juveniles to feed for 3 hours during each meal before the water system was opened again for its progressive renewal.

Microalgae species were obtained from *in situ* cultivation of phytoplankton. The sediment was collected in the Tarragona water consortium (CAT) at the Ametlla de Mar, where water from the Ebro River is depurated. These fine riverine sediments are the same than those naturally arriving to pen shell populations in Ebro Delta Bays, but for facility, they were directly collected at the CAT. Sediments were autoclaved, and then filtered through a 200 µm mesh size net; only the fractions below this grain size were retained for the feeding of juvenile pen shells. Tanks were cleaned weekly to eliminate the excess of sediments and feces.

2.2 Experimental design and diet treatments

In May 2018 juvenile pen shells were distributed in five 50 L tanks (with 40 L of water, 8 juveniles per tank) using a PVC structure that enabled maintaining them in a standing position. Experimental diets were tested from May 2018 until the death of individuals (most of them in August-September). In a sixth 50 L tank, eight additional juveniles were kept under the same diet and water renewal conditions used during the acclimation period, so they could be used as a “control” for deviations from initial conditions. A second control of eight juveniles located

177 in situ at the same site of collection but at greater depth (ca. 60 cm), was also monitored
178 during the dietary experimental period, to serve as background comparison.
179 Experimental diets consisted of different species of manufactured phytoplankton gel (Easy
180 Reefs, Fitoplancton Marino) with a fixed number of cells per ml based on the patent of a
181 cytostatic formula that keeps them alive but inhibits cell division (see values per ml in Table 1).
182 Diet A consisted of *T. chuii* gel (7 to 14 ml along the experimental period), and diet B of a two
183 species gel mix of *T. chuii* and T-ISO (Diet B: 3.5 to 7 ml of each one). For diet C, since no
184 species *Chaetoceros* is commercially available in gel, another diatom, *P. tricornatum*, was used
185 instead in a mix with *T. chuii* and T-ISO (2.4 to 4.7 ml of each one). Individuals on diet D were
186 feed with 2 g dry weight (DW) of riverine sediments with 13.5 %OM and < 200 µm size per day.
187 At last, diet E consisted of a mix of 5 to 10 ml of *T. chuii* and 0.5 g of riverine sediments.
188 Individuals were fed in the morning and allowed to eat during 24 h, after which the water of
189 the tanks was totally renovated and individuals fed again. Every week, a water sample was
190 taken from each tank prior to water renovation in order to ensure that there was still
191 phytoplankton available in the water column; if not, the volume of gel was increased to ensure
192 that individuals were always supplied *ad libitum*.
193
194 **2.3 Mortality and growth rates**
195 Shell length and width of the 48 juvenile pen shells maintained in the tanks were measured
196 to the nearest 0.1 mm with digital Vernier calipers at the end of each study month. Then,
197 growth rates were estimated as size differences (mm) between each consecutive pair of
198 measurements of pen shell size. The width of the 8 in situ control individuals was measured
199 three times using plastic calipers: once in April at the end of the acclimation period of the
200 juveniles maintained in the tanks, and just before starting the experimental period, a second
201 time in July 2018, during the experimental period, and a third time in late October 2018 after

the experimental period. Using this sampling configuration, the growth trend could be established for these juveniles.

The number of dead individuals was registered throughout the stabling period (i.e. from late January to October 2018, when only one individual of Diet E was left). A tissue sample of each dead juvenile was kept in absolute ethanol for later detection of pathogens reported to be associated to pen shell mortality such as *H. pinnae*, *Mycobacterium* spp. (Catanese et al., 2018; Carella et al., 2019), and *Vibrio* sp. (Rodríguez et al., 2018) using standard molecular procedures (PCR and DNA sequencing).

2.4 Molecular analyses

Extraction of DNA was conducted with the Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA, USA), and for all samples the A260/280 ratios were examined by spectrophotometry to evaluate purity and concentration. Samples for detection of *H. pinnae* were run with the specific primers for (pairs HPNF1/HPNR3 and HPNF3/HPNR3) and PCR conditions indicated by Catanese et al., (2018) and López-Sanmartín et al., (2019).

For *Mycobacteria* spp. we used the specific primers (mycgen-f/ mycgen-r) described by Böddinghaus al., (1990) and PCR conditions indicated by Carella et al., (2019) for detecting its presence in *P. nobilis*.

The detection of *V. mediterranei* was conducted using specific primers for the 16 *atpA* gene (Vib-atpA-F: 5'-CAATTGAAGCTAAACTTACGTC-3' and Vib-atpA-R: 5'-CCGTGGCTTAGCTGACGCTTAG-3') and PCR conditions used by Andree et al., (submitted). Each 25 µL polymerase chain reaction contained 800 µM dNTP´s, 2 mM $MgCl_2$ and 0.6 uM of each primer. The thermal cycler program used was 40 cycles of 95ºC for 1 min, 56ºC for 30 sec, and 72ºC for 45 sec, preceded by 5 min at 95 ºC, and followed by 10 min at 72 ºC. The resulting product was a fragment of 914 bp.

228 **2.5 Ingestion rates, microalgae preferences, and absorption efficiencies**

229 Fifteen random individuals counting three individuals from each experimental tank (Diets A,
230 B, C, D, and E), were chosen monthly from May to August(i.e., months with at least 3 surviving
231 individuals of each diet, excepting Diet D, which only survived for 2 experimental months, from
232 May to June) and transferred to 15 individual 5 L aerated containers. Individuals within each
233 container were given 1/8 of their corresponding diet and allowed to eat during 8 h (intestinal
234 passage time; Trigos et al., 2015). After that time, individuals were returned to their tanks, and
235 the water within each container homogenized by vigorous shaking in order to disaggregate
236 possible pseudofeces and a small sample of water (ca. 20 ml) taken and fixed with Lugol's
237 iodine solution for later cell counting under the microscope. In the case of diets D and E, both
238 containing sediments, the entire volume of water (ca. 5 L) was filtered for determination of
239 uningested material.

240 The DW of each microalgae species (*T. chuii*, T-ISO, and *P.tricornatum*) was estimated by
241 filtering three replicates of 1 ml of each gel type through pre-weighed Whatman glass fiber
242 filters 0.2 µm pore size using abundant distilled water for washing the salts. Filters were
243 allowed to dry for 24 h and then weighed again for determination of DW. Then, the result was
244 divided by the fixed number of cells in each gel type guaranteed by the manufacturer (see
245 Table 1), to obtain the DW of each single cell of each species.

246 Samples of 20 ml from the 5 L containers were homogenized and a 3 ml subsample, placed
247 within a Hydro-Bios combined plate chambers, allowed to sediment for an hour and then cells
248 were counted under an inverted microscope. Samples with high cell densities were counted in
249 10 optical fields of view (fov; 0.23 mm^2 each), and samples with lower cell densities in 3
250 replicate transects (14.04 mm^2 each), all of them at x40 magnification. Then numbers
251 extrapolated to the surface and volume in the entire plate chamber, to numbers per ml, and to
252 uningested total cells in each 5 L container. Hence consumption rates were calculated as the
253 difference between the numbers of cells supplied (ml of gel supplied x No. of cells per ml for

each species) and the number of cells remaining in the 5 L containers after the 8 hours. Consumption values were expressed in mg DW by multiplying the number of cells left by the DW of each microalgae species. In the case of Diet E, containing both sediment and microalgae, the DW of uningested phytoplankton was subtracted from the DW of the filtered material in the 5 L containers in order to obtain the DW of uningested sediment. For all combined diets (B, C, and E), the DW of each consumed microalgae and sediment fraction was added to obtain a total ingested DW (mg).

Percent consumption rates of Diet C (i.e., % decline between initial and final counts of each species per ml) obtained from May to August (N= 12) were used to investigate food preference of juvenile pen shells for each type of microalgae (*T. chuii*, T-ISO, and *P. tricornatum*).

Absorption efficiencies of each diet were also estimated once a month as the decline in OM content from food to feces (Prado et al., 2012). The day of measurements, pen shells were allowed to feed for 8 hours (time for intestinal digestion; Trigos et al., 2014), and then tanks cleaned, the water changed and animals left undisturbed until the next day when all feces produced were collected from the bottom of the tank.

2.6 Data analyses

The effect of Diet in the monthly growth of individuals (including shell length and width) was investigated with a two-way RM-MANOVA (Diet and Month fixed factors with 6 levels, including controls, and 4 levels, respectively). Analyses only included May to August months due to the large number of individuals lost in September 2018. Growth patterns among tanks during the acclimation period (from late January to April) were also investigated with a two-way RM-MANOVA (Tank and Month fixed factors with 6 and 3 levels, respectively).

Differences in the total ingested material per diet at each month of the experiment (May to August) were investigated with a two-way factorial ANCOVA (Diet Fixed factor with 5 levels, and Month fixed factor with 4 levels), using the size of individuals as a covariate. For the

control diet, monthly differences between tanks during the initial acclimation period (February to April) were also investigated with a two-way ANCOVA, with shell size as a covariate. Significant groupings were investigated with Student-Newman-Keuls (SNK) post hoc analyses.

Differences in absorption efficiencies were investigated with a 2-way ANOVA with Diet and Month as fixed factors (5 and 6 levels, respectively) and SNK post hoc analyses.

For food preferences, the Friedman ANOVA by ranks (Conover 1980) and Kendall's concordance coefficient (Kendall 1955) were used to assess significant differences in consumption rates among diets and the degree of agreement among the rankings. Then, the Wilcoxon matched pairs (WMP) post hoc comparisons were used to assess significant differences in % consumption rates among microalgae species (see Cronin et al., 2002 and Prado and Heck 2012 for similar approaches).

Data were all tested for ANOVA assumptions of normality (Chi-squared test) and homogeneity of variances (Cochran's test). All statistical analyses were performed using the Statistica software.

3 Results

3.1 Mortality and growth rates

Mortality rates were nearly zero during the 3-month acclimation period (February to May) and the first month of the experiment (June). In July seven of the individuals on Diet D (only riverine sediments) died. Later in August, and particularly September (warmest months with temperatures of ca. 25-26°C) the highest mortalities for Diets A (1 species), B (2 species), C (3 species), and E (1 species and sediment) were registered. The last individual of Diet D also died in August (Fig. 1). Compared to the other experimental diets, individuals from the control diet were the more resistant, with only one death in June and two in October (see Fig. 1), which was closer treatment to the 100% survival of juveniles in the field.

All juvenile pen shells that died during the experiment showed positive results for the presence of *V. mediterranei* except for individuals E3 and E8. In contrast, other pathogens commonly causing large mortality event in *P. nobilis* such as *H. pinnae* and *Mycobacteria* sp. reported negative results for all individuals investigated.

RM-MANOVA results for combined growth rates (shell length and width) showed significant effects of Diet, Month, and their interaction (Di x Mo) (Table 2a). Among diets, patterns were due to higher growths in controls than in the remaining treatments (6.43 ± 0.92 mm length and 1.97 ± 0.47 mm width vs. 0.93 ± 0.31 mm length and 0.28 ± 0.12 mm width per month, respectively). Monthly growth rates (including controls) showed higher values in May (2.43 ± 0.40 mm length and 0.75 ± 0.11 mm width) and July (2.29 ± 0.36 mm length and 0.35 ± 0.12 mm width), and lower in June (1.03 ± 0.30 mm length and 0.41 ± 0.09 mm width) and August (1 ± 0.40 mm length and 0.46 ± 0.20 mm width). The Di x Mo interaction was mostly due to slightly higher growth rates of some of the diets in February and July (see Fig. 2a,b). For the acclimation period, RM-MANOVA showed no significant tank effects (Fig. 2a,b), although slightly higher growth was recorded in April (Table 2b). In the field, the growth of juveniles was considerably higher reaching average width values of 2.8 ± 0.5 mm throughout the study period (ca. 40% higher than controls).

Based on the size-length relationship for several healthy field individuals (N= 5), an average of 95 ± 1.2 mg of DW was estimated for individuals during the period from May to August (R^2 = 0.934; p= 0.001; y= 0.8702x -6.5211).

3.2 Ingestion rates, microalgae preferences, and absorption efficiencies

ANCOVA results for ingestion rates showed significant effects of Diet, Month, and their interaction (Di x Mo) as well as a significant effect of the shell size covariate (Table 3a). SNK analyses showed that Diets A, B, and C (1, 2 and 3 species) were not significantly different between them (12.3 ± 1.1 to 13.9 ± 0.9 mg DW ingested in 8 h trials), whereas diets D and E

including sediments had significantly higher ingestion rates (49.7 ± 6.8 and 34.5 ± 3.3 mg DW ingested in 8 h) (Fig. 3). Temporally, ingestion rates showed a Gauss curve, with highest values in June-July (27.4 ± 5.2 and 23.2 ± 4.7 mg DW, respectively) and lowest in May and August (18.7 ± 3.4 and 18.2 ± 2.1 mg DW), but patterns were due to the effects of Diets D and E, whereas diets based on phytoplankton displayed similar values across time, particularly from June to August (Fig. 3).

ANOVA results for absorption efficiencies showed significant effects of both Diet and Month (Table 3b). Absorption rates were highest for diets B and C with two and three species of phytoplankton (67.36 ± 0.90, and 65.37 ± 1.17%) followed by diet A (1 species: 45.26 ± 1.63%), and lowest for diets E (1 species and riverine sediment: 5.03 ± 0.59%) and D (only riverine sediment: 2.8 ± 0.43%). Monthly patterns were not strong and were due to slightly lower rates in August (Fig. 4).

Results of Friedman's test showed significant effects of microalgae species on consumption rates (see Table 5). According to the Wilcoxon matched pairs test, juvenile pen shells showed highest preference for T-ISO, closely followed for *P. tricornatum*, and lowest for *T. chuii* (Fig. 5).

4 Discussion

Juveniles which fed on microalgae gels (diets A-C), riverine sediments (diet D), or a combination of gels and sediments (diet E) consistently showed lower growth rates than controls feed with a mix of three species of phytoplankton cultured *in situ* mixed with riverine sediments rich in OM (ca. 6 mm vs. <1 mm shell growth· month^{-1}). This suggests that individuals were subjected to severe nutritional impairment (Raubenheimer, 1992; Marshall et al., 2010), with only 10.4% of the juveniles surviving the captivity period from late January to November 2018. In addition, there were also important differences in survival rates between controls (50% survival), and the rest of the treatments (2.5% survival). In all instances, mortality of juvenile pen shells was ultimately associated to the presence of *V. mediterranei*,

357 which has been suggested as a major cause of death in captive individuals of *P. nobilis* (Prado
358 et al. 2019), whereas all analyses showed negative results for *H. pinnae* and *Mycobacteria* sp.
359 (Catanese et al., 2018; Carella et al., 2019). *V. mediterranei* (an earlier synonym of *V. shiloi*) is a
360 common pathogen of the coral *Oculina patagonica* (Kushmaro et al., 2001), but can be also
361 found in bivalves such as mussels and clams (Tarazona et al., 2014) that are extensively
362 cultured in the Alfacs Bay. More recently, it has also been identified from wild individuals of *P.*
363 *nobilis* (Rodríguez et al., 2018; Andree et al. submitted) and in the adults from the MAPAMA
364 rescue project at IRTA that were held under the same diet as the control juveniles and
365 experienced similar mortalities (ca. 58%) by November 2018 (Prado et al., 2019). In recent
366 challenge experiments with juvenile pen shells conducted by Andree et al., (submitted),
367 authors demonstrated the pathogenicity capacity of the bacteria, with over 80% mortalities
368 recorded at low doses of 10^3 CFU in less than three weeks. Long-term stabled individuals in
369 this study were subjected to persistent growth limitation associated to dietary captivity
370 conditions and appear to lack the necessary fitness to tackle the development of *V.*
371 *mediterranei* under optimal temperature conditions (Prado et al., 2019).
372 Although individuals which fed on the control diet showed the highest survival and growth
373 (47.5% and 85% higher, respectively), rates were still low compared to those of field
374 individuals of the same age still remaining in deeper areas of the collection site in the Alfacs
375 Bay (100% survival, and 30 to 40% higher growth than controls). This suggests that captive
376 individuals were subjected to varying degrees of physiological stress due to nutritional
377 imbalances which impaired host condition and increased the risk of developing associated
378 pathologies (Pettersen et al., 2010; Ragg et al., 2010; Prado et al., 2019). Davenport et al.,
379 (2011) found a total of 29 taxa of phytoplankton and several groups of zooplankton (copepods,
380 bivalve and gastropod larvae, and ciliates) in the gut contents of *P. nobilis* whereas most
381 bivalve diets under captivity conditions are simplified to a combination of 2 or 3 species of
382 phytoplankton for practical reasons (FAO, 2006; this study). Although the nutritional

information provided by the manufacturer appears to provide a balanced combination of proteins, lipids, carbohydrates and vitamins (Table 1), phytoplankton gels (diets A-C) clearly provided a lower physical fitness than microalgae cultured in situ at IRTA's facilities. The reasons for this result are largely unknown, and given the relatively novelty of the product, no previous information on the performance of the gels seems to be available in the literature. However, similar negligible growth has been observed in captive juveniles of the congeneric species *P. rudis* fed on the same gels (Hernandis-Caballero, pers. communication) suggesting that they do provide a maintenance diet, but that they are inadequate for the growth of juvenile bivalves. Moreover, additional feeding trials with phytoplankton gel (T-iso) during the early larval development of *P. nobilis* (D-veliger stage) also resulted in 100% mortality within 48 h (P. Prado, unpublished data). Only one individual under diet E (*T. chuii* geland riverine detritus) was able to persist alive up to 8 months in IRTA facilities after the end of the experiment, but without any visible growth whereas the remaining controls continued growing at the same previous rates.

The mortality of individuals on diet D (riverine detritus) occurred even earlier than under treatments with phytoplankton gels, suggesting that sediment alone is also an inadequate diet for juvenile pen shells. In fact, individuals fed only with detritus showed the lowest nutritional performance with higher ingestion (up to 5.5 times higher) and lower absorption rates (by approx. 63%) than those feed on microalgae gels with higher content of organic matter (77 to 89% vs. 13% OM). *P. nobilis*, has been shown to ingest large quantities of undetermined detritus (ranging from approx. 50% to 95%, depending on the study; Davenport et al., 2011; Trigos et al., 2014), with particularly high values indicated for small individuals living at a close proximity (in the order of few centimeters) to the sediment (Davenport et al., 2011). Our results, confirm that juvenile *P. nobilis* can ingest large quantities of detrital material (up to 47.7 mg DW in 8 h), but also evidence that individuals under this diet can experience nutritional imbalance, possibly resulting from the lack of some essential amino acids,

409 unsaturated fatty acids, or other required elements that are present in microalgae (Knauer and
410 Southgate 1999). According to Davenport et al., (2011), other items such as phytoplankton,
411 and micro and mesozooplankton were also observed within stomach contents of *P. nobilis* and
412 could be essential for a balanced diet. Daily ingestion rates relative to body weight could be
413 estimated thanks to the size-length relationship established for several field individuals and
414 ranged from 0.3 to 0.42% of the body DW for individuals fed phytoplankton gels at up to 1.6%
415 of the body DW for individuals fed riverine sediments. These values are considerably lower
416 than feeding rations commonly used in bivalve hatcheries and implemented at IRTA for
417 feeding adult *P. nobilis* (2-4% of body weight; Helm et al., 2004) and could be partly due to
418 captivity stress or to latent disease, as suggested by some individuals that were not eating
419 during the feeding trials.

420 Absorption efficiencies of individuals based on differences in ash content between food and
421 feces ranged from approx. 45 to 67% for individuals feed on phytoplankton, to values as low as
422 3% in individuals fed riverine sediments. Studies in other species of bivalves have reported
423 similar values of absorption efficiencies for individuals fed on different species of
424 phytoplankton: 78.6% in the Atlantic deep-sea scallop *Placopecten magellanicus* (Cranford and
425 Hargrave, 1994), 52 to 65% in the blue mussel *Mytilus edulis* (Kiørboe et al., 1980), and approx.
426 70% in the horse mussel *Modiolus modiolus* (Navarro and Thompson, 1994). Yet, variable
427 absorption efficiencies were observed among microalgae diets (A, B, and C), with patterns
428 suggesting lower rates for the Chlorophyta *T. chuii*, and similar values for the diatom *P.*
429 *tricornatum* and the haptophyte clon T-ISO. This ability of bivalves to sort algal species within
430 their gut has also been indicated by other authors. For instance, Shumway et al., (1985)
431 reported a clear evidence for a preferential absorption of the cryptomonad flagellate
432 *Chroomonas salina* compared to the dinoflagellate *Prorocentrum minimum* and the diatom *P.*
433 *tricornutum* in several species of bivalves. Similarly, Bricelj et al., (1984) showed the
434 preferential absorption of a chrysophyte, as compared to two chlorophytes and two

435 cyanobacteria, in the suspension-feeding bivalve *Mercenaria mercenaria.* In regards to the
436 effects of suspended sediments, the dietary supply resulted on decreased absorption rates of
437 the overall ingested material (3 and 5%, respectively for diets D and E) suggesting a poor
438 adaptation to feed exclusively on detrital material, and failure to compensate for lower OM
439 contents despite enhanced ingestion rates. In fact, different species of bivalves might have
440 varying capacities to absorb organic matter from suspended sediments. For instance, the
441 oyster *Crassostrea gigas*, has been shown to exhibit increasing absorption efficiencies with
442 enhanced organic content of ingested sediments, and displayed values above 40% for OM
443 contents of approx. 13% (Barillé et al., 1997) such as those supplied during the experiment. In
444 contrast, the cockle *Cerastoderma edulis* has exhibited a similar pattern to that observed here
445 in *P. nobilis*, featuring decreasing absorption rates at increasing concentrations of silt particles
446 mixed with the microalgae *T. suecica* (Iglesias et al., 1992). Yet, considering the strong
447 preference of *P. nobilis* for smaller microalgae (see later) further research is needed to
448 evaluate the effect of sediments at size ranges much smaller than 200 µm.

449 *P. nobilis* individuals also showed consistent ability to discriminate among microalgae
450 species during monthly feeding trials, with preference for less voluminous microalgae (T-ISO
451 featuring 5-6 µm length x 2-4 µm width closely followed by *P. tricornatum* featuring 10-12 µm
452 length x 3.5 µm width) compared to more voluminous cells of *T. chuii* (12-14 µm length x 9-10
453 width). This finding is coherent with the observations by Davenport et al., (2011) who
454 indicated that *P. nobilis* is capable of significant selection of phytoplankton species with an
455 apparent preference for small dinoflagellates vs. large diatom species (especially when
456 occurring in chains), particularly in large and medium sized individuals. In bivalves, particle
457 sorting and rejection of undesirable particles prior to ingestion is thought to be facilitated by
458 the dimensions and ultrastructure of the ctenidia and labial palps (Ward et al., 1997, 1998). In
459 *P. nobilis* labial palps are remarkably complex and feature ridges and grooves provided with
460 cilia that allow the movement of water and may contribute to the selection and retention of

461 particles (Gilmour, 1974). In addition, the pen shells feature prominent waste tracts that help
462 remove unwanted material (pseudofaecal bolus) from the labial palps towards the exhalant
463 aperture (Liang and Morton, 1988) and might further help in the selection process. Among
464 other factors that could have influenced observed preference rankings of microalgae,
465 nutritional features provide poor information since contents indicated by the manufacturer
466 were very similar among the three species of microalgae (see Table 1). In the particular case of
467 diet E, a higher retention of *T. chuii* with enhanced nutritional content and smaller particle size
468 compared to riverine detritus is also hypothesized to account for a more prolonged period of
469 survival.

470 To conclude, our results suggest that juveniles maintained under conditions of captivity
471 were subjected to different levels of nutritional stress (Raubenheimer, 1992; Marshall et al.,
472 2010). Control diets based on three species of microalgae cultured *in situ* appeared to be the
473 most adequate in terms of growth and long-term survival of individuals, but performance was
474 still poor compared to available records from field animals. In fact, long-term maintenance of
475 adult individuals under the control diet also resulted in mortality rates of 88% after 20 months
476 of captivity (Prado et al., 2019). The development of new diets nutritionally similar to that
477 available in the field and the finding of new food supplements promoting disease resistance
478 (e.g., Burgents et al., 2004; Talpur and Ikhwanuddin, 2012) are a research priority for the
479 optimal maintenance of the species under long-term conditions of captivity and potential
480 domestication for captive rearing. Although the nutritional reasons behind the low state of
481 physical fitness obtained with phytoplankton gels are uncertain, based on our results their use
482 for bivalve rearing is not advised. Diets exclusively based on sediments also returned the
483 lowest growth and may lead to reduced absorption rates when combined with phytoplankton.
484 Observed differences in metrics related to nutritional performance suggest that small-size
485 microalgae such as T-iso and *P. tricornutum* are preferentially selected and ingested at higher
486 rates, as well as more readily absorbed, and provide the basis for further studies.

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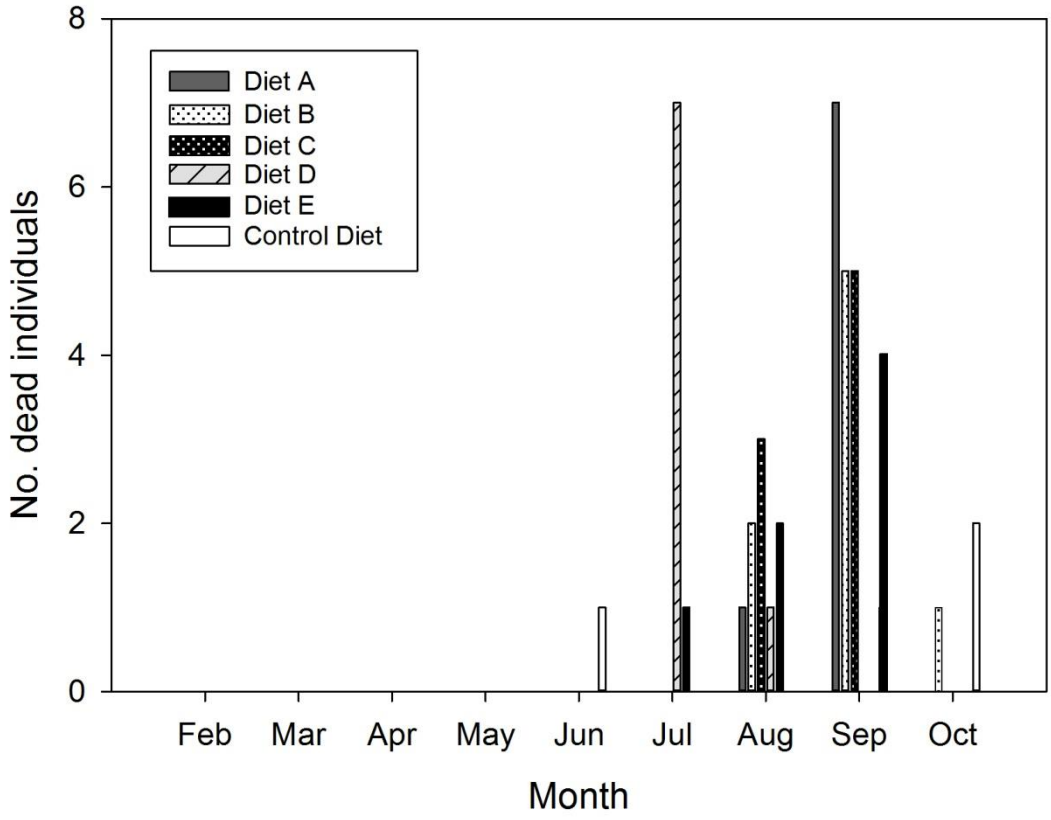
686

687 **Fig. 1.** Frequency of death individuals along the stabling period (starting in February 2018). The
688 acclimation period lasted from February to April and the experimental period from May until
689 the death of individuals.
690
691 **Fig. 2.** Monthly growth rates of individuals in terms of A) length and B) width during the
692 stabling period including the initial acclimation period from February to April (all individuals
693 feed the same diet) and the experimental period from May until the decease of most
694 individuals in August-September. Note that the growth of controls and one surviving individual
695 from Diet E are also indicated for September and October although those months were not
696 included in the RM-ANOVA due to the low replication. During the acclimation period letters
697 indicate Tanks and during the experimental period Diets. Error bars are SE.
698
699 **Fig. 3.** Ingestion rates (mg DW) of experimental diets from May to August 2018 over 8 h
700 feeding trials. The fractions of each diet item are indicated. Error bars are SE.
701
702 **Fig. 4.** Absorption rates of the 5 different diets during the experimental period from May to
703 August. Error bars are SE.
704
705 **Fig. 5.** Food preference of microalgae species based on percent consumption rates of initial
706 food rations after 8 h trials. Mean consumption, SE, and SD are indicated (N= 12).
707



708

709 Fig. 1

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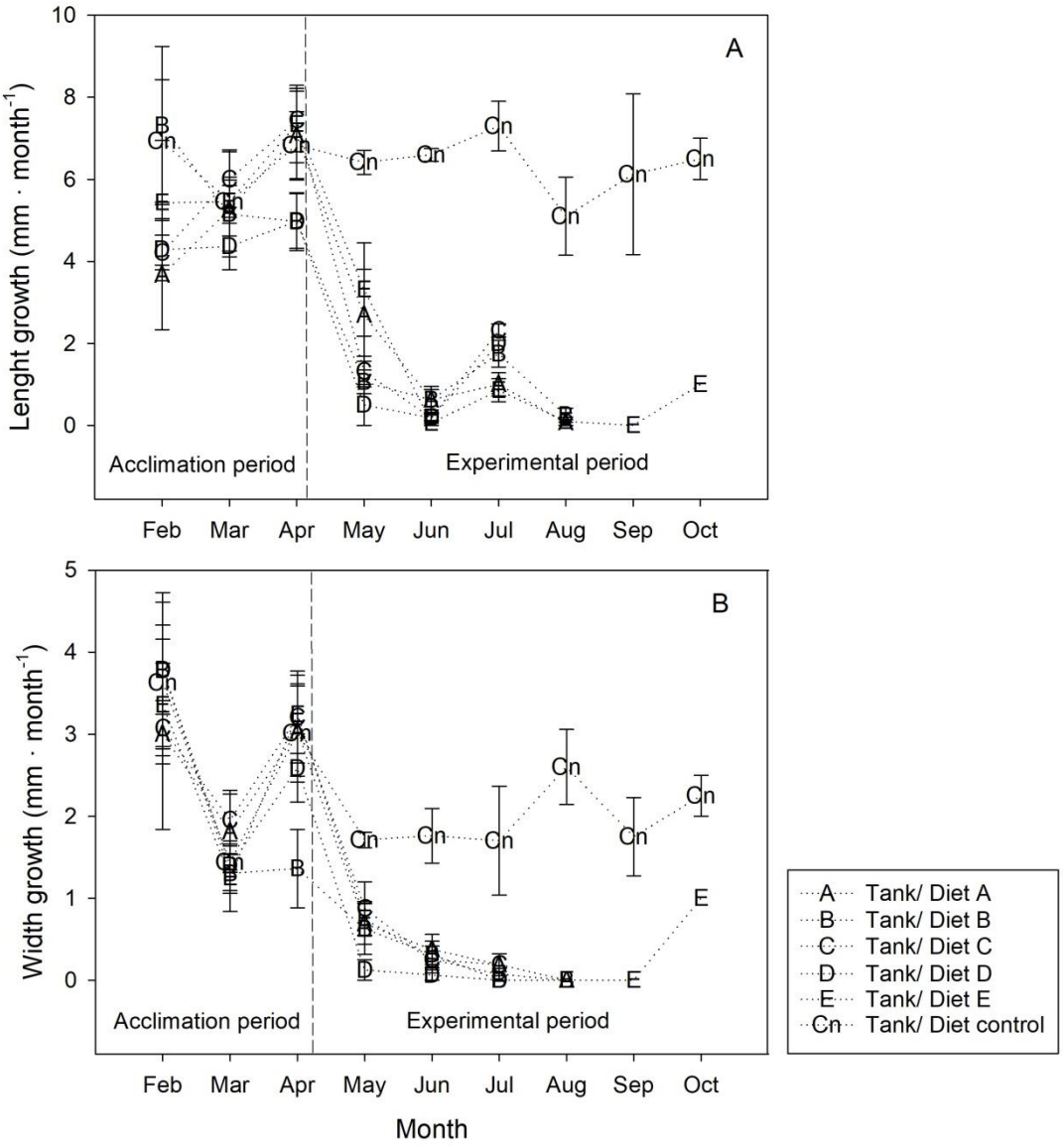

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712 Fig. 2

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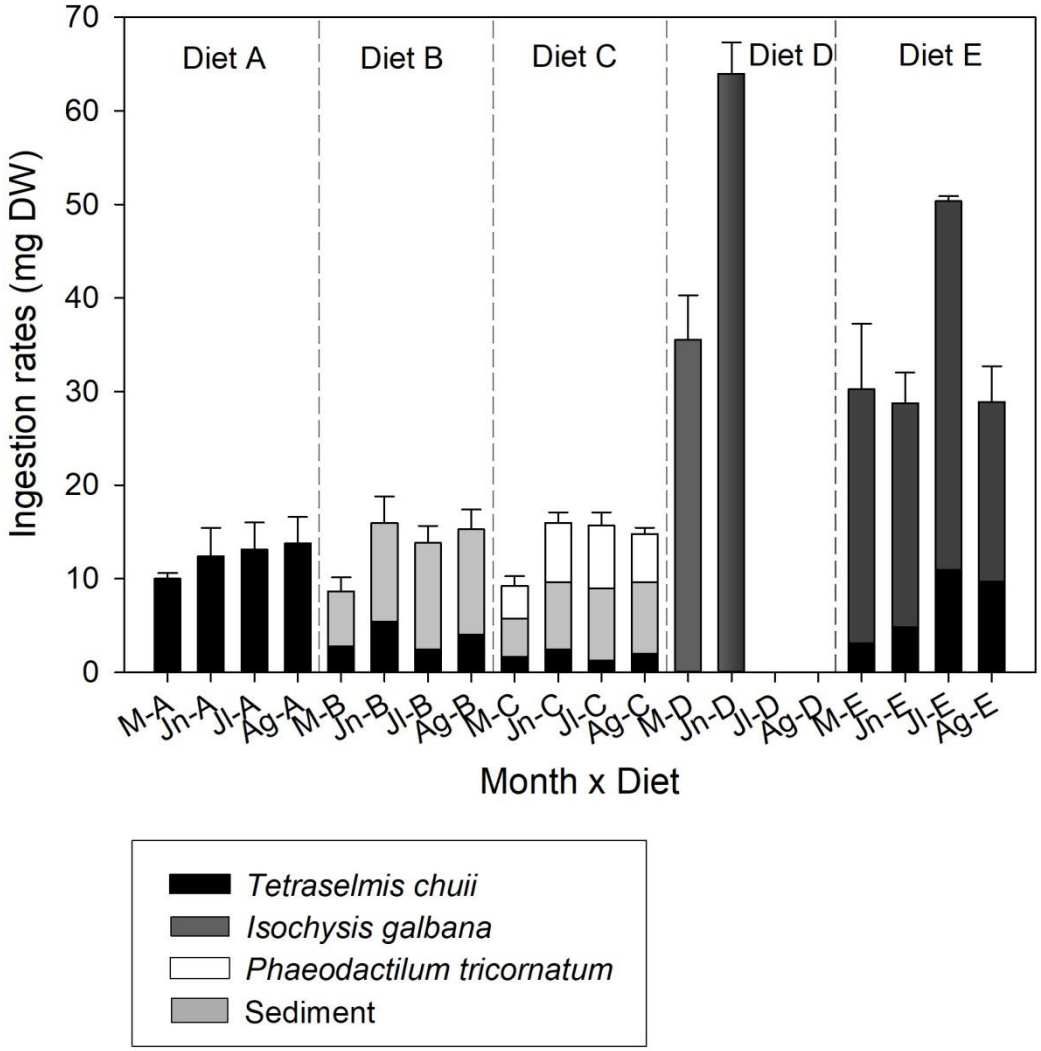

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716 Fig. 3

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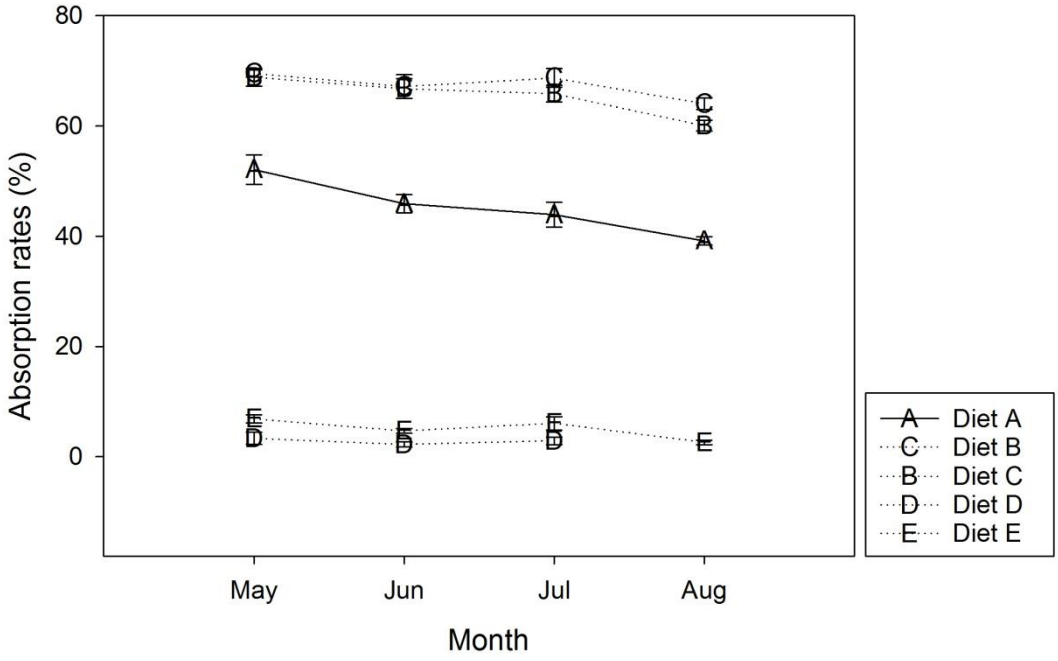

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720 Fig. 4

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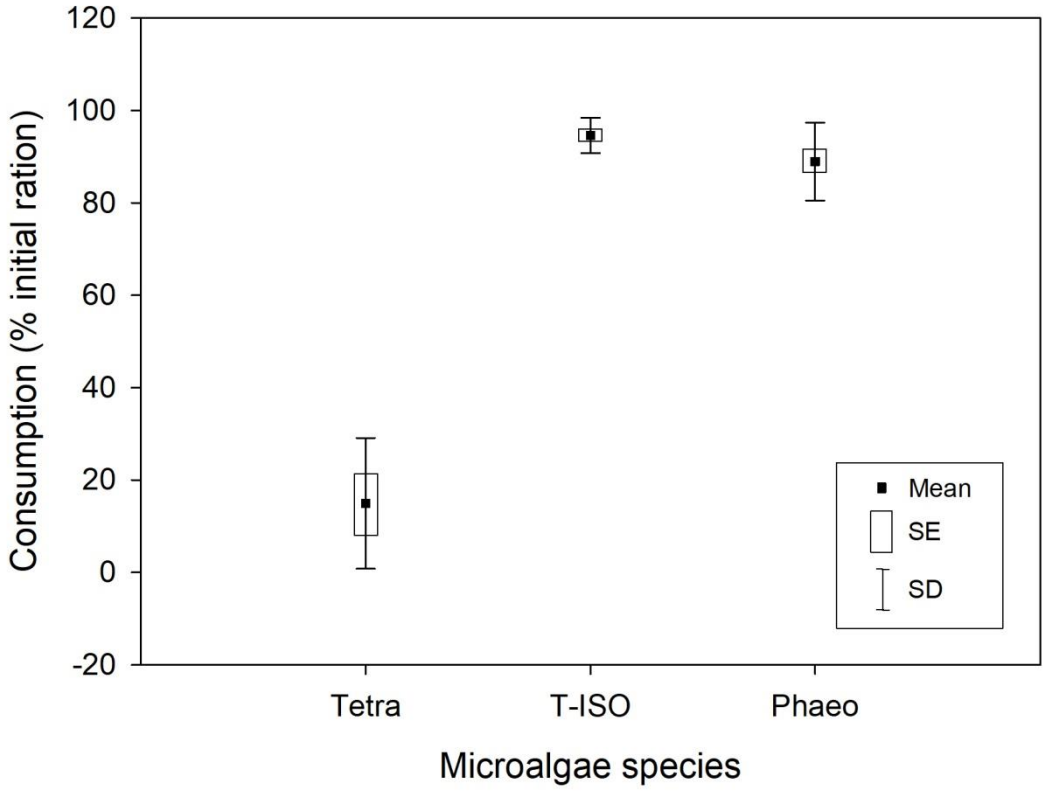

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724 Fig. 5

725 **Table 1.** Number of cells per ml, the resulting DW per cell, and nutritional features of each species gel, including proteins, carbohydrates, lipids, fatty acids,

726 amino acids and vitamins as indicated by the manufacturer.

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Species	Cel/ ml	DW cell (mg)	Prot	Carbs	Lipids	Fatty acids	Amino acids	Vitamins
T. chuii	$0.1225 \cdot 10^{9}$	$1.79 \cdot 10^{-7}$	41 %	20 %	17%	EPA, ARA, DHA	Isoleucine, Leucine, Lysine, Methione, Phenylalanine, Proline, Threonine, Tryptophan, Valine, Alanine, Arginine, Aspartic acid, Glutamic acid, Glicine, Histidine, Tyrosine	Folic acid, Vit A, Vit B1 (thiamin), Vit B2 (riboflavin), Vit B3 (niacin), Vit B5 (pantothenic acid), Vit B6 (pyridoxine), Vit B12 (cobalamin), Vit E (tocopherol), Vit C (Ascorbic acid)
T-Iso	$1.25 \cdot 10^{9}$	$1.1 \cdot 10^{-8}$	45 %	22 %	18%			
P. tricornutum	$1.0415 \cdot 10^{9}$	$1.21 \cdot 10^{-8}$	33 %	30 %	22%	EPA		

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Table 2. A) Two-way RM-MANOVA results for differences in growth rates during the experimental period (May to August) and B) during the acclimation period (February to April). Statistically significant results are indicated in **Bold**.

A) Growth rates experimental period				
RM-MANOVA	**df Effect/ Error**	**Wilk's λ**	***F***	***p***
Diet (Di)	10, 76	0.0934	17.259	**0.0000**
Month (Mo)	6, 34	0.2185	20.259	**0.0000**
Di x Mo	30, 138	0.1367	2.964	**0.0000**
SNK Diet		Cont> A= B= C= D= E		
SNK Month		May= Jul > Jun =Aug		
B) Growth rates acclimation period				
RM-MANOVA	**df Effect/ Error**	**Wilk's λ**	***F***	***p***
Tank (T)	10, 64	0.7927	0.7882	0.6399
Month (Mo)	4, 30	0.4387	9.5958	**0.0437**
T x Mo	20, 100	0.6146	0.7939	0.7151
SNK Time		Apr≥ Feb= Mar		

Table 3. A) Two-way ANCOVA results for differences in ingestion rates among Diets and Months using length (shell size) as a covariate. B) Two-way ANOVA results for differences among Diets and Months. Statistically significant results are indicated in **Bold**.

A) Ingestion rates				
ANCOVA	**df**	**MS**	***F***	***p***
Length (mm)	1	518.6	12.23	**0.0010**
Diet (Di)	4	2074.0	79.29	**0.0000**
Month (Mo)	3	206.7	7.90	**0.0003**
Di x Mo	9	107.2	4.10	**0.0010**
Error	36	26.16		
SNK Diet		E> D> C= B= A		
SNK Month		Jun≥ Jul= May= Aug		
B) Absorption rates				
ANOVA	**df**	**MS**	***F***	***p***
Diet (Di)	4	10042.06	1683.609	**0.0000**
Month (Mo)	3	36.83	6.174	**0.0047**
Di x Mo	12	9.27	1.554	0.1528
Error	40	5.96		
SNK Diet		C= B> A> E> D		
SNK Month		May= Jun= Jul≥ Aug		

762 **Table 4.** Friedman's ANOVA χ^2 and Kendall's coefficient of concordance (*W*) for ranked
763 ingestion rates (%) of the three microalgae species. In Wilcoxon matched pairs (WMP) post hoc
764 comparisons, significant differences between pairs of microalgae are indicated: ***p< 0.001.
765 Tetra: *T. Chuii*; T-ISO: *I. galbana*; Phaeo: *P. tricornutum*.
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N= 12, df= 2		Friedman's ANOVA χ^2 24.00		Kendall's (*W*) 1***
	Ave. rank	Sum of ranks	Mean	SD
Tetra	1	12	14.95	14.14
T-ISO	3	36	94.58	3.84
Phaeo	2	24	88.92	8.44
WMP post hoc:		T-ISO> Phaeo > Tetra		