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Flocculation of *Artemia* induced by East Asian common Octopus *octopus sinensis* paralarvae under culture conditions

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ABSTRACT

Artemia are potential food organisms for the mass culture of common octopus paralarvae but cause poor paralarval growth and mortality. To understand problems arising from *Artemia* use, we focused on *Artemia* flocculation in paralarval culture tanks; *Artemia* get caught up with each other, exhibit disrupted swimming, are deposited on the tank bottom and eventually die. To clarify whether paralarvae induce the flocculation of food organisms or not, we cultured newly hatched *Artemia* nauplii, 3-day-old metanauplii and decapod crustacean zoeae with or without paralarvae at different growth stages (weight). Flocculation occurred only when *Artemia* were cultured with paralarvae; metanauplii had a higher susceptibility for flocculation than nauplii. Flocculated *Artemia* proportion increased with increasing paralarval weight. Scanning electron microscopy revealed that flocculated metanauplii had deformed setules on their setae, with hook-shaped tips and adhesion of neighbouring tips, suggesting that flocculation may occur via a mechanism similar to the 'hook-and-loop fastener'. As octopus paralarvae exhibit external digestion, digestive enzymes secreted by paralarvae may deform *Artemia* setules and result in flocculation. As flocculation did not occur when metanauplii were cultured in water in which paralarvae were cultured and then removed, causative enzymes were probably rapidly inactivated after secretion.

1. Introduction

The East Asian common octopus *Octopus sinensis* is distributed throughout the temperate western Pacific Ocean, primarily in the coastal waters of Japan, Korea and China (Amor et al., 2017; Gleadall, 2016). This species is an important fishery resource and has a high commercial value and high growth rate; thus, it has been selected for aquaculture along with the Atlantic and Mediterranean common octopus *Octopus vulgaris* (Iglesias et al., 2007; Vaz-Pires et al., 2004). *Octopus sinensis* and *O. vulgaris* are closely related species and have similar life cycle characteristics such as a meroplanktonic stage (paralarval stage) in the first month after hatching; therefore, until recently, they were classified into one species, *O. vulgaris* (Kaneko et al., 2011; Warnkle et al., 2004). Intensive studies have been conducted to develop culture technology for these common octopus paralarvae for approximately 50 years since 1960s (Iglesias et al., 2007; Itami et al., 1963). Recently, Dan et al. (2018) demonstrated that using the upwelling culture system may improve paralarval survival of *O. sinensis* while maintaining adequate water flow in a culture tank. In addition, supplying swimming crab (*Portunus trituberculatus*) zoeae in combination with the upwelling culture system has great potential to produce benthic juvenile octopuses while achieving high survival and growth rates (Dan et al., 2019). However, in terms of mass production of juveniles for aquaculture in an industrial scenario, securing a large number of crab zoeae is still a bottleneck because it is estimated that approximately 18 million zoeae are needed to produce 10,000 benthic juvenile octopuses (Dan et al., 2019).

Artemia is a potential food for the mass culture of octopus paralarvae because sufficient *Artemia* can be obtained from commercially available cysts and the paralarvae exhibit aggressive feeding behaviour towards *Artemia* nauplii and metanauplii (Iglesias et al., 2006). Nevertheless, it has been well recognised that supplying *Artemia* as a main food source for octopus paralarvae results in poor growth and mass mortality of the paralarvae (Dan et al., 2019; Garrido et al., 2016: Iglesias et al., 2007). Many authors have revealed the lack of important nutrients in *Artemia* such as n-3 highly unsaturated fatty acids and

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amino acids, which are probably essential for common octopus paralarvae (Fuentes et al., 2011; Garrido et al., 2016; Hamasaki and Takeuchi, 2000, 2001; Hamasaki et al., 1991; Iglesias et al., 2007; Navarro and Villanueva, 2000, 2003; Reis et al., 2014; Vaciano et al., 2011; Villanueva and Bustamante, 2006; Villanueva et al., 2004, 2009). However, many attempts to supply nutritionally enriched *Artemia* have not yet achieved stable juvenile octopus production (Dan et al., 2019; Fuentes et al., 2011; Garrido et al., 2016; Iglesias et al., 2007; Navarro and Villanueva, 2000, 2003; Reis et al., 2014; Vaciano et al., 2011; Villanueva and Bustamante, 2006; Villanueva et al., 2004, 2009).

We have recently noticed that there is an additional problem in the use of *Artemia* as a food source for *O. sinensis* paralarvae during culture trials. In the culture tanks where *Artemia* and paralarvae coexisted, *Artemia* became tangled up with each other and flocculated. Since the flocculated *Artemia* were not able to swim normally, they deposited on the tank bottom and eventually died. This phenomenon could be easily observed, particularly in small-scale (< 10-L) paralarval culture tanks, because deposited *Artemia* flocks were visible on the tank bottoms. The flocculation of *Artemia* may degrade their nutritional value via disruption of their swimming and feeding. This also decreases the rate of encounters between paralarvae and *Artemia* due to deposition of the latter on the tank bottom, thus reducing the dietary efficiency of *Artemia* for paralarvae. Octopus paralarvae have been known to have a characteristic feeding behaviour which includes secreting digestive fluid from the salivary gland via a buccal mass (i.e. external digestion) (Hernández-García et al., 2000). There is a possibility that the digestive fluid of paralarvae induces *Artemia* flocculation via degradation of the exoskeleton of *Artemia*. However, very little is known regarding the cause and underlying mechanism of *Artemia* flocculation in paralarval culture tank.

The present study aims to define the problem in octopus paralarval culture using *Artemia*, in terms of the flocculation of food organisms induced by paralarvae. To evaluate the flocculation potency of paralarvae on food organisms, newly hatched *Artemia* nauplii, 3d-old *Artemia* metanauplii and decapod crustacean zoeae, which are commonly used as feeds for octopus paralarvae, were cultured with or without paralarvae at different growth stages, and the flocculation rate was examined. In addition, to clarify the effect of water-soluble residuals of digestive fluid secreted by paralarvae as a possible causative agent for flocculation, flocculation of *Artemia* was examined in water in which paralarvae had been cultured and then removed. Micromorphology of the flocculated *Artemia* were also examined using scanning electron microscopy, to give information of underlying mechanism of the flocculation.

2. Materials and methods

2.1. Broodstock and hatchlings

Four adult female *O. sinensis* with a body weight of 1562, 2080, 2092 and 2100 g were collected from local fisheries using octopus traps between May 3 and 12, 2016 in the central area of the Seto Inland Sea, off Hiroshima, Japan (34°20′N, 133°14′E). The females were reared in a cylindrical fibreglass tank (3.3 kL; diameter, 2384 mm; depth, 1000 mm) with flow-through water systems and sufficient aeration at the Research Center for Marine Invertebrates, National Research Institute of Fisheries and Environment of Inland Sea, Japan Fisheries Research and Education Agency, Onomichi, Hiroshima, Japan, according to the method described by Dan et al. (2018). They were fed frozen shrimp (*Trachysalambria curvirostris*) and clams (*Ruditapes philippinarum*) once a day. Eight shelters (entrance diameter, 120 mm; depth, 285 mm; Sunpoly Co. Ltd., Yamaguchi, Japan) were located in the tank. Two females started spawning in their shelters, on May 19 (brood A) and June 10 (brood B). They were then transferred into 500-L cylindrical polyethylene tanks with shelters and maintained individually. Paralarvae hatched on June 22, 24, 25 and 26 (brood A) and on June 26, 29 and 30 and July 1 (brood B), were used for culture in experiments 1 and 2. Water temperature and salinity during the broodstock management and egg incubation periods were 21.1 ± 2.2 °C and 32.7 ± 1.1 ppt (mean ± standard deviation), respectively. To produce flocculated *Artemia* for observation using scanning electron microscopy, paralarvae hatched on 10 November 2018 with the same procedure were used (experiment 3).

2.2. Artemia and decapod crustacean zoeae

To produce newly hatched *Artemia* nauplii and metanauplii as food for paralarval culture and to test the flocculation potency of paralarvae, *Artemia* (Utah Strain; Pacific Trading Co. Ltd., Fukuoka, Japan) were hatched in seawater at 25 °C for 24 h. Then, newly hatched *Artemia* nauplii were cultured for 3d to produce matanauplii in 100-L or 500-L polyethylene cylindrical tanks at a stocking density of three individuals mL^{-1} and at 25 − 27 °C. Culture water was not renewed during the culture period and was supplied with the microalga *Nannochloropsis oculata* (Marine Alfa; Marinetech Co. Ltd., Aichi, Japan), whose cell walls had been physically broken to aid digestibility for the *Artemia* nauplii (Dan et al., 2016), at a density of 3×10^6 cells mL^{-1} three times a day.

Natural zooplankton, including decapod crustacean zoeae, were collected with light-traps placed in two 5300 m^2 ponds (34° 22′44 N, 133°16′13 E) with a sand bottom and connected to the sea via channels and gates, at a facility at the Momoshima Laboratory of the National Research Institute of Fisheries and Environment of Inland Sea, Japan Fisheries Research and Education Agency, Onomichi, Hiroshima, Japan, according to the method described by Dan et al. (2018). The natural zooplankton were stocked daily in a 500-L polyethylene cylindrical tank. The amount of natural zooplankton collected daily was 757 ± 566 thousand individuals, and the proportion of decapod crustacean zoeae was 47.1 ± 23.3 %. The decapod crustacean zoeae were visually selected and used for experiment 1. In accordance with previous studies, mud shrimp (*Callianassa* spp.) zoeae occupied a greater part (72.5 ± 17.5 %) of the collected decapod crustacean zoeae community (Dan et al., 2018), while brachyuran crab zoeae occupied the remaining part (27.5 ± 17.5 %) (Fig. S1).

The total lengths of newly hatched *Artemia* nauplii, metanauplii, and decapod crustacean zoeae measured four times for 15 individuals during the experimental period were 579 ± 108, 1449 ± 117 and 1628 ± 559 μm, respectively.

2.3. Paralarval culture

Newly hatched paralarvae were cultured using two systems to produce the test paralarvae at different growth stages. In the culture using 1-L white plastic beakers, paralarvae were stocked at a density of 15 paralarvae L^{-1}. As food for paralarvae, natural decapod crustacean zoeae were supplied at a density of 300 individuals L^{-1}. To prevent starvation of zoeae, newly hatched *Artemia* and the microalga *N. oculata* (Marine Alfa) were supplemented once a day at a density of 1.0 individuals mL^{-1} and 2×10^6 cells mL^{-1}, respectively. The beakers were placed in a shallow-water bath, and the temperature was maintained at 25 °C with a heater. The beakers were lightly aerated to provide oxygen and turbulence to prevent the zoeae and *Artemia* from settling. The fluorescent lights were turned on from 6:30 am to 5:30 pm. Each morning, paralarvae were transferred using a large-mouthed pipette to newly prepared beakers containing fresh seawater and food, and the dead animals were removed. The paralarvae hatched from brood A on June 25 and 26 and from brood B on June 29 and 30 and July 1, were cultured until reaching 5 days after hatching (DAH), and then used for experiment 1 (Table 1).

To produce large paralarvae, they were cultured using 500-L polyethylene cylindrical tanks. Paralarvae hatched from brood A on June 22 and 24 and brood B on June 26 were stocked separately in three culture

Table 1
Brood, hatching date, types of paralarval culture tank, number of test individuals of *Octopus sinensis* paralarva, and food organisms (*Artemia* nauplii, metanauplii and natural decapod crustacean zoeae) used in experiment 1.

Test culture No.	DAH	Brood	Hatching date	Type of culture tank	Number of paralarvae tested per each food	Number of supplied food organisms per paralarva
1	0	A	25 June	–	5	60
2	0	A	26 June	–	5	60
3	0	B	29 June	–	5	60
4	0	B	30 June	–	5	60
5	0	B	1 July	–	5	60
6	5	A	25 June	1-L	3	100
7	5	A	26 June	1-L	3	100
8	5	B	29 June	1-L	3	100
9	5	B	30 June	1-L	3	100
10	5	B	1 July	1-L	3	100
11	12	A	24 June	500-L	9	100
12	12	B	26 June	500-L	6	100
13	20	A	22 June	500-L	9	100

tanks with flow-through water systems (water exchange rate: 100 % d^{-1}) at a density of 1000 individuals $tank^{-1}$. The *Artemia* metanauplii cultured for 3d (with the above-mentioned method) were supplied once a day to maintain a density of 0.5 individuals mL^{-1}, and the microalga *N. oculata* (Marine Alfa) were supplemented twice a day at a density of 2×10^6 cells mL^{-1}. The tank bottom was siphoned daily for cleaning and to remove dead animals. The cultures were terminated at 18 DAH for brood A (hatched on June 24) and brood B (hatched on June 26) and at 20 DAH for brood A (hatched on June 22), because paralarvae could not be sampled due to mortality.

2.4. Experiment 1: Effects of paralarvae on the flocculation of Artemia nauplii and metanauplii and decapod crustacean zoeae

To test flocculation potency of paralarvae on food organisms, we prepared treatment groups with different combinations of paralarvae and food types as follows; paralarvae cultured with newly hatched *Artemia* nauplii, 3d-old *Artemia* metanauplii and natural decapod crustacean zoeae. In addition, newly hatched *Artemia* nauplii, 3d-old *Artemia* metanauplii and natural decapod zoeae were cultured without paralarvae as a control treatment. The test cultures were conducted in small polystyrene dishes (diameter; 60 mm, height; 22 mm) filled with 30 mL of sterilised seawater, which was filtered through a 0.5 μm pore size membrane. The walls of the dishes were masked individually using white tape, and they were placed in a white box and covered with translucent sheets while keeping the light intensity < 800 lx using fluorescent lights turned on from 6:30 am to 5:30 pm, to reduce the stress to paralarvae and enhance their food consumption (Márquez et al., 2007). Culture water salinity was 32.0 ± 1.0 ppt. Paralarvae at 0, 5, 12 and 20 DAH were sampled from culture beakers or tanks and were then stocked in dishes individually. After 10 min of acclimation, each food organism was introduced into the test dishes. At 0 DAH, five paralarvae hatched on June 25 and 26 from brood A and on June 26 and 30 and July 1 from brood B were applied for each treatment group (5 individuals × 3 food types × 5 different hatches = total 75 individuals) (Table 1). At 5 DAH, three individuals from the same hatch with 0 DAH were used (total 45 individuals). At 12 DAH, nine paralarvae hatched on June 24 (brood A) and six paralarvae hatched on June 26 (brood B) were used (total 45 individuals). At 20 DAH, nine paralarvae hatched on June 22 from brood B were used (total 27 individuals). The amount of tested food organisms was 60 individuals-$dish^{-1}$ for paralarvae at 0 DAH, and 100 individuals for paralarvae at 5, 12 and 20 DAH, taking increase in consumption by paralarvae into account. After the 24-h test period (started from 1 pm), paralarvae were removed using a large-mouthed pipet, and then dry weight was recorded individually at 1, 6, 13 and 21 DAH, according to the method described by Dan et al. (2018). The food organisms retained in the test dishes were then counted using a Pasteur pipette. Because feeding behaviour of paralarvae was observed in the dishes, the decrease in total number of food organisms during the test culture was treated as the number of animals consumed by the paralarvae. Flocculation of the food organisms was assessed by counting the number of animals that became entangled with each other (more than two individuals) using a Pasteur pipette. Flocculation rate was calculated as the number of flocculated animals/number of total residual animals × 100.

2.5. Experiment 2: Effect of paralarval culture water on the flocculation of Artemia

To obtain seawater that might include residuals of digestive fluid of paralarvae as products of external digestion, eighteen paralarvae cultured in 500-L tanks until reaching 18 DAH were sampled and restocked in 30-mL dishes individually. Then, they were cultured for 24 h with 100 individuals of 3d-old *Artemia* metanauplii (pre-culture). After the pre-culture period, paralarvae were removed and their dry weights were recorded. Residual *Artemia* metanauplii were also removed from the dishes while determining flocculation rate and the number of animals consumed by paralarvae. One hundred recently prepared 3d-old *Artemia* metanauplii were then stocked into each one of eighteen dishes to test the flocculation potency of seawater including residual of paralarval digestible fluid. After the 24-h test period, the flocculation rate of the metanauplii was determined. As a control treatment without the possible effect of paralarval digestive fluid, 3d-old *Artemia* metanauplii were also cultured in eighteen dishes filled with fresh seawater and without paralarva for 24 h, and then counted in the same way as the treatment group.

2.6. Experiment 3: Observation of the microstructure of intact and flocculated Artemia using scanning electron microscopy

Flocculated *Artemia* metanauplii were prepared by culturing newly hatched paralarvae with 3d-old metanauplii in 30-mL dishes for approximately half an hour after which they were flocculated sufficiently. Intact metanauplii were also obtained by culturing them without paralarvae. The intact and flocculated metanauplii were sampled by using a pipette and stocked into 1.5-mL tubes. The samples in culture fluid were fixed with an equal amount of 4% paraformaldehyde (PFA) and 4% glutaraldehyde (GA) in 0.1 M cacodylate buffer pH 7.4 at incubation temperature and placed in a refrigerator in order to lower the temperature to 4 °C for 3 h. Then, they were fixed with 2% GA in 0.1 M cacodylate buffer pH 7.4 at 4 °C for 3 h. After fixing, the samples were washed four times with 0.1 M cacodylate buffer for 30 min each, followed by post fixation with 2% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer at 4 °C for 4 h. During these treatments, flocculated metanauplii were separated into individuals probably due to physical agitation in the solution.

The samples were dehydrated in graded ethanol solutions (50 %, 70 %, 90 % and 100 %). The schedule was as follows: 50 % and 70 % for 30 min each at 4 °C, 90 % for 30 min at room temperature, and four changes of 100 % for 30 min each at room temperature. After this dehydration process, the samples were continuously dehydrated with 100 % ethanol at room temperature overnight. The samples were substituted into tert-butyl alcohol at room temperature. The schedule was as follows: 50:50 mixture of ethanol and tert-butyl alcohol for 1 h, three changes of 100 % tert-butyl alcohol for 1 h each followed by being frozen at 4 °C. The frozen samples were vacuum-dried. After drying, the samples were coated with a thin layer (30 nm) of osmium by using an osmium plasma coater (NL-OPC80A, Nippon Laser & Electronics Laboratory, Nagoya, Japan).

The samples were examined with a scanning electron microscope (JSM-7500 F; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 3.0 kV. Digital images were taken directly into the computer.

2.7. Data analysis

All statistical analyses were performed using R (R3.5.3; R Core Team, 2019) with a 5% significance level. The difference in flocculation rate of each food organism in experiment 1 was assessed by a generalised linear mixed effects model (GLMM) with the binomial family (logit link) using the *glmer* function in the lme4 package (Bates, 2010; Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009), in which the flocculated or non-flocculated (dispersed) individuals at the end of the test cultures were included as the two-vector response variable, and the different food organisms (as a categorical fixed factor) and paralarval dry weight in mg were included as the explanatory variable. In this analysis, the paralarval culture method (1-L or 500-L tanks), brood (A or B) and hatching date were different among test cultures in 30-mL dishes (Table 1), therefore, the identity of the test cultures was included as a random intercept effect. Because flocculation was not observed in the groups with supplying natural decapod crustacean zoeae and control treatment (see the Results section), these groups were not included in the analyses.

To evaluate the statistical significance of the explanatory variables, the Wald Chi-square test (GLMM analysis) was performed using the *Anova* function (type II) implemented in the car package (Fox and Weisberg, 2011).

3. Results

3.1. Experiment 1: Effects of paralarvae on flocculation of Artemia nauplii, metanauplii and decapod crustacean zoeae

The mean dry weight of the paralarvae measured immediately after the test cultures were 0.40 ± 0.03 mg at 1 DAH (test cultures started at 0 DAH), 0.78 ± 0.08 mg at 6 DAH (5DAH), 1.04 ± 0.06 mg at 13 DAH (12 DAH) and 1.62 ± 0.11 mg at 21 DAH (20 DAH).

In the control treatment in which food organisms were cultured without paralarvae, flocculation was not observed for all food types. In the groups in which food organisms were cultured with paralarvae, number of food consumption by a paralarva ranged from 12.9–23.6 individuals for natural decapod crustacean zoeae, from 18.3–31.4 individuals for newly hatched *Artemia* nauplii and 10.3–21.2 individuals for 3d-old *Artemia* metanauplii (Fig. 1a). Flocculation also did not occur in the group culturing natural decapod crustacean zoeae with paralarvae at any growth stage (Fig. 1b). On the other hand, the newly hatched *Artemia* nauplii and 3d-old metanauplii flocculated when they were cultured with paralarvae at all growth stages, and the flocculation rate was significantly higher in the group culturing 3d-old metanauplii (range, 2.2–47.7%) than those with newly hatched nauplii (range, 0.7–10.5%) (χ^2 = 497.6, df = 1, $p <$ 0.0001). In these groups culturing *Artemia* nauplii and metanauplii, the flocculation rates tended to be higher with increasing paralarval dry weight (χ^2 = 16.47, df = 1, $p <$ 0.0001).

It could be observed that flocculated newly hatched *Artemia* nauplii attached each other with their swimming setae on the second antennae (Fig. 2a). On the other hand, 3d-old metanauplii attached each other not only with the setae on their second antennae but also with their thoracic limbs (Fig. 2b) (see, video in the supplementary data).

3.2. Experiment 2: Effect of paralarval culture water on flocculation of Artemia

During the pre-culture to produce seawater containing paralarval digestible fluid, the paralarvae consumed *Artemia* metanauplii in all dishes and the number of consumed metanauplii was 19.8 ± 8.9 individuals. The dry weight of paralarvae was 1.31 ± 0.27 mg. Flocculation of metanauplii were observed in all pre-culture dishes and the flocculation rate was 42.9 ± 12.2 % (Fig. 3). On the other hand, during subsequent test culture without paralarvae but with pre-culture water, only two individuals got caught each other only in one dish, and flocculation was not observed in other seventeen dishes. Thus, flocculation rate was low at 0.11 ± 0.47 %. In the control group, only three individuals of metanauplii formed a flock in one dish, and the flocculation rate was 0.28 ± 0.83 %.

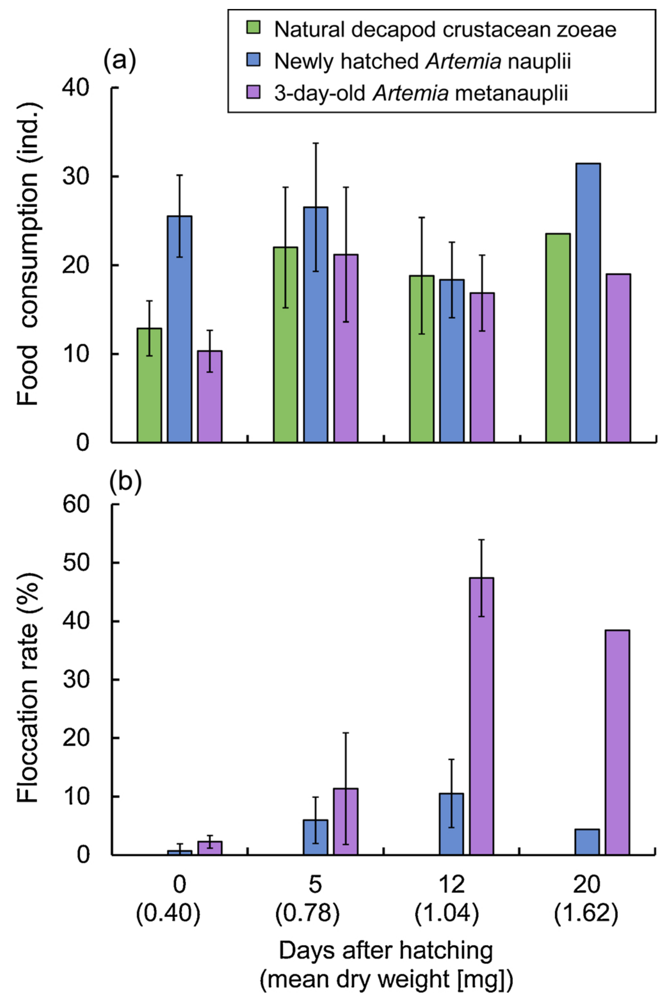

Fig. 1. Mean food consumption by a paralarva (a) and mean flocculation rate (b) of newly hatched *Artemia* nauplii, 3-day-old metanauplii and natural decapod crustacean zoeae cultured with East Asian common octopus (*Octopus sinensis*) paralarvae at different growth stages in experiment 1. Vertical bars indicate standard deviations.

3.3. Experiment 3: Observation of microstructure of intact or flocculated Artemia using scanning electron microscope

Metanauplii had plumose setae on their second antennae and thoracic limb (Fig. 4a, b), though setae on antennae had no setules on the base half of the setae (Fig. S2a, b). The setules form in one line on the top half of the setae on antennae, but in double lines on the setae of thoracic limbs (Figs. 4c, d, g, h, S2a, b). A difference in morphology was found in these plumose setae between metanauplii cultured with and without paralarvae. The setules on setae of metanauplius cultured without paralarvae were straight towards the tips, though some neighbouring setules attached each other (Fig. 4c, d). On the other hand, flocculated metanauplius cultured with paralarvae had setules with curved tips, and many setules attached to each other with

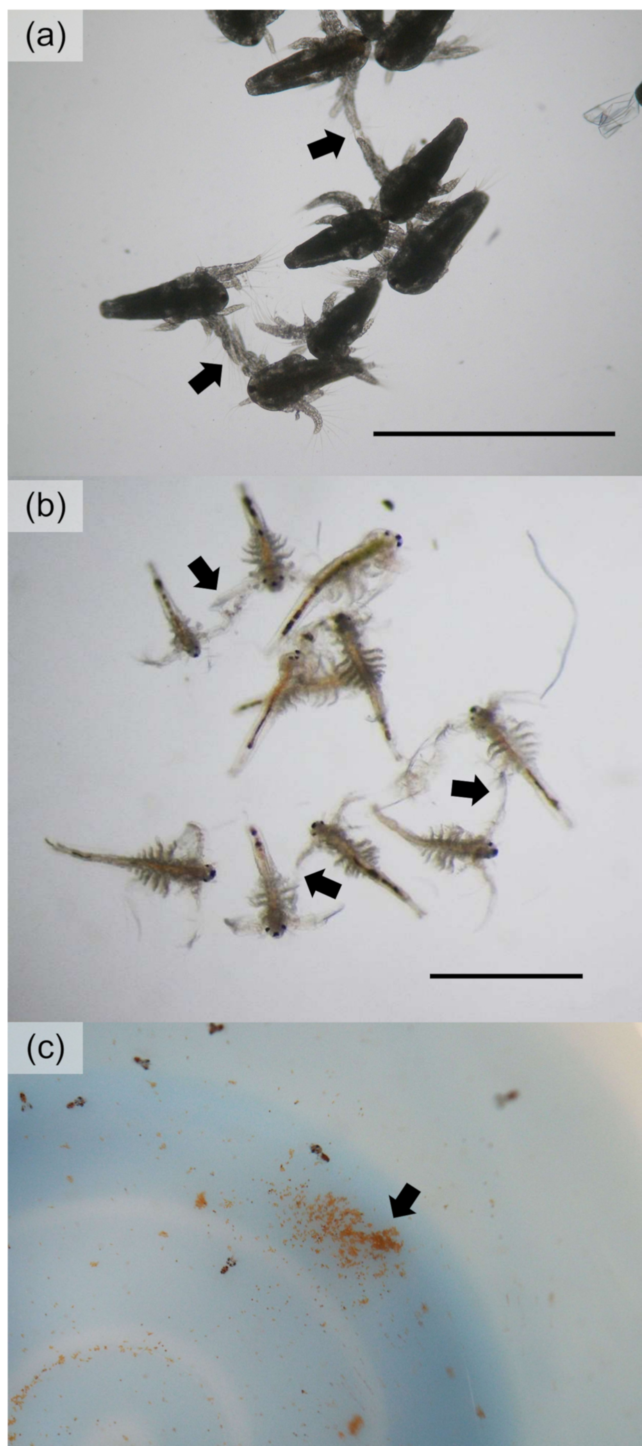

Fig. 2. Photographs of flocculated newly hatched *Artemia* nauplii (a), 3d-old metanauplii (b) and flocks of newly hatched nauplii (c) observed in culture water of East Asian common octopus (*Octopus sinensis*) paralarvae. The arrows indicate attachment of setae of the second antennae and thoracic limbs of different animals (a, b), and flocks deposited on the tank bottom (c).

neighbouring setules (Fig. 4g, h).

4. Discussion

In this study, flocculation of newly hatched *Artemia* nauplii and 3d-old metanauplii were observed when they were cultured with *O. sinensis* paralarvae, while they did not flocculate when cultured without paralarvae. In addition, coexisting paralarvae did not induce flocculation of natural decapod crustacean zoeae. This implies that the cause of the flocculation of *Artemia* is attributed to the coexisting paralarvae, and *Artemia* have greater susceptibility to flocculation than decapod crustacean zoeae. During the paralarval culture trials, it could be rarely observed the flocculation of decapod zoeae such as swimming crab *Portunus trituberculatus* zoeae and the mud shrimp *Callianassa* spp. zoeae (Dan et al., 2018, 2019; Dan, personal communication). Thus, flocculation may be a specific phenomenon for *Artemia*. It has been well known that *Artemia* nauplii and metanauplii have less dietary effect on paralarval growth and survival than decapod zoeae (Dan et al., 2019; Fuentes et al., 2011; Garrido et al., 2016; Iglesias et al., 2007; Navarro and Villanueva, 2000, 2003; Reis et al., 2014; Vaciano et al., 2011; Villanueva and Bustamante, 2006; Villanueva et al., 2004, 2009). Since the flocculation disrupts swimming of individual *Artemia*, induces deposition on the tank bottom and results in death (Fig. 2c), flocculation is likely to be one of the causes of poor dietary value of *Artemia* for culturing paralarvae.

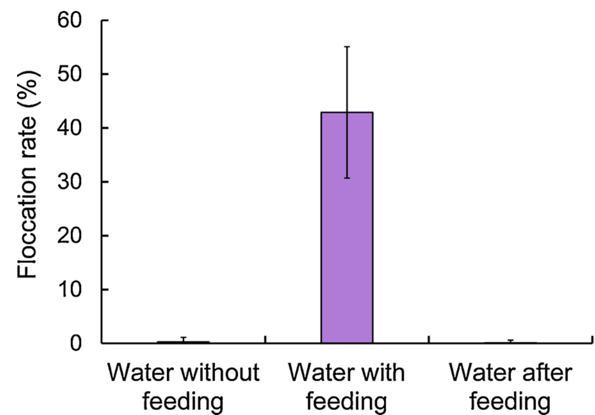

Fig. 3. Mean flocculation rate of 3d-old *Artemia* metanauplii cultured in water without East Asian common octopus (*Octopus sinensis*) paralarvae (without feeding), water with paralarvae (feeding) or water that had cultured paralarvae (after feeding). Vertical bars indicate standard deviations.

Why do *Artemia* get entangled with each other? Microstructural observation using the scanning electron microscope clarified that setules on setae of antennae and thoracic limbs of the flocculated metanauplius were deformed. Because *Artemia* flocculation occurred by attaching their setae of antennae and thoracic limbs, it can be inferred that deformity of setules on setae has close relationships with the flocculation of *Artemia*. Deformed setules had characteristic hook-like curved tips and adhesion of the several neighbouring setules tips formed enclosed spaces between the setules. It is generally known that, when tiny hooks and small loops (enclosed spaces) touched together, they are locked with each other with intertwining. This phenomenon is widely applicated by humans to adhere different things together, known as 'hook-and-loop fastener' or 'VELCRO®' inspired by morphology of epizoochorous seeds of plants (Römermann et al., 2005). Thus, it is likely that the hook-and-loop fastening of the deformed setules on setae is a cause of the flocculation of *Artemia*.

In experiment 1, 3d-old *Artemia* metanauplii had greater susceptibility to flocculation compared with newly hatched nauplii. *Artemia* have one pair of the second antennae during nauplius to facilitate swimming, and then they develop limbs on their thoracic trunks having plumose setae to reinforce propulsion for filter feeding and gas change as they growth (Williams, 1994, 2007). Indeed, four pairs of thoracic limbs with plumose setae could be observed on the thoracic trunk of metanauplii by electron microscopy. Therefore, 3d-old metanauplius had broader region, at which deformed setules potentially cause flocculation, than newly hatched nauplius. Although decapod crustacean zoeae of *Thalassinidea* and Brachyura also have plumose natatory setae

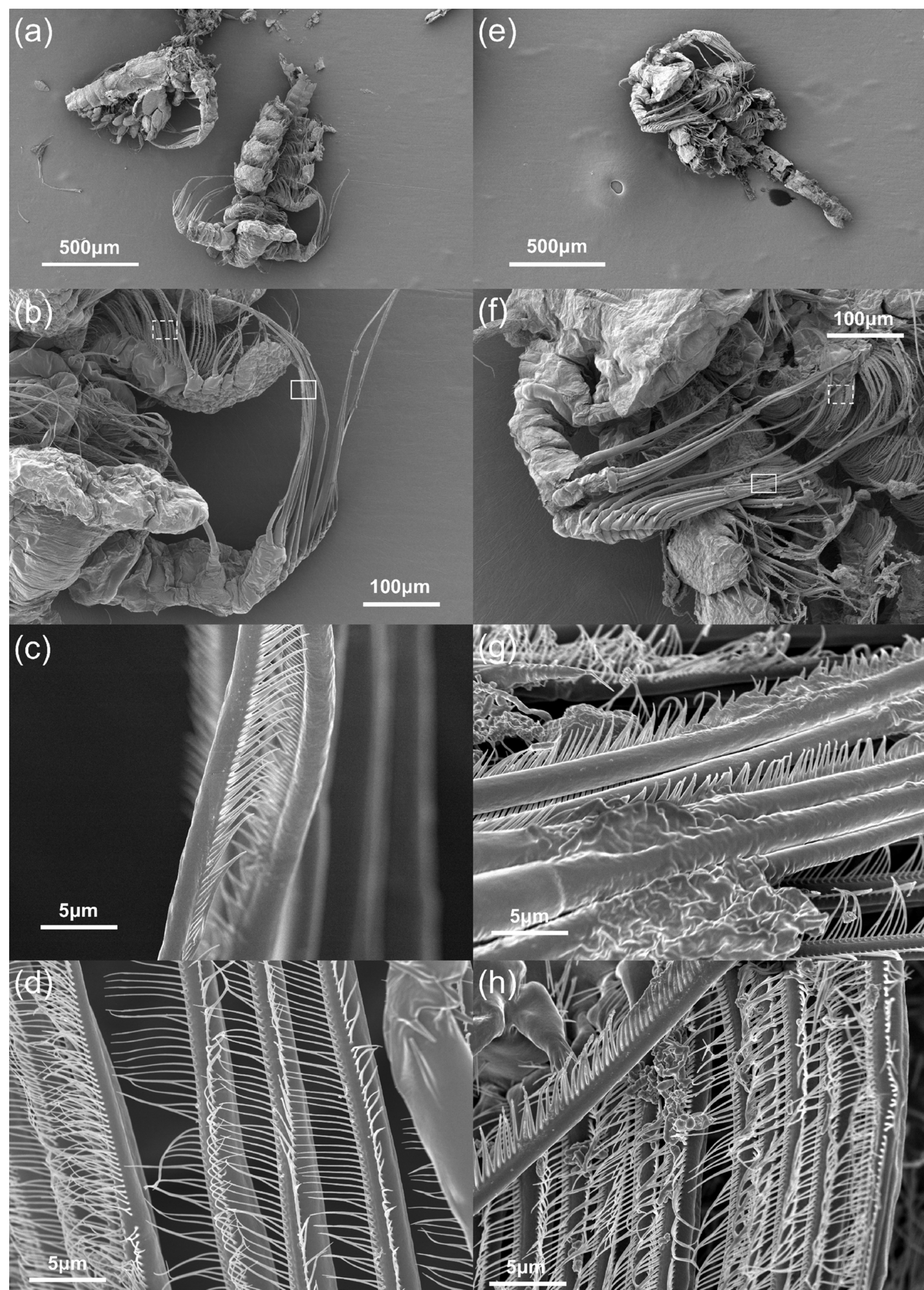

Fig. 4. Scanning electron microscopic photographs of intact (a − d) and flocculated (e − h) 3d-old *Artemia* metanauplii. Solid-line squares in (b) and (f) represent regions of setae of second antennae magnified in (c) and (f). Broken-line squares represent regions of setae of thoracic limbs magnified in (d) and (h).

with long setules on maxillipeds (Anger, 2001; Pohle and Telford, 1981), *Artemia* nauplii and metanauplii had dense short setules, which might be compatible for hook-and-loop fastening, on their setae of antennae and/or thoracic limbs (Figs. 4, S2). In addition, the zoeae have thicker exoskeleton than *Artemia* (Iglesias et al., 2006). These differences in morphology between zoea, *Artemia* nauplius and metanauplius are likely to be reasons for the difference in susceptibility to flocculation between the food organisms.

Paralarvae of *O. vulgaris* exhibit characteristic feeding behaviour that is a similar ingestion process with their juvenile and adult forms (Hernández-García et al., 2000; Nixon, 1984; Nixon and Mangold, 1969). Once they catch the decapod crustacean zoeae, they penetrate the zoeal exoskeletons using a beak and secrete mixture of digestive fluid from salivary grand and inject into the zoeal body (Hernández-García et al., 2000; Nande et al., 2017). Then, the paralarvae ingest the dissolved edible content and they discard the empty exoskeletons of zoea (i.e. external digestion). To perform external digestion, the *O. vulgaris* paralarvae can produce mixture of digestive enzymes such as esterase, acid phosphatase, protease and acetyl-glycosaminidase in the posterior salivary grand (Boucaud-Camou and Roper, 1995). On the other hand, when *O. vulgaris* paralarvae feed on *Artemia*, the paralarvae exhibit internal ingestion, as pieces of *Artemia* appendages were found in paralarval digestive tract (Iglesias et al., 2006). Iglesias et al. (2006) hypothesised that a thinner exoskeleton of *Artemia* is a reason of the differences in ingestion process between zoeae and *Artemia*. Taking these findings in to account, a possible cause of flocculation of *Artemia* is that the thinner exoskeletons of *Artemia* may have high susceptibility to enzymes secreted by paralarvae, particularly in delicate

microstructure of the setules. Thus, the crustacean chitin structure of *Artemia* setules was deformed in association with paralarval feeding behaviour. Although the deformity in exoskeleton microstructure of decapod crustacean zoeae was not assessed microscopy in the present study, there is a possibility that digestive enzyme of paralarvae has an ability to denature the zoeal exoskeletons as a function to help penetration by a beak, as suggested by Hernández-García et al. (2000). This hypothesis deserves further investigation, because it might relate to the feeding strategy of octopus paralarvae.

In experiment 2, the water that might include residuals of digestive fluid secreted by paralarvae did not induce flocculation of *Artemia*. Taking the fact that paralarvae had induced flocculation of *Artemia* during the pre-culture period into consideration, it can be inferred that the enzymes were inactivated in a short time after secretion. This implies that the causative matter of flocculation does not accumulate in the paralarval culture water, and thus, the concentration may depend primarily on secretion amount of the digestive fluid by paralarvae in the culture tank, i.e. paralarval body size, stocking density of paralarvae and frequency of feeding behaviour. Indeed, larger (heavier) paralarvae had greater potency of flocculation than smaller paralarvae in experiment 1. To control the flocculation of *Artemia*, lower stocking density of paralarvae seems to be better; lower *Artemia* supply density may be also effective to reduce encounter opportunity of *Artemia* together. Further study is needed to develop the technology of feeding method for *Artemia* by octopus paralarvae while preventing flocculation of *Artemia*.

5. Conclusions

The *O. sinensis* paralarvae may have potential to induce flocculation of coexisting *Artemia* via deformation of the setules and thus reducing dietary value of *Artemia* by themselves. Flocculation of *Artemia* was likely caused by hook-and-loop fastening of the deformed setules on setae, and metanauplii having developed trunk limbs with many setules had greater susceptibility to flocculation compared with newly hatched nauplii. Because potency of *Artemia* flocculation depended on size of *O. sinensis* paralarvae and causative matter did not accumulate in the culture water, lower culture stocking density of paralarvae is recommended to reduce the flocculation of *Artemia*.

Data availability statement

The dataset generated during the current study is available in the Mendeley Data repository (http://doi.org/10.17632/x8gr5wv2kv.3).

Ethics statement

This study has been approved by the committee on the ethics of animal experiments at the National Research Institute of Fisheries and Environment of Inland Sea, Japan Fisheries Research and Education Agency (permission number: 2016 − 11).

Author statement

Shigeki Dan: Conceptualization, Methodology, Writing - Original draft preparation. **Arata Takasugi**: Investigation. Data curation, Visualization, **Hiraku Iwasaki**: Investigation. **Shodai Shibasaki**: Investigation. **Kazuhiro Yamashita**: Investigation. **Katsuyuki Hamasaki**: Supervision, Writing- Reviewing and Editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100330.

References

Amor, M.D., Norman, M.D., Roura, A., Leite, T.S., Gleadall, I.G., Reid, A., Perales-Raya, C., Lu, C.C., Silvey, C.J., Vidal, E.A.G., Hochberg, F.G., Zheng, X., Strugnell, J.M., 2017. Morphological assessment of the *Octopus vulgaris* species complex evaluated in light of molecular-based phylogenetic inferences. Zool. Scr. 46, 275–288. https://doi.org/10.1111/zsc.12207.

Anger, K., 2001. Morphology. In: In: Vonk, R. (Ed.), The Biology of Decapod Crustacean Larvae. Crustacean Issues, vol. 14. A. A. Balkema, the Netherlands, pp. 13–40.

Bates, D.M., 2010. lme4: Mixed-effect Modelling With R. Springer, Berlin. http://lme4.r-forge.r-project.org/book/.

Boucaud-Camou, E., Roper, C.F., 1995. Digestive enzymes in paralarval cephalopods. Bull. Mar. Sci. 57, 313–327.

Dan, S., Oshiro, M., Ashidate, M., Hamasaki, K., 2016. Starvation of *Artemia* in larval rearing water affects post-larval survival and morphology of the swimming crab, *Portunus trituberculatus* (Brachyura, Portunidae). Aquaculture 452, 407–415. https://doi.org/10.1016/j.aquaculture.2015.06.004.

Dan, S., Iwasaki, H., Takasugi, A., Yamazaki, H., Hamasaki, K., 2018. An upwelling system for culturing common octopus paralarvae and its combined effect with supplying natural zooplankton on paralarval survival and growth. Aquaculture 495, 98–105. https://doi.org/10.1016/j.aquaculture.2018.05.036.

Dan, S., Iwasaki, H., Takasugi, A., Shibasaki, S., Yamazaki, H., Oka, M., Hamasaki, K., 2019. Effects of co-supply ratios of swimming crab *Portunus trituberculatus* zoeae and *Artemia* on survival and growth of East Asian common octopus *Octopus sinensis* paralarvae under an upwelling culture system. Aquaculture 50, 1361–1370. https://doi.org/10.1111/are.14013.

Everitt, B.S., 2005. An R and S-Plus Companion to Multivariate Analysis. Springer-Verlag, London.

Everitt, B.S., Hothorn, T., 2009. A Handbook of Statistical Analyses Using R. CRC Press, New York.

Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression, second ed. Sage Publications, California.

Fuentes, L., Sánchez, F.J., Lago, M.J., Iglesias, J., Pazos, G., Linares, F., 2011. Growth and survival of *Octopus vulgaris* (Cuvier 1797) paralarvae fed on three *Artemia*-based diets complemented with frozen fish flakes, crushed zooplankton and marine microalgae. Sci. Mar. 75, 771–777. https://doi.org/10.3989/scimar.2011.75n4771.

Garrido, D., Martin, V.M., Rodríguez, C., Iglesias, J., Navarro, J.C., Estévez, A., Hontoria, F., Becerro, M., Otero, J.J., Pérez, J., Varó, I., Reis, D., Riera, R., Sykes, A.V., Almansa, E., 2016. Meta-analysis approach to the effects of live prey on the growth of *Octopus vulgaris* paralarvae under culture conditions. Rev. Aquacult. 10, 3–14. https://doi.org/10.1111/raq.12142.

Gleadall, I.G., 2016. *Octopus sinensis* d'Orbigny, 1841 (Cephalopoda: octopodidae): valid species name for the commercially valuable East Asian common octopus. Species Divers. 21, 31–42. https://doi.org/10.12782/sd.21.1.031.

Hamasaki, K., Takeuchi, T., 2000. Effects of the addition of *Nannochloropsis* to the rearing water on survival and growth of planktonic larvae of *Octopus vulgaris*. Saibai Giken 28, 13–16 in Japanese.

Hamasaki, K., Takeuchi, T., 2001. Dietary value of *Artemia* enriched with ω-Yeast or shark eggs as feed for planktonic larvae of *Octopus vulgaris*. Saibai Giken 28, 65–68 in Japanese.

Hamasaki, K., Fukunaga, K., Yoshida, Y., Maruyama, K., 1991. Effect of marine microalgae *Nannochloropsis* sp. on survival and growth on rearing pelagic paralarvae of *Octopus vulgaris*, and results of mass culture in the tank of 20 m^3. Saibai Giken 19,

75–84 in Japanese.
Hernández-García, V., Martín, A.Y., Castro, J., 2000. Evidence of external digestion of crustacean in *Octopus vulgaris* paralarvae. J. Mar. Biol. Assoc. U.K. 80, 559–560. https://doi.org/10.1017/S0025315400002320.
Iglesias, J., Fuentes, L., Sánchez, F.J., Otero, J.J., Moxica, C., Lago, M.J., 2006. First feeding of *Octopus vulgaris* Cuvier, 1797 paralarvae using *Artemia*: effect of prey size, prey density and feeding frequency. Aquaculture 261, 817–822. https://doi.org/10.1016/j.aquaculture.2006.08.002.
Iglesias, J., Sánchez, F.J., Bersano, J.G.F., Carrasco, J.F., Dhont, J., Fuentes, L., Linares, F., Muñoz, J.L., Okumura, S., Roo, J., van der Meeren, T., Vidal, E.A.G., Villanueva, R., 2007. Rearing of *Octopus vulgaris* paralarvae: present status, bottlenecks and trends. Aquaculture 266, 1–15. https://doi.org/10.1016/j.aquaculture.2007.02.019.
Itami, K., Izawa, Y., Maeda, S., Nakai, K., 1963. Note on the laboratory culture of the octopus larvae. Bulletin of the Japanese Society of Scientific Fisheries 29, 514–520 in Japanese with English abstract.
Kaneko, N., Kubodera, T., Iguchi, A., 2011. Taxonomic study of shallow-water octopuses (Cephalopoda: Octopodidae) in Japan and adjacent waters using mitochondrial genes with perspectives on octopus DNA barcoding. Malacologia 54, 97–108. https://doi.org/10.4002/040.054.0102.
Márquez, L., Quintana, D., Almansa, E., Navas, J.I., 2007. Effect of visual conditions and prey density on feeding kinetics of paralarvae of *Octopus vulgaris* from a laboratory spawning. J. Molluscan Stud. 73, 117–121. https://doi.org/10.1093/mollus/eym003.
Nande, M., Presa, P., Roura, Á., Andrews, P.L.R., Pérez, M., 2017. Prey capture, ingestion, and digestion dynamics of *Octopus vulgaris* paralarvae fed live zooplankton. Front. Physiol. 8, 573. https://doi.org/10.3389/fphys.2017.00573.
Navarro, J.C., Villanueva, R., 2000. Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture 183, 161–177. https://doi.org/10.1016/S0044-8486(99)00290-2.
Navarro, J.C., Villanueva, R., 2003. The fatty acid composition of *Octopus vulgaris* paralarvae reared with live and inert food: deviation from their natural fatty acid profile. Aquaculture 219, 613–631. https://doi.org/10.1016/S0044-8486(02)00311-3.
Nixon, M., 1984. Is there external digestion by *Octopus*? J. Zool. 202, 441–447. https://doi.org/10.1111/j.1469-7998.1984.tb05094.x.
Nixon, M., Mangold, K., 1969. The early life of *Octopus vulgaris* (Cephalopoda: octopodidae) in the plankton and at settlement: a change in lifestyle. J. Zool. 239, 301–327. https://doi.org/10.1111/j.1469-7998.1996.tb05453.x.
Pohle, G., Telford, M., 1981. Morphology and classification of decapod crustacean larval setae: a scanning electron microscope study of *Dissodactylus crinitichelis* Moreira, 1901 (Brachyura: pinnotheridae). Bull. Mar. Sci. 31, 736–752.
R Core Team, 2019. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/.
Reis, D.B., García-Herrero, I., Reira, R., Felipe, B.C., Rodríguez, C., Sykes, A.V., Martín, M.V., Andrade, J.P., Almansa, E., 2014. An insight on *Octopus vulgaris* paralarvae lipid requirements under rearing conditions. Aquac. Nutr. 21, 797–806. https://doi.org/10.1111/anu.12205.
Römermann, C., Tackenberg, O., Poschlod, P., 2005. How to predict attachment potential of seeds to sheep and cattle coat from simple morphological seed traits. Oikos 110, 219–230. https://doi.org/10.1111/j.0030-1299.2005.13911.x.
Vaciano, E., Iglesias, J., Lago, M.J., Sánchez, F.J., Otero, J.J., Navarro, J.C., 2011. Fatty acid composition of polar and neutral lipid fractions of *Octopus vulgaris* Cuvier, 1797 paralarvae reared with enriched on-grown *Artemia*. Aquac. Res. 42, 704–709. https://doi.org/10.1111/j.1365-2109.2010.02605.x.
Vaz-Pires, P., Seixas, P., Barbosa, A., 2004. Aquaculture potential of the common octopus (*Octopus vulgaris* Cuvier, 1797): a review. Aquaculture 238, 221–238. https://doi.org/10.1016/j.aquaculture.2004.05.018.
Villanueva, R., Bustamante, P., 2006. Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. Aquaculture 261, 225–240. https://doi.org/10.1016/j.aquaculture.2006.07.006.
Villanueva, R., Riba, J., Ruíz-Capillas, C., González, A.V., Baeta, M., 2004. Amino acid composition of early stages of cephalopods and effect of amino acid dietary treatments on *Octopus vulgaris* paralarvae. Aquaculture 242, 455–478. https://doi.org/10.1016/j.aquaculture.2004.04.006.
Villanueva, R., Escudero, J.M., Deulofeu, R., Bozzano, A., Casoliva, C., 2009. Vitamin A and E content in early stages of cephalopods and their dietary effects in *Octopus vulgaris* paralarvae. Aquaculture 286, 277–282. https://doi.org/10.1016/j.aquaculture.2008.09.032.
Warnkle, K., Söller, R., Blohm, D., Saint-Paul, U., 2004. A new look at geographic and phylogenetic relationships within the species group surrounding *Octopus vulgaris* (Mollusca, Cephalopoda): indications of very wide distribution from mitochondrial DNA sequences. J. Zoolog. Syst. Evol. Res. 42, 306–312. https://doi.org/10.1111/j.1439-0469.2004.00277.x.
Williams, T.A., 1994. Locomotion in developing *Artemia* larvae: mechanical analysis of antennal propulsors based on large-scale physical models. Biol. Bull. 187, 156–163. https://doi.org/10.2307/1542238.
Williams, T.A., 2007. Structure and development of setae on the thoracic limbs of the anostracan crustacean, *Thamnocephalus platyurus*. Arthropod Struct. Dev. 36, 63–76. https://doi.org/10.1016/j.asd.2006.08.008.
Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M., 2009. Mixed Effects Models and Extensions in Ecology With R. Springer Publishing. New York. .