Invertebrate Biology

Invertebrate Biology 137(1): 66–77.
© 2018, The American Microscopical Society, Inc.
DOI: 10.1111/ivb.12206

Effect of multiple spawning on female reproductive output and offspring quality in a freshwater caridean shrimp with direct development

Agustina Marciano, Carolina Tropea, and Laura S. López Greco[a]

Facultad de Ciencias Exactas y Naturales, Departamento de Biodiversidad y Biología Experimental, Laboratorio de Biología de la Reproducción y el Crecimiento de Crustáceos Decápodos, Instituto de Biodiversidad y Biología Experimental y Aplicada (IBBEA), Universidad de Buenos Aires, CONICET, C1428EGA Buenos Aires, Argentina

Abstract. The decline with age in components of fitness is variable among different taxa and includes changes in fertility and brood quality. In this study, we selected individuals of *Neocaridina davidi*, a freshwater shrimp with direct development, to analyze juvenile quality and female reproductive performance over successive spawnings, both of which are correlated with female age. Given the high costs of reproduction in species with direct development, we hypothesized that female reproductive performance and juvenile quality decrease in later spawns. Two experiments were performed. In Experiment 1, we evaluated the reproductive performance of females of *N. davidi* and the quality of juveniles (through a food restriction test) over the first six successive spawnings. In Experiment 2, we analyzed the lipid and protein contents in juveniles from the third, fourth, and fifth spawns, after feeding them daily or starving them for 8 d or 12 d after hatching. Female mortality was observed throughout Experiment 1, along with a decrease in the proportion of ovigerous females over successive spawns. However, the interval between spawnings and the number and size of newly hatched juveniles were similar among spawns. Moreover, females that spawned many times had a reproductive efficiency similar to those that spawned few times, as evidenced by a similar percentage of broods successfully hatched and a similar percentage of broods with more than 28 juveniles among all spawns. Overall, these results may indicate a partial effect of multiple spawning on female reproductive performance. Growth, survival, and biochemical composition of food-restricted juveniles showed similar or even higher values in later spawns as compared to the first spawns. This is, to our knowledge, the first empirical demonstration in a decapod crustacean with direct development that, although the percentage of ovigerous females decreases over time, other reproductive variables and juvenile performance do not decline in successive spawnings, at least for the initial six consecutive spawns.

Additional key words: successive spawnings, juvenile quality, food restriction, biochemical composition, *Neocaridina davidi*

Reproductive output is one of the central attributes of life history, and knowledge of age-specific reproduction may enhance the understanding of population performance and dynamics (Tsujimoto et al. 2016). The decline in fitness components with age is highly variable among different groups of organisms and includes changes in fertility and brood quality (Jones et al. 2014). In some crabs and penaeid shrimps, certain variables related to offspring quality, such as fecundity, hatching rate, size, and larval resistance to starvation, decrease over successive spawnings, which correlate with female age (Wouters et al. 1999; Kobayashi 2001; Coman & Crocos 2003; Ji et al. 2006; Nan et al. 2006; Darnell et al. 2009; Andrés et al. 2010; Wu et al. 2010; Verísimo et al. 2011).

The exposure of offspring to a physiologically stressful situation, such as physical manipulation, starvation, or changes in temperature or salinity, is a commonly used tool to evaluate offspring quality in crustaceans (Racotta et al. 2003; Smith et al. 2004). Resistance of larvae and juveniles to temporary starvation is a key factor for their successful

[a]Author for correspondence.
E-mail: laura@bg.fcen.uba.ar

development in nature, especially in freshwater habitats (Anger 2001), and depends directly on the energy budget transferred by females to the oocytes during vitellogenesis (Gardner 2001). Reserve allocation for embryonic development is particularly important in decapods with direct development due to the absence of free-living larval stages (Herring 1974), enabling maternal influence on progeny to be greater and more direct as compared to crustaceans with indirect development. Moreover, the impact of energetic investment in brood production may be more relevant in multiple-spawning species, potentially leading to female reproductive exhaustion and, consequently, different brood quality over successive spawnings (Marsden et al. 1997; Wouters et al. 1999). In this context, it is surprising to find little information about the effect of multiple spawning on reproduction of freshwater decapods with direct development.

Neocaridina davidi (Bouvier 1904), or "red cherry shrimp" (Caridea, Atyidae), is an excellent biological model in which to address effects of multiple spawning. These shrimps are easily maintained in the laboratory and have a short life cycle: Under laboratory conditions, juveniles hatch after an incubation period of 15 d and reach sexual maturity at an approximate weight of 50 mg, attained from day 50 of life, depending on stocking density. Females spawn repeatedly during a life span of 1–2 years, with short intervals between spawnings (Tropea & López Greco 2015; Tropea et al. 2015), which allowed us to analyze juvenile quality and female reproductive performance over successive spawnings. Given the high costs of reproduction in species with direct development, we hypothesized that female reproductive performance and juvenile quality, evaluated in terms of resistance to food restriction, decrease in later spawns.

Methods

Experimental rationale

Two experiments were performed. In Experiment 1, we evaluated the reproductive performance of females and quality (through a food restriction test) of juveniles of *N. davidi* over the first six successive spawnings, during a 210-d experimental period (May to November 2014). In Experiment 2, we analyzed the biochemical composition of juveniles from the third, fourth, and fifth spawns, after exposing them to certain food restriction treatments, over 280 d (June 2015 to April 2016).

Experimental Animals

The shrimps used in both experiments were obtained from a reproductive stock provided by a commercial supplier (Acuamanus Aquarium, Buenos Aires, Argentina). The stock was maintained in plastic aquaria of 31 × 24 × 19 cm, containing 10 L of dechlorinated tap water (pH 7.2–7.4), under continuous aeration and a constant temperature of 27°C±1°C. The photoperiod was 14-h light: 10-h dark. Java moss (*Vesicularia sp.*) was provided for shelter. Shrimps were fed daily *ad libitum* a balanced food for tropical fish (Tetradiskus, TETRA®), with the following approximate composition: minimum crude protein 47.5%, minimum crude fat 6.5%, maximum crude fiber 2.0%, maximum moisture 6.0%, minimum phosphorus 1.5%, and minimum ascorbic acid 100 mg kg^{-1}. Water was completely replaced once a week. These rearing conditions were based on Tropea et al. (2015) and Tropea & López Greco (2015). Juveniles obtained from the reproductive stock were used in Experiments 1 and 2 after reaching sexual maturity, when females showed visible ovaries through the cephalothorax and became larger and more intensely colored than males.

Experimental design

General considerations for both experiments. Each mature female was placed in a plastic aquarium of 18 × 12.5 × 12 cm with two mature males, to increase mating probability. They were maintained under the same conditions of water quality, photoperiod, temperature, and feeding as described above. Each aquarium was a replicate. Aquaria were visually searched daily for ovigerous females. The total number of newly hatched juveniles (or first-stage juveniles, JI) was counted for each spawn, and broods with more than 28 JI were selected for further study. A sample of 10 JI was taken from each of these broods and weighed after blot-drying the sample with absorbent paper for removal of excess water. The JI wet weight (initial wet weight) was calculated to the nearest 0.1 mg by dividing the sample weight by the number of JI. Then, a stereomicroscope was used to measure the cephalothorax length (initial cephalothorax length) from the tip of the rostrum to the posterior end of the cephalothorax of all JI from the sample. A mean value of the initial cephalothorax length was then calculated for each sample. The JI were counted, weighed, and measured as described above for each successive spawning of experimental females.

Another sample of 18 JI was then taken to evaluate juvenile quality through a food restriction test, which lasted 32 d. Each JI was placed in a 250-cm^3 plastic container with a piece of onion bag mesh (1 × 1 cm), and a small stone (0.5 × 0.2 × 1 cm) that juveniles used as substrate and shelter. Water was under continuous aeration, at a temperature of 27°C±1°C, and was completely replaced once a week. Juveniles were fed according to restriction treatments that consisted of starvation periods of increasing duration. These treatments are described in more detail for the two experiments (see below). Juveniles were checked daily for mortality. This general experimental design was applied in both Experiments 1 and 2, with specific details described in the following sections.

Experiment 1: female reproductive performance and juvenile quality. A total of 19 females (mean weight 56.7±9.8 mg [41.3–74.4 mg]) and 38 males (mean weight 32.9±4.0 mg [28.3–48.2 mg]), previously obtained from the reproductive stock, were randomly selected for Experiment 1. They were stocked and maintained as described in the general experimental design for the evaluation of female reproductive performance, with one female and two males in each aquarium. Each aquarium was a replicate, and 19 replicates were used.

The quality of juveniles from the first six successive spawnings of experimental females was evaluated through a food restriction test that included the following treatments: DF (daily feeding during the 32-d period), S4 (food deprivation for 4 d after hatching), S8 (food deprivation for 8 d after hatching), S12 (food deprivation for 12 d after hatching), S16 (food deprivation for 16 d after hatching), and CS (continuous starvation during the 32-d period). Food deprivation for 16 d coincides with the point of no return of 50% (PNR_{50}) for juveniles of *N. davidi* (Pantaleão et al. 2015). Eighteen JI were selected from each brood and were equally and randomly assigned to these food restriction treatments (three JI per treatment). After the corresponding starvation days, juveniles were fed daily *ad libitum* Tetradiskus, TETRA®, until day 32 (Fig. 1A). At that time, we measured final wet weight to the nearest 0.1 mg and final cephalothorax length in order to calculate the percentage of mass increase (%MI) and length increase (%LI), respectively.

Experiment 2: juvenile quality. Thirty females and 60 males previously obtained from the reproductive stock were selected for Experiment 2, with a mean weight of 58.7±9.3 mg (43.5–79.2 mg) and 36.9±6.1 mg (28.5–50.3 mg), respectively. They were stocked and maintained as described in the general experimental design to obtain juveniles from later spawns. Each aquarium was a replicate, and 30 replicates were used.

The quality of juveniles from the third, fourth, and fifth spawnings of experimental females was evaluated through a food restriction test that included the following treatments: DF (daily feeding during the 32-d period), S8 (food deprivation for 8 d after

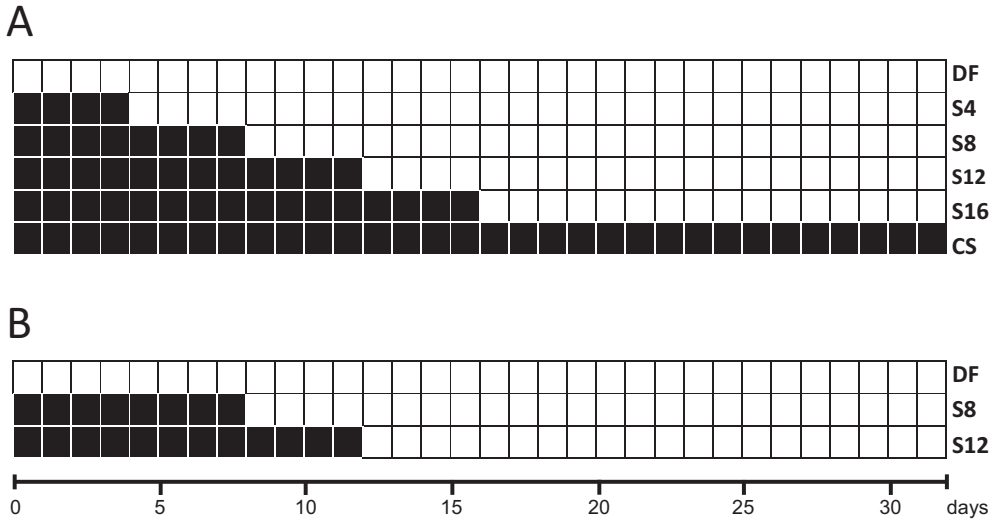

Fig. 1. Scheme of the treatments applied to newly hatched juveniles of *Neocaridina davidi* in the food restriction test. **A.** Experiment 1: DF, daily feeding during a 32-d period; CS, continuous starvation during a 32-d period; S4, food deprivation for 4 d after hatching; S8, food deprivation for 8 d after hatching; S12, food deprivation for 12 d after hatching; and S16, food deprivation for 16 d after hatching. **B.** Experiment 2: DF, daily feeding during the 32-d period; S8, food deprivation for 8 d after hatching; and S12, food deprivation for 12 d after hatching. Black cells represent the starvation days and white cells the feeding days.

hatching), and S12 (food deprivation for 12 d after hatching). Eighteen JI were selected from each brood and were equally and randomly assigned to these food restriction treatments (six JI per treatment). After the corresponding starvation days, juveniles were fed daily *ad libitum* Tetradiskus, TETRA®, until day 32 (Fig. 1B). At that time, we measured wet weight to the nearest 0.1 mg and calculated the % MI. Juveniles were then sacrificed after being cold-anesthetized at −20°C for 15 min and stored at −70°C for biochemical analyses.

Protein and lipid concentrations (expressed in μg mg^{-1}) were determined spectrophotometrically in homogenates of 32-day-old juveniles, according to the method described by Folch et al. (1957), which was modified by Frings & Dunn (1970), and according to the method described by Bradford (1976). Because single juveniles were too small to be analyzed individually, we divided each spawn within each food restriction treatment into four replicate groups per spawn number (3, 4, and 5) and food restriction treatment (DF, S8, and S12). In all cases, calculations were performed on a wet-weight basis.

To determine protein concentrations, samples weighing 11–29 mg were homogenized in 4:1 volume (μL): weight (mg) of 50 mmol L^{-1} Tris-HCl buffer (pH 7.5) and then centrifuged at 10,000 *g* for 30 min in a refrigerated centrifuge (4°C). Supernatants were diluted 1:8 (volume: volume) with distilled water. Total proteins were quantified by the Coomassie Blue dye method. Bovine serum albumin was used as standard (1 mg mL^{-1}), and the absorbance was read at 595 nm. For lipid determination, samples weighing 16–256 mg were homogenized in 20:1 volume (μL): weight (mg) of a mixture of chloroform and methanol (2:1 volume: volume), then mixed and centrifuged with 0.9% NaCl to separate the lipid fraction. Supernatants were discarded, and the final volume of chloroform–methanol in each tube was recorded. Aliquots of 10–40 μL were boiled for 10 min, after the addition of 1 mL of H_2SO_4. Total lipids were quantified by the sulfo-phospho-vanillin method. Extra-virgin olive oil (lipid concentration: 920 mg mL^{-1}) diluted in absolute ethanol (final lipid concentration: 1 mg mL^{-1}) was used as standard, and the absorbance was read at 530 nm.

Statistical analyses. The following variables were statistically compared among successive spawnings to evaluate female reproductive performance (Experiment 1): total number of JI per spawn per female; interval between spawnings (number of days between consecutive spawning events for females that spawned six or more times); and initial cephalothorax length of JI. In addition, female reproductive performance was qualitatively characterized over successive spawnings through the following variables: percentage of ovigerous females ([number of ovigerous females/total number of females]×100); percentage of broods successfully hatched; and percentage of broods with more than 28 juveniles.

The following variables were statistically compared among successive spawnings to evaluate juvenile quality at the end of the food restriction test (Experiments 1 and 2): % MI=([final weight–initial weight]/initial weight)×100; % LI; protein concentration (μg proteins/mg juvenile); lipid concentration; survival of juveniles at day 10 (Experiment 1) and day 32 (Experiments 1 and 2) of the 32-d period.

All of these variables, except survival, were analyzed through repeated-measures analysis of variance (ANOVA) within general linear mixed-effect models, using spawn number as the repeated measure factor (with six levels in Experiment 1 and three levels in Experiment 2), food restriction treatment as a fixed factor (with six levels in Experiment 1 and three levels in Experiment 2), and female as the random factor. Data were tested for normality and homoscedasticity with Shapiro–Wilk and Levene's median tests, respectively. The Akaike information criterion was used to select the best model, and the Di Rienzo, Guzmán y Casanoves (DGC) test was applied when significant differences were found (Di Rienzo et al. 2002). Survival was analyzed through two-way ANOVA within general linear mixed-effect models, with no random factor. Orthogonal contrasts were performed when significant differences were found.

Results are presented as means±SE. All tests were carried out at the 95% significance level with Infostat® 2016 software (Infostat Group, FCA UNC, Córdoba, Argentina) (Di Rienzo et al. 2016).

Results

Experiment 1: female reproductive performance

All females spawned at least two consecutive times, but fewer females spawned four, five, and six consecutive times. The percentage of broods successfully hatched was 100% for the first, third, fifth, and sixth spawns, and 80% and 69.2% for the second and fourth spawns, respectively. The percentage of broods with more than 28 JI was as follows: 47.4% in first spawns, 83.3% in second spawns, 53.9% in third spawns, 66.7% in fourth spawns, 71.4% in fifth spawns, and 50.0% in sixth spawns. Female mortality reached 37% toward the end of the experiment (Fig. 2).

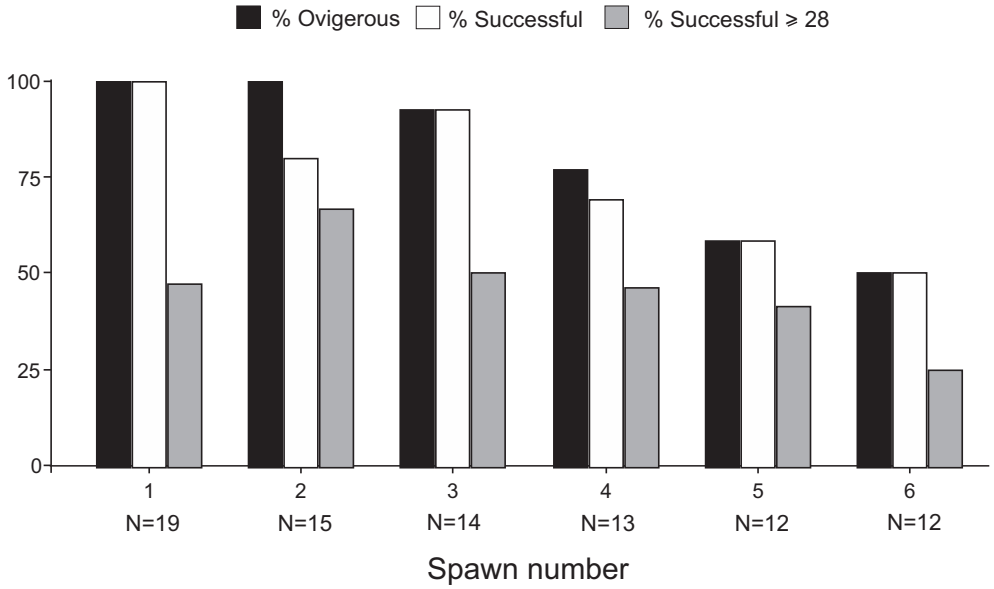

Fig. 2. Reproductive performance in females of *Neocaridina davidi,* evaluated as the percentage of ovigerous females, percentage of broods successfully hatched, and percentage of successfully hatched broods with ≥28 juveniles. The reported *N* corresponds to the initial number of females for each spawn number.

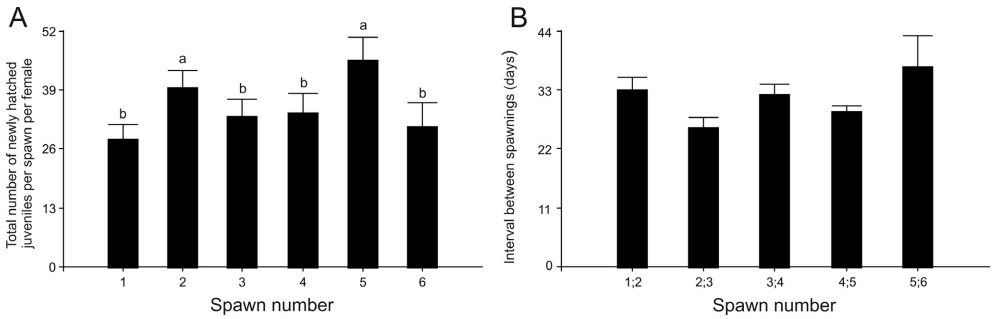

Fig. 3. A. Total number of newly hatched juveniles per spawn per female in the first six successive spawns of *Neocaridina davidi*. **B.** Interval (days) between successive spawnings of *Neocaridina davidi* that spawned six times. Values (mean ±SE) with different letters are significantly different ($p<0.05$).

Figure 3A shows the number of JI per female per spawn in the first six consecutive spawnings of experimental females. This variable was 26% higher in the second and fifth spawns compared to the other spawn numbers (p=0.027, F=2.83, *df*=5). The interval between spawnings was variable with spawn number but not statistically significant (p=0.204, F=1.6, *df*=4; Fig. 3B). Finally, the initial cephalothorax length of JI was similar (p=0.689, F=0.61, *df*=5) among spawn numbers, with a mean value of 1.29±0.04 mm.

Juvenile quality

Experiment 1. The percentage of mass increase (%MI) and length increase (%LI) were compared among the first six consecutive spawnings and the food restriction treatments DF, S4, and S8. As juvenile survival was very low in the most extreme treatments (S12, S16, and CS), the number of replicates was insufficient for statistical analyses. There was no significant interaction between the effect of spawn number and food restriction treatment on either %MI (p=0.879, F=0.51, *df*=10) or %LI (p=0.982, F=0.29, *df*=10), so these factors were analyzed separately. Percentage mass increase was 16% greater in juveniles fed daily than in juveniles starved for 4 and 8 d (p=0.028, F=3.81, *df*=2; Fig. 4A), while no significant differences were observed in %LI among food restriction treatments (p=0.087, F=2.54, *df*=2; Fig. 4C), independent of spawn number. On the other hand, %MI was 63.5% less ($p<0.001$, F=7.82, *df*=5) in juveniles from the first spawn (Fig. 4B), while %LI was 15%

greater (p=0.016, F=3.05, *df*=5) in juveniles from the third and fifth spawns, independent of food restriction treatment (Fig. 4D).

Survival was compared among the first six consecutive spawnings and the food restriction treatments DF, S4, S8, S12, S16, and CS at days 10 and 32. Because there was a significant interaction between the effects of spawn number and food restriction treatment on survival at day 10 (p=0.015, F=1.80, *df*=25) and at day 32 (p<0.001, F=2.70, *df*=25), neither factor could be analyzed separately. Therefore, and based on the objective of this study, we decided to compare juvenile survival among consecutive spawns within each food restriction treatment. A trend of lower survival among DF juveniles from the fourth spawn was observed at day 10, which was statistically significant (p=0.033, F=4.71, *df*=1) at day 32 (Fig. 5A). At both days 10 and 32, there was no significant difference in survival (p=0.479, F=3.2, *df*=1) among juveniles from consecutive spawns when they were starved for 4 d (Fig. 5B). Within S8 treatment, survival was 50% lower in juveniles from the first and second spawns compared to later spawns at day 10 (p=0.009, F=8.83, *df*=1). A similar pattern was observed at day 32 (Fig. 5C). When juveniles were starved for 12 d, their survival tended to increase with spawn number at both days 10 and 32 of the test (p=0.008, F=14.86, *df*=1), with juveniles from the fifth and sixth spawns (at day 10) and the sixth spawn (at day 32) showing a 55% higher survival (Fig. 5D). Survival was 54% lower in S16 juveniles from the first, second, and fifth spawns compared to those from the third and sixth spawns (p=0.021, F=6.62, *df*=1) at day 10 of the test. Survival was low for all spawn numbers at day 32 (0–27%), except for the sixth spawn, which showed the highest values (89%) (p=0.014, F=21.41, *df*=1; Fig. 5E). Finally, CS juveniles from the first, second, and fourth spawns showed 48% lower survival than those from the fifth and sixth spawns at day 10, with the latter showing a survival of 100% (p=0.012, F=7.58, *df*=1). No juveniles from the first four consecutive spawnings survived at day 32, while juveniles from the sixth spawn showed higher survival than those from the fifth spawn (p=0.001, F=20.74, *df*=1; Fig. 5F). For all the food restriction treatments, juvenile survival was qualitatively lower at day 32 than at day 10, this difference becoming more pronounced with the increase in the number of starvation days. Unlike the other spawns, juveniles from the sixth spawn showed similar (S4, S8, and S12 treatments) or slightly lower (DF, S16 and CS treatments) survival at day 32 with respect to that at day 10 of the test.

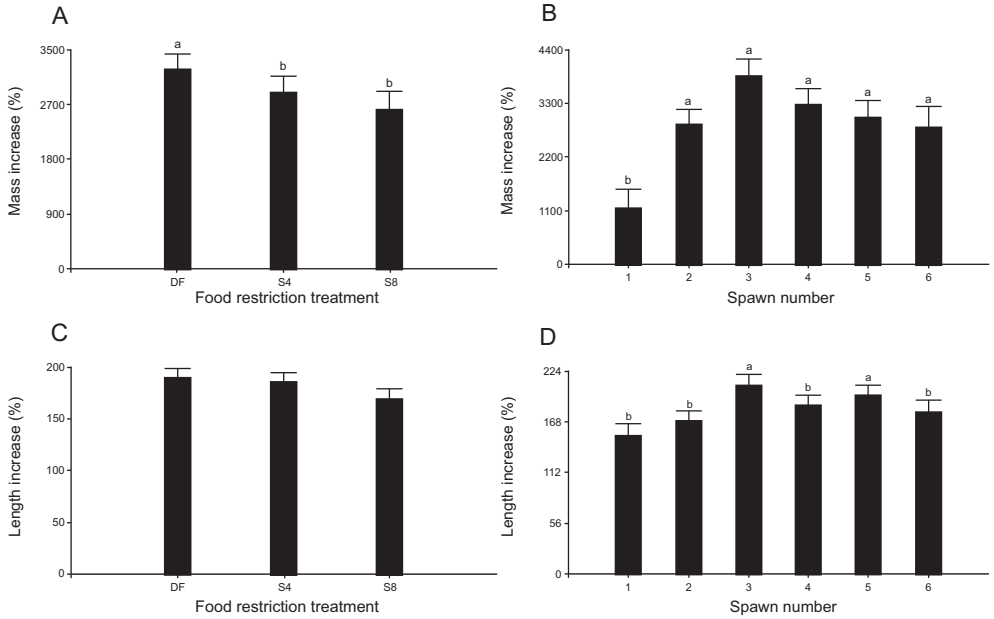

Fig. 4. Percentage of mass increase (**A,B**) and cephalothorax length increase (**C,D**) in *Neocaridina davidi* juveniles. Two panels (A,C) show data from six successive spawns, averaging values from the food restriction treatments DF (daily feeding during a 32-d period), S4 (food deprivation for 4 d after hatching), and S8 (food deprivation for 8 d after hatching). Two other panels (B,D) show data from juveniles exposed to the food restriction treatments DF, S4, and S8, averaging values from six successive spawns. Values (mean±SE) with different letters are significantly different ($p < 0.05$).

Experiment 2. In this experiment, we analyzed %MI, survival, and biochemical composition of juveniles from the third, fourth, and fifth spawns exposed to DF, S8, and S12 treatments. We evaluated these spawn numbers because, in Experiment 1, survival at day 10 and at day 32 of the food restriction test was higher in juveniles from the third and subsequent spawns than in juveniles from the first two consecutive spawns. Sixth spawnings were not included in the analysis because the number of juveniles at the end of the experiment was insufficient for biochemical determinations. The S4 treatment was not evaluated due to similarity in juvenile survival with DF in Experiment 1. Also, S16 and CS treatments were not evaluated because survival was very low for juveniles exposed to these treatments in Experiment 1.

The number of broods used in the food restriction test (i.e., broods with more than 28 juveniles) was as follows: 14 from third spawns, 13 from fourth spawns, and nine from fifth spawns. There was no significant interaction between the effect of spawn number and food restriction treatment on %MI ($p=0.761$, $F=0.47$, $df=4$), survival at 32 d ($p=0.958$, $F=0.64$, $df=4$), lipids ($p=0.062$, $F=2.57$, $df=4$), or proteins ($p=0.196$, $F=1.82$, $df=4$), so these factors were

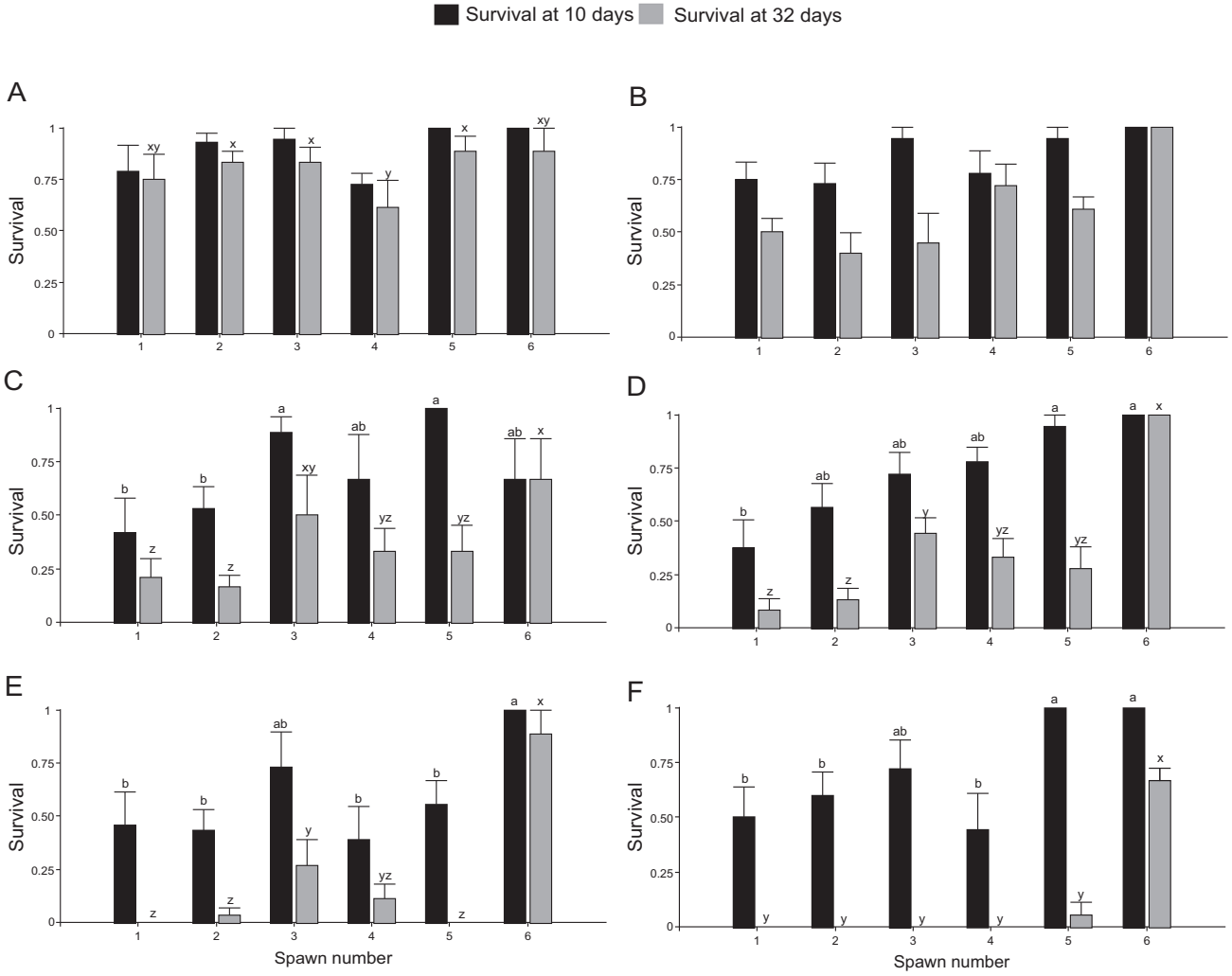

Fig. 5. Survival in juveniles of *Neocaridina davidi* from six consecutive spawns exposed to the food restriction treatments DF (**A**), S4 (**B**), S8 (**C**), S12 (**D**), S16 (**E**), and CS (**F**), at days 10 and 32 of the food restriction test. Different lower-case letters (a,b) indicate statistically significant differences among spawns at day 10, and different lower-case letters (x,y,z) indicate statistically significant differences among spawns at day 32 ($p < 0.05$). DF, daily feeding during a 32-d period; S4, food deprivation for 4 d after hatching; S8, food deprivation for 8 d after hatching; S12, food deprivation for 12 d after hatching; S16, food deprivation for 16 d after hatching; and CS, continuous starvation during a 32-d period.

analyzed separately. The %MI was 29% higher ($p=0.034$, $F=3.59$, $df=2$) in juveniles from the third spawn than in those from the fourth and fifth spawns (Fig. 6A), and 42% higher ($p<0.001$, $F=3.03$, $df=2$) in juveniles fed daily than in juveniles starved for 8 d and 12 d (Fig. 6B). Juvenile survival at day 32 of the test increased with spawn number, from near 50% in third spawns to 75% in fifth spawns ($p<0.001$, $F=3.64$, $df=2$; Fig. 7A), and decreased with the number of starvation days, from 70% in DF and S8 to 49% in S12 ($p<0.001$, $F=2.89$, $df=2$; Fig. 7B). There were no significant differences in protein concentrations ($p=0.839$, $F=0.18$, $df=2$) or lipid concentrations ($p=0.257$, $F=1.43$, $df=2$) in juveniles among consecutive spawns. There were also no significant differences in protein concentrations ($p=0.179$, $F=2.02$, $df=2$) or lipid concentrations ($p=0.478$, $F=0.76$, $df=2$) among treatments at the end of the experiment, with mean values of 58.0±4.5 μg proteins per mg juvenile and 4.0±0.5 μg lipids per mg juvenile (Table 1).

Discussion

This study is, to our knowledge, the first to use food restriction to analyze juvenile quality over successive spawnings in a freshwater decapod species with direct development. Growth and survival in food-restricted juveniles from later spawns were similar to or even greater than in juveniles from the first spawns, indicating no negative effect of multiple spawning on juvenile performance under adverse conditions.

The use of stress tests is based on the assumption that the physiological state of organisms determines their ability to survive under unfavorable environmental conditions. In this context, the organism performance may be taken as an indirect indicator of its quality (Anger 2001; Racotta et al. 2003). The most common stress tests used to evaluate the quality of crustacean larvae and juveniles include the exposure to low salinity, high ammonium concentrations, and food restriction (Palacios et al. 1999; Djunaidah et al. 2003; Arcos et al. 2005; Howard & Hentschel 2005; Zhang et al. 2015). The physiological stress triggered by starvation is particularly important in early stages of development, because it can lead to severe nutrient deficiencies (Anger 2001; Wahle 2003). In this sense, a decrease in larval biomass was shown to peak during the initial period of famine in some crab species. However, the rate of biomass loss then levels off due to a reduction in the metabolic rate, a physiological effect of prolonged starvation (Anger 2001). In addition, Stumpf et al. (2010), Calvo et al. (2012), and Sacristán et al. (2016) reported a delay in molting, reduced growth, and lower survival in juveniles of the freshwater crayfish *Cherax quadricarinatus* as the starvation period increased.

Results obtained in Experiment 1 of the present study showed less growth in juveniles of *N. davidi* from the first and second spawns compared to the other spawns, independent of their feeding regime. In addition, survival at day 10, under the more extreme food restriction treatments (S8, S12, S16, and CS), was higher in juveniles from the third and subsequent spawns compared to the first and second spawns. This was also true at day 32, with juveniles from the sixth spawns showing highest survival when starved for 12 (S12), 16 (S16), and 32 (CS) d after hatching. These results indicate that juvenile performance, as measured by growth and survival,

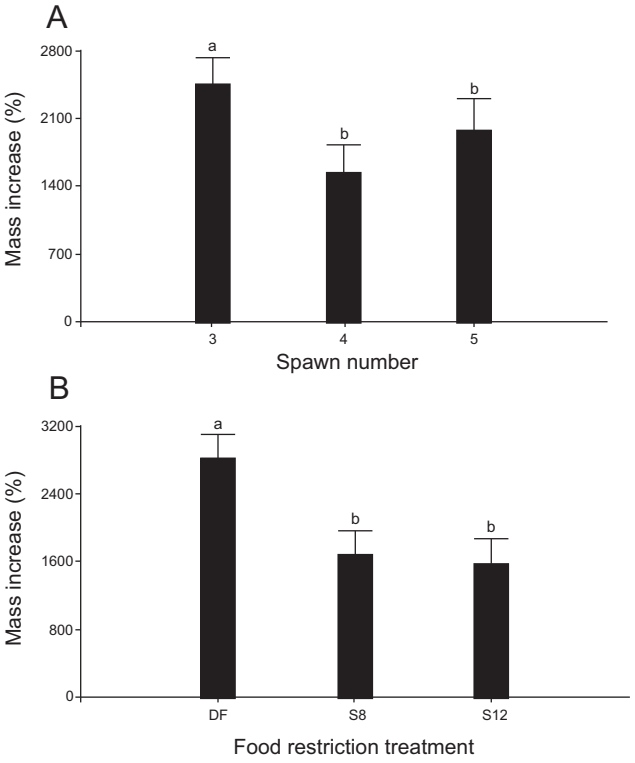

Fig. 6. A. Percentage of mass increase in juveniles of *Neocaridina davidi* from spawns 3, 4, and 5, averaging values from the food restriction treatments DF (daily feeding during a 32-d period), S8 (food deprivation for 8 d after hatching), and S12 (food deprivation for 12 d after hatching). **B.** Percentage of mass increase in juveniles of *N. davidi* exposed to the food restriction treatments DF, S8, and S12, averaging values from spawns 3, 4, and 5. Values (mean±SE) with different letters are significantly different ($p<0.05$).

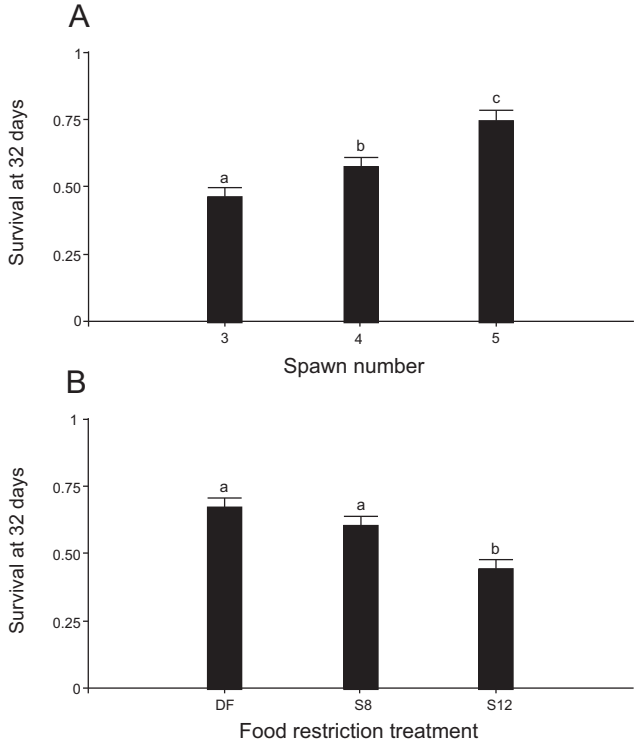

Fig. 7. A. Survival of juveniles of *Neocaridina davidi* from spawns 3, 4, and 5, at day 32 of the food restriction test, averaging values from the food restriction treatments DF (daily feeding during a 32-d period), S8 (food deprivation for 8 d after hatching), and S12 (food deprivation for 12 d after hatching). **B.** Survival of juveniles of *N. davidi* exposed to the food restriction treatments DF, S8, and S12, at day 32 of the food restriction test, averaging values from spawns 3, 4, and 5. Values (mean±SE) with different letters are significantly different ($p<0.05$).

did not decline in later spawns and was even higher in some cases. In other words, multiple spawning had no negative effects on juvenile quality, at least over the initial six successive spawns evaluated. Contrary to our results, previous studies in decapods with indirect development have reported a decrease in larval quality as a consequence of multiple spawning. For example, larvae from the third spawn of the marine crab *Scylla paramamosain* showed a more rapid decline in survival during starvation exposure compared to larvae from the first and second spawns (Islam & Yahya 2016). Likewise, in studies of *Litopenaeus vannamei,* Palacios et al. (1999) and Arcos et al. (2005) observed lower resistance to changes in water salinity in larvae from later spawns, indicating a certain degree of maternal physiological exhaustion.

Berkeley et al. (2004) proposed that the increase in the amount of lipids transferred by the female to oocytes was the mechanism that allowed for greater resistance to starvation in larvae from older females of the fish *Melanops sebastes*. This may not be the case in *N. davidi*, as Tropea & López Greco (2015) found lower lipid concentration and energy contents in eggs from the fourth and fifth spawns, which indicates a decrease in maternal provisioning as a result of multiple spawning. However, embryo consumption of yolk components may differ in successive spawnings, leading to different biochemical compositions of juveniles at hatching, which may ultimately determine their quality. Future studies are necessary to ascertain the biochemical composition of eggs throughout embryonic development and of newly hatched juveniles, over consecutive spawns, in order to understand how biochemical composition affects juvenile resistance to food restriction.

On the other hand, food deprivation for 4 d and 8 d after hatching had a clear effect on juvenile growth when all spawn numbers were considered in the analysis, as evidenced by a lower percentage of mass increase in juveniles in these treatments compared to juveniles fed daily. Food deprivation had a similar effect on survival, which clearly decreased at day 32 when starvation exceeded 4 d, and reached minimum values in juveniles starved for 16 d (S16) and 32 d (CS) (Experiment 1). Likewise, Anger & Dawirs (1981) observed a decline in growth and survival in larvae of *Hyas araneus* when the onset of feeding was delayed. The fact that juvenile survival at day 10 was in all cases greater than at day 32 indicates that the effect of food deprivation on juvenile physiological state was not immediate, and became evident after at least 10 d. When the first and second spawns were not considered in the analysis (Experiment 2), juveniles starved for 8 d had similar survival to that of juveniles fed daily. Moreover, no differences were found in the biochemical composition of juveniles among DF, S8, and S12 treatments and among the third, fourth, and fifth spawns at day 32. Overall, these results suggest that juveniles of *N. davidi* from later spawns are capable of coping with starvation periods of at least 8 d, with no decrease in survival and a significant increase in mass (but lower than the control). Larvae and juveniles of decapod crustaceans have specific dietary requirements for proteins, lipids, carbohydrates, minerals, and vitamins. These components are catabolized and stored in reserve tissues when food is abundant, proteins being the major source of energy during starvation periods (Barclay et al. 1983; Anger 2001). We cannot rule out a possible effect of food deprivation on juvenile biochemical reserves, because protein and lipid

Table 1. Biochemical composition of juveniles of *Neocaridina davidi* from the third, fourth, and fifth spawns at the end of the food restriction test. For all treatments, N=4.

	Proteins (μg mg^{-1})	Lipids (μg mg^{-1})
Food restriction treatment[a,b]		
DF	56.41±3.52	4.83±0.63
S8	59.22±7.54	3.62±0.39
S12	58.43±2.39	3.59±0.38
Spawn number[c]		
3	57.98±3.25	4.40±0.59
4	61.40±3.10	3.74±0.41
5	54.69±7.29	3.91±0.42

[a]Value for each food restriction treatment represents mean±SE of spawns 3, 4, and 5.
[b]DF, fed daily (control); S8, food deprivation for 8 d after hatching; S12, food deprivation for 12 d after hatching.
[c]Value for each spawn number represents mean±SE from the food restriction treatments DF, S8, and S12.

levels were not measured immediately following starvation. What is clear from our results is that such effect, if any, was reversed during the subsequent feeding period with a high-protein diet. Feeding may have allowed for energy storage and synthesis of new body tissues, as suggested by Stumpf et al. (2014) when reporting the same phenomenon in *C. quadricarinatus*.

The resistance to food restriction shown by juveniles of *N. davidi* was expected, based on the previous study by Pantaleão et al. (2015), and it may be related to the abbreviated development of larvae in this species, a strategy commonly associated with unpredictable environments, such as high latitudes (Thorson 1950), sea depth (Pond et al. 1997), and freshwater environments (Magalhães & Medeiros 1998). In particular, the unstable conditions associated with freshwater habitats have selected for certain reproductive characteristics, including an increase in egg size and proportional decrease in egg number, reduction in the number of larval stages, and enhanced larval and juvenile resistance to extended starvation (Jalihal et al. 1993; Anger 1995). In this context, starvation tolerance may be explained by the energy accumulated by larvae and juveniles from the remnant egg yolk, which may allow them to survive during the first days after hatching without external food sources (Anger 2001). As the energy reserves are consumed, juvenile performance may deteriorate, leading to a decrease in survival and growth if the starvation period continues. Our results, and those previously obtained in the crayfish *C. quadricarinatus* (Stumpf et al. 2010) and in the marine caridean shrimp *Exopalaemon carinicauda* (Zhang et al. 2015), are in agreement with this interpretation.

With respect to female reproductive performance, a decrease in the proportion of ovigerous females was observed with time. This result, along with female mortality recorded throughout the experiment, may reflect the physiological exhaustion in females of *N. davidi* as a consequence of multiple spawning, considering the high energetic costs associated with reproduction in species with direct development. However, the interval between spawnings and the number and size of newly hatched juveniles were similar among spawn numbers. Moreover, the percentage of broods successfully hatched and percentage of broods with more than 28 juveniles were similar among females that spawned many times (at least six) and those that spawned few times, which reflect a high reproductive efficiency of females. Results regarding the number of hatched juveniles coincide with those previously reported in penaeid shrimps (Arcos et al. 2003, 2005; Palacios & Racotta 2003), and in the marine crab *Maja brachydactyla* (Andrés et al. 2010), but differ from results obtained by Islam & Yahya (2016) in the mud crab *S. paramamosain*. These authors observed a lower fecundity (number of eggs) in the third spawn compared to the first and second spawns, along with a lower proportion of viable eggs. Kobayashi (2001) also observed a decrease in fecundity in the estuarine crab *Eriocheir japonica*, in terms of number of eggs and larvae. Female reproductive exhaustion due to successive spawnings has been reported for other decapod species, with negative consequences on offspring quality (Primavera 1985; Harrison 1990). This may be due to the insufficient time between spawnings for storage of reserves in the hepatopancreas and their transport to the ovary. Our results indicate a partial effect of multiple spawning on reproductive performance in females of *N. davidi*: Not all females spawned many times, but brood production was not different among those that spawned many or few times.

In conclusion, our study shows for the first time in a freshwater decapod with direct development that juvenile quality, evaluated in terms of survival and growth under food restriction conditions, did not decline over consecutive spawns. Although the percentage of ovigerous females decreased with time, many other reproductive variables were similar in successive spawnings. These results suggest that reproduction is partially affected by multiple spawning, at least for the initial six consecutive spawns.

Acknowledgments. This study is part of the undergraduate thesis by Agustina Marciano, which was funded by a scholarship from the University of Buenos Aires, Argentina. Laura S. López Greco is grateful to Agencia Nacional de Promoción Científica y Tecnológica (PICT 2012, project 1333), CONICET (PIP 2012-2014, project 112-201101-00212), and UBACYT (2014-2017 project 20020130100186BA) for financial support. We thank Amir Dyzenchauz (IBBEA, CONICET-UBA) for language revision.

References

Andrés M, Estévez A, Simeó CG, & Rotllant G 2010. Annual variation in the biochemical composition of newly hatched larvae of *Maja brachydactyla* in captivity. Aquaculture 310: 99–105.

Anger K 1995. The conquest of freshwater and land by marine crabs: adaptations in life-history patterns and larval bioenergetics. J. Exp. Mar. Biol. Ecol. 193: 119–145.

——— 2001. The Biology of Decapod Crustacean Larvae. Balkema Publishers, Lisse. 424 pp.

Anger K & Dawirs RR 1981. Influence of starvation on the larval development of *Hyas araneus* (Decapoda, Majidae). Helgol. Meeresunters 34: 287–311.

Arcos FG, Ibarra AM, Palacios E, Vazquez Boucard C, & Racotta IS 2003. Feasible predictive criteria for reproductive performance of white shrimp *Litopenaeus vannamei*: egg quality and female physiological condition. Aquaculture 228: 335–349.

Arcos FG, Palacios E, Ibarra AM, & Racotta IS 2005. Larval quality in relation to consecutive spawnings in white shrimp *Litopenaeus vannamei*. Aquac. Res. 36: 890–897.

Barclay MC, Dall W, & Smith DM 1983. Changes in lipid and protein during starvation and the moulting cycle in the tiger prawn, *Penaeus esculentus* Haswell. J. Exp. Mar. Biol. Ecol. 68: 229–244.

Berkeley SA, Chapman C, & Sogard SM 2004. Maternal age as a determinant of larval growth and survival in a marine fish, *Sebastes melanops*. Ecology 85: 1258–1264.

Bradford MM 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Calvo NS, Tropea C, Anger K, & López Greco LS 2012. Starvation resistance in juvenile freshwater crayfish. Aquat. Biol. 16: 287–297.

Coman GJ & Crocos PJ 2003. Effect of age on the consecutive spawning of ablated *Penaeus semisulcatus* broodstock. Aquaculture 219: 445–456.

Darnell MZ, Rittschof D, Darnell KM, & McDowell RE 2009. Lifetime reproductive potential of female blue crabs *Callinectes sapidus* in North Carolina, USA. Mar. Ecol. Prog. Ser. 394: 153–163.

Di Rienzo JA, Guzmán AW, & Casanoves F 2002. A multiple-comparisons method based on the distribution of the root node distance of a binary tree. J. Agric. Biol. Environ. Stat. 7: 129–142.

Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, & Robledo CW 2016. InfoStat Versión. Grupo InfoStat FCA, UNC, Argentina.

Djunaidah IS, Wille M, Kontara EK, & Sorgeloos P 2003. Reproductive performance and offspring quality in mud crab (*Scylla paramamosain*) broodstock fed different diets. Aquac. Int. 11: 3–15.

Folch J, Lees M, & Sloane Stanley GH 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497–509.

Frings CS & Dunn RT 1970. A colorimetric method for determination of total serum lipids based on the sulfophosphovanillin reaction. Amer. J. Clin. Pathol. 53: 89–91.

Gardner C 2001. Composition of eggs in relation to embryonic development and female size in giant crabs *Pseudicarcinus gigas* (Lamarck). Mar. Freshw. Res. 52: 333–338.

Harrison KE 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. J. Shellfish Res. 9: 1–28.

Herring PJ 1974. Size, density and lipid content of some decapod eggs. Deep Sea Res. 21: 91–94.

Howard SC & Hentschel BT 2005. Effects of short term food variability on the plasticity of age and size at metamorphosis of porcelain crab larvae. Limnol. Oceanogr. 50: 1960–1971.

Islam ML & Yahya K 2016. Successive reproductive performance and amino acid profiles in the newly hatched larvae of green mud crab (*Scylla paramamosain*) under captive condition. Int. J. Fish. Aquat. Stud. 4: 270–278.

Jalihal DR, Sankolli KN, & Shenoy S 1993. Evolution of larval developmental patterns and the process of freshwaterization in the prawn genus *Macrobrachium* Bate, 1868 (Decapoda, Palaemonidae). Crustaceana 65: 365–376.

Ji Y, Wu XG, Cheng YX, Wille M, & Sorgeloos P 2006. Effects of different diets on reproductive performance and HUFA composition of Chinese mitten crab (*Eriocheir sinensis*) broodstock during second spawning. J. Fish. Sci. China 13: 92–99.

Jones OR, Scheuerlein A, Salguero-Gómez R, Camarda CG, Schaible R, Casper BB, Dahlgren JP, Ehrlén J, García MB, Menges ES, Quintana-Ascencio PF, Caswell H, Baudisch A, & Vaupel JW 2014. Diversity of ageing across the tree of life. Nature 505: 169–173.

Kobayashi S 2001. Fecundity of the Japanese mitten crab *Eriocheir japonica* (de Haan). Benth. Res. 56: 1–7.

Magalhães C & Medeiros N 1998. The larval development of palaemonid shrimps from the Amazon region reared in the laboratory. VII. Abbreviated development of *Pseudopalaemon amazonensis* Ramos-Porto, 1979 (Crustacea: Decapoda: Caridea). Acta Amazon 28: 433–448.

Marsden GE, McGuren J, Hansford SW, & Burke MJ 1997. A moist artificial diet for prawn broodstock: its

effect on the variable reproductive performance of wild caught *Penaeus monodon*. Aquaculture 149: 145–156.

Nan TZ, Cheng YX, Wu XG, Wang JQ, Wang LJ, & Liu BI 2006. Comparison on the first and second berried crab on embryo and larval quality (Z1) of *Eriocheir sinensis*. J. Shanghai Fish. Univ. 15: 41–46.

Palacios E & Racotta IS 2003. Effect of number of spawns on the resulting spawn quality of 1 year old pond reared *Penaeus vannamei* (Boone) broodstock. Aquac. Res. 34: 427–435.

Palacios E, Perez Rostro CI, Ramirez JL, Ibarra AM, & Racotta IS 1999. Reproductive exhaustion in shrimp (*Penaeus vannamei*) reflected in larval biochemical composition, survival and growth. Aquaculture 171: 309–321.

Pantaleão JAF, Barros-Alves SDP, Tropea C, Alves DF, Negreiros-Fransozo ML, & López Greco LS 2015. Nutritional vulnerability in early stages of the freshwater ornamental "red cherry shrimp" *Neocaridina davidi* (Bouvier, 1904). (Caridea: Atyidae). J. Crustacean Biol. 35: 676–681.

Pond D, Dixon D, & Sargent J 1997. Wax ester reserves facilitate dispersal of hydrothermal vent shrimps. Mar. Ecol. Prog. Ser. 146: 289–290.

Primavera JH 1985. In: A review of maturation and reproduction in closed thelycum penaeids. Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps. Taki Y, Primavera JH, Llobrera JA, eds., pp. 47–64. Aquaculture Department, Asian Fisheries Development Center, Iloilo, Philippines.

Racotta IS, Palacios E, & Ibarra AM 2003. Shrimp larval quality in relation to broodstock condition. Aquaculture 227: 107–130.

Sacristán HJ, Ansaldo M, Franco Tadic LM, Fernández Giménez AV, & López Greco LS 2016. Long-term starvation and posterior feeding effects on biochemical and physiological responses of midgut gland of juveniles of *Cherax quadricarinatus* (Parastacidae). PLoS ONE. dx. https://doi.org/10.1371/journal.pone.0150854.

Smith GG, Ritar AJ, Johnston D, & Dunstan GA 2004. Influence of diet on broodstock lipid and fatty acid composition and larval competency in the spiny lobster, *Jasus edwardsii*. Aquaculture 233: 451–475.

Stumpf L, Calvo NS, Pietrokovsky S, & López Greco LS 2010. Nutritional vulnerability and compensatory growth in early juveniles of the 'red claw' crayfish *Cherax quadricarinatus*. Aquaculture 304: 34–41.

Stumpf L, Tropea C, & López Greco LS 2014. Recovery growth of *Cherax quadricarinatus* juveniles fed on two high-protein diets: effect of daily feeding following a cyclic feeding period on growth, biochemical composition and activity of digestive enzymes. Aquaculture 433: 404–410.

Thorson G 1950. Reproductive and larval ecology of marine bottom invertebrates. Biol. Rev. 25: 1–45.

Tropea C & López Greco LS 2015. Female growth and offspring quality over successive spawnings in a caridean shrimp *Neocaridina davidi* (Decapoda, Atyidae) with direct development. Biol. Bull. 229: 243–254.

Tropea C, Stumpf L, & López Greco LS 2015. Effect of temperature on biochemical composition, growth and reproduction of the ornamental red cherry shrimp *Neocaridina heteropoda heteropoda* (Decapoda, Caridea). PLoS ONE 10: e0119468.

Tsujimoto M, Komori O, & Imura S 2016. Effect of lifespan and age on reproductive performance of the tardigrade *Acutuncus antarcticus*: minimal reproductive senescence. Hydrobiologia 772: 93–102.

Verísimo P, Bernárdez C, González Gurriarán E, Freire J, Muiño R, & Fernández L 2011. Changes between consecutive broods in the fecundity of the spider crab, *Maja brachydactyla*. ICES J. Mar. Sci. 68: 472–478.

Wahle RA 2003. Revealing stock–recruitment relationships in lobsters and crabs: is experimental ecology the key? Fish. Res. 65: 3–32.

Wouters R, Gomez L, Lavens P, & Calderon J 1999. Feeding enriched *Artemia* biomass to *Penaeus vannamei* broodstock: its effect on reproductive performance and larval quality. J. Shellfish Res. 18: 651–656.

Wu XG, Cheng YX, Zeng CS, Wang CL, & Cui ZX 2010. Reproductive performance and offspring quality of the first and the second brood of female swimming crab, *Portunus trituberculatus*. Aquaculture 303: 94–100.

Zhang C, Li Z, Li F, & Xiang J 2015. Effects of starvation on survival, growth and development of *Exopalaemon carinicauda* larvae. Aquac. Res. 46: 2289–2299.