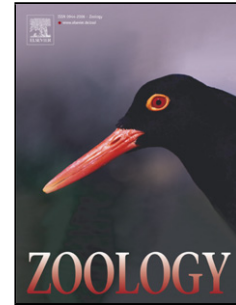


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Brothers are better than nothing: first report of incestuous mating and inbreeding depression in a freshwater decapod crustacean

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Highlights

- The probability of mating with brothers and non-brothers was similar
- The number of hatched juveniles, but not laid eggs, was lower in inbred clutches
- Incestuous mating negatively affected survival of late juvenile stages
- Inbreeding depression was higher for survival than growth under optimal conditions
- Food deprivation magnified the effect of inbreeding on juvenile growth

Abstract

This study aimed at evaluating the effect of one generation of full-sibling mating on traits related to the fitness of the gregarious freshwater shrimp *Neocaridina davidi*, both under optimal and stressful (i.e. starvation) laboratory conditions. Females were maintained either with their brothers (Inbreeding treatment) or non-brothers

(Outbreeding treatment), and the first and second broods were used to evaluate egg production and juvenile quality, respectively. The latter was analyzed in a 60-day period following hatching under optimal rearing conditions, and in a 20-day period following hatching under food deprivation conditions. All surviving females from both treatments mated and spawned, indicating that mating with brothers was as likely as mating with non-brothers. Different evolutionary scenarios are discussed for this outcome. With respect to offspring production, inbreeding had no effect on fecundity and fertilization success, but negatively affected the number of hatched juveniles. These results suggest that egg loss was higher in inbred clutches, possibly due to lower embryonic survival. On the other hand, the effect of inbreeding on growth was absent for embryos and for juveniles under optimal rearing conditions, while it was significant for juveniles starved for 10 consecutive days following hatching. Inbreeding depression for survival was only detected in juveniles from stages S8-S9 under optimal rearing conditions. Overall, the present results show that *N. davidi* fitness decreases after one generation of full-sibling mating. The use of a potentially weak food deprivation protocol and/or the evaluation of only one inbred generation could explain the absence or subtle inbreeding depression for some of the evaluated traits. No evident relationship between life-history traits and the existence and magnitude of inbreeding was found when comparing our results with those previously reported in high-fecundity marine crustaceans and low-fecundity terrestrial crustaceans.

Key words: caridean shrimps; inbreeding depression; growth; nutritional stress; survival

1. Introduction

Mating between closely related individuals often results in a decline of offspring fitness through lower viability or fecundity. This phenomenon, generally referred to as inbreeding depression, is explained in many cases by the increased expression of deleterious recessive alleles (Charlesworth and Charlesworth, 1987; Pusey and Wolf, 1996; Keller and Waller, 2002). The most common estimates of inbreeding depression include life-history traits, which are closely related to fitness (e.g. fecundity, offspring survival), and morphological traits, which are indirectly associated with fitness (e.g. adult body weight). Some of these traits have been shown to decrease due to inbreeding in many vertebrate species, such as flycatchers (Kruuk et al., 2002), guppies (Pitcher et al., 2008) and lemurs (Charpentier et al., 2008). Negative effects of inbreeding have also been reported for various traits in invertebrate groups, such as spiders (Bilde et al., 2007), cockroaches (Lihoreau et al., 2007), sea urchins (Anderson and Hedgecock, 2010), scallops (Zheng et al., 2012), beetles (Liu et al., 2014), and terrestrial isopods (Fortin et al., 2018). However, this phenomenon is poorly documented in aquatic crustaceans, with few studies showing lower survival, growth, or resistance to diseases in inbred progenies of some copepod, penaeid shrimp and crab species (Palmer and Edmands, 2000; Keys et al., 2004; Moss et al., 2007; Luo et al., 2014; Gao et al., 2015). The occurrence of inbreeding and the levels of inbreeding depression under natural conditions vary across taxa, populations and ecological factors, with mammals and birds accounting for most reported cases. Data from these groups show in general a lower birth weight, juvenile survival and reproductive success due to inbreeding (reviewed in Pusey and Wolf, 1996; Keller and Waller, 2002). Population size, limited natal dispersal and active mate choice for relatives are some of the proposed factors to explain inbreeding in the wild (Shields, 1993; Duarte et al., 2003; Jaimeson et al., 2009). Theoretically, incestuous matings may increase the inclusive fitness of females, with

this benefit possibly overriding the effects of inbreeding depression (Parker, 1979; Kokko and Ots, 2006; Szulkin et al., 2013). In that case, a preference for mating with relatives may evolve, as demonstrated empirically both in vertebrate (e.g. Cohen and Dearborn, 2004; Sommer, 2005; Thünken et al., 2007; Richard et al., 2009) and invertebrate (e.g. Schjorring and Jager, 2007; Thurin and Aron, 2009; Robinson et al., 2012) groups.

The fitness costs associated with inbreeding are predicted to be amplified by stressors such as food shortage, extreme temperatures, or presence of pathogens (Armbruster and Reed, 2005; Fox and Reed, 2010; Bijlsma and Loeschcke, 2012). An important stressor in freshwater habitats is transient food deprivation resulting from temporally and spatially variable plankton production. In this context, the resistance of early developmental stages of freshwater decapod crustaceans to the absence of food is a key factor for their successful development (Anger, 2001). Inbreeding may, nonetheless, increase the sensitivity to such stressful condition for many fitness-related traits of the offspring, such as survival.

When the costs associated to inbreeding depression and those associated to investment in reproduction are high, incest avoidance mechanisms may be favored (Kokko and Ots, 2006; Parker, 2006; Metzger et al., 2010). These mechanisms include the dispersal of one or both sexes (Perrin and Goudet, 2001; Lebigre et al., 2010), and the recognition and rejection of related mates through kinship-related cues (Holmes and Sherman, 1983; Pusey and Wolf, 1996). Kinship recognition is particularly important in gregarious or social species that are typically poor dispersers (Shellman-Reeve, 2001; Lihoreau and Rivault, 2010). It is known that some decapod crustaceans are capable of distinguishing familiar from unfamiliar conspecifics and eventually reject those individuals that do not belong to the social group (Karavanich and Atema, 1998; Duffy

et al., 2002; Rufino and Jones, 2001; Detto et al., 2006; Tierney et al., 2013; Chak et al., 2015). But the question remains whether they are capable of distinguishing between related and unrelated individuals and whether they choose unrelated mates to breed.

The caridean shrimp *Neocaridina davidi* is a gregarious freshwater crustacean with no specific social structure and no reported pattern of male or female dispersal from the natal group. Like most decapod crustaceans with direct development, females of this species show a high investment in reproduction, since large energy reserves must be provided for the developing embryo (Herring, 1974). Moreover, they show post-fertilization maternal care consisting of egg ventilation and cleaning, which is a general feature within caridean shrimps and other pleocyemate taxa. This increases offspring fitness by protecting embryos against hypoxia and pathogens (Förster and Baeza, 2001; Baeza and Fernández, 2002). The potential encounters of related males and females in nature, along with the high energetic costs associated with reproduction, may have favored the development of inbreeding avoidance mechanisms in this species to maximize offspring fitness. However, an important step prior to the study of incest avoidance in this and other decapod species is to determine the occurrence and magnitude of inbreeding depression.

In this study, we evaluated the effect of one generation of full-sibling mating on *N. davidi* traits directly and indirectly related to fitness to test the hypothesis that inbreeding has significant consequences on the fitness of this shrimp species. Females were raised either with brothers or non-brothers and fecundity, along with juvenile survival and growth under optimal and stressful (i.e. starvation) laboratory conditions, were analyzed. All these traits were predicted to decrease in clutches produced by full-siblings as compared to clutches produced by non-siblings.

2. Materials and Methods

2.1. Experimental specimens

The female and male shrimps used were obtained from a reproductive stock provided by Decorpez Aquaria, Buenos Aires, Argentina. Each female was placed with one male in a plastic aquarium measuring 18 x 12.5 x 12 cm ($n = 13$), filled with dechlorinated tap water ($\text{pH} = 6.8\text{-}7.2$; $^{\circ}\text{dGH} < 3$; $\text{NO}_3^- = 10\text{-}25 \text{ mg l}^{-1}$; $\text{NO}_2^- = 0\text{-}1 \text{ mg l}^{-1}$) under continuous aeration and at a constant temperature of $27 \pm 1 \text{ }^{\circ}\text{C}$. The photoperiod was 14L:10D. Java moss (*Vesicularia* sp.) was provided as substratum. Shrimps were fed daily *ad libitum* balanced food for tropical fish (Tetracolor®, Tetra GmbH, Melle, Germany).

The first brood (hereinafter called B1) of each female was placed in individual aquaria measuring 33.5 x 25 x 19 cm (Fig. 1). Juveniles were reared under similar experimental conditions as their parents and were sexed with the help of a stereomicroscope when they reached a weight of 15-25 mg. Sex was defined based on the morphology of the first and second pair of pleopods, which are clearly different between males and females from that size (Shih and Cai, 2007). Immature females were separated from immature males to guarantee their virginity at the onset of the experiment. They were raised until reaching a body weight of 30-40 mg in the case of males and 50-70 mg in the case of females.

Since ovaries are visible through the translucent cuticle throughout the entire maturation process, we were able to determine ovarian developmental stage with the aid of a stereomicroscope. Those females with stage III ovaries were selected as they were close to attain sexual maturity (Tropea et al., 2018). In addition, 4-10 mature males (i.e. with visible spermatophores in the distal vas deferens) were selected from each brood and used for the experiment described below (104 females and 104 males in total).

2.2. Experimental design

Eight sibling females from each B1 (mean weight: 64.71 ± 2.91 mg) were placed in quartets in plastic aquaria (18 x 12.5 x 12 cm), which were randomly assigned to one of the following breeding treatments (Fig. 1):

- *Inbreeding*: the female quartet was maintained with four mature brothers weighing 36.21 ± 0.91 mg;
- *Outbreeding*: the female quartet was maintained with four mature non-brothers weighing 35.64 ± 0.76 mg, which were randomly selected from different B1.

Each aquarium was a replicate and 13 replicates were used *per* treatment. Shrimps were maintained under the same conditions of water quality, photoperiod, and feeding, as described above, for the entire experimental period. All replicates had the same shrimp density, which was previously shown to be optimal for the species rearing under laboratory conditions (Vazquez et al., 2017).

All aquaria were cleaned and water was completely replaced once a week. In addition, they were visually searched daily for ovigerous females. The total number of ovigerous females was recorded for each treatment to calculate the proportion of females that had mated with brothers and the proportion that had mated with non-brothers, as follows:

$100 * (\text{number of ovigerous females} / \text{number of surviving females})$. The first brood of all the females from each replicate was used to evaluate egg number and fertilization success, while the second brood of three out of the four females from each aquarium was used to evaluate juvenile quality (Fig. 1).

2.2.1. Egg number and fertilization success (first brood)

Once detected, all ovigerous females were weighed (wet weight, precision: 0.1 mg) and eggs were gently removed from their pleopods at day 5-6 of the incubation period, when embryos were clearly visible (Tropea et al., 2019). Both the *total number of eggs* and the *number of fertilized eggs* (i.e. eggs with visible embryo) were recorded for each brood.

2.2.2. Juvenile quality (second brood)

The second clutch of three females *per* aquarium was used to evaluate juvenile quality. Two of them were exposed to stressful conditions during a 20-day period (i.e. food restriction test), while another second clutch *per* replicate was reared under optimal laboratory conditions during a 60-day period (Fig. 1). Tropea et al. (2015) have shown that *N. davidi* males and females are sexually mature from an approximate body weight of 30 mg and 50 mg, respectively, which is reached within two months after hatching when reared at a density similar to that of the present study. Hence, juveniles were raised during 60 days in an attempt to evaluate some reproductive variables of inbred and outbred offspring, such as ovarian maturation.

The experimental period and the food treatments (see below) of the nutritional stress test were defined based on previous results on starvation resistance of *N. davidi* juveniles (Pantaleão et al., 2015; Marciano et al., 2018). The mentioned authors have demonstrated that mortality is above 70% when juveniles are starved for more than 20 days. In addition, Marciano et al. (2018) have demonstrated that there is an inflexion point in the survival of juveniles at day 32 when they are initially starved between eight and 12 consecutive days, with respect to daily fed juveniles.

Newly hatched juveniles from all the selected broods were first counted to record the *number of newly hatched juveniles per female*. The *postorbital cephalothorax length*

was then measured in a sample of 5 juveniles *per* brood with the aid of an ocular ruler fitted to a Zeiss® stereomicroscope (50 units = 1 mm, at 50x). This measure was taken from behind the eye to the posterior end of the cephalothorax, and was used to calculate juvenile length gain, as explained below.

2.2.2.1. Juvenile performance under stressful conditions

For the food restriction test, 12 newly hatched juveniles were randomly chosen from each of the two selected second broods *per* aquarium. They were individually placed in 250-cm³ plastic containers with a piece (2 x 2 cm) of onion bag mesh and a small stone (0.5 x 0.5 x 0.3 cm) as substrata. The containers were equally and randomly assigned to one of the three following treatments (four juveniles *per* treatment): DF (daily feeding with Tetracolor® for 20 days following hatching), S10 (food deprivation for 10 days following hatching and daily feeding with Tetracolor® for the subsequent 10 days), and CS (food deprivation for 20 days following hatching) (Fig. 1).

All containers were cleaned and water was completely replaced three times a week.

Deaths were recorded at each cleaning event to calculate for each treatment the *percentage of surviving juveniles at days 10 and 20* of the test. Also, the postorbital cephalothorax length at days 10 and 20 was measured in each surviving juvenile as described above. This information was used to calculate *length gain between days 0 and 10 and between days 10 and 20*, as follows: $100 * ((fPO - iPO) / iPO)$, where fPO and iPO are the final and initial postorbital cephalothorax lengths, respectively. Finally, juvenile *wet weight* was recorded (precision: 0.1 mg) at day 20 after blot-drying shrimps with tissue paper for the removal of excess water. Ten-day-old and 20-day-old juveniles were presumably at stage 5 (S5) and stage 8 (S8) of development, respectively, meaning they had molted five and eight times after hatching, according to Pantaleão et al. (2017).

2.2.2.2. Juvenile performance under optimal laboratory conditions

For the evaluation of growth and survival under optimal laboratory conditions, 10 newly hatched juveniles were randomly chosen from another selected second brood *per* aquarium (Fig. 1). They were placed in a plastic aquarium measuring 18 x 12.5 x 12 cm, under similar experimental conditions as their parents. The wet weight (precision: 0.1 mg) and postorbital cephalothorax length of each juvenile, along with *mortality*, were recorded at days 30 and 60 of the 60-day growth period, as described above. *Weight gain* was calculated for the period 30–60 days as follows: $100 * ((fW - iW) / iW)$, where fW and iW are the final and initial wet weights, respectively. *Length gain* was calculated for the periods 0–30 and 30–60 days as follows: $100 * ((fPO - iPO) / iPO)$, where fPO and iPO are the final and initial postorbital cephalothorax lengths, respectively.

Thirty-day-old and 60-day-old juveniles were presumably at stage 11 (S11) and at stage > 16 (S > 16) of development, respectively, meaning they had molted 11 and more than 16 times after hatching, according to Pantaleão et al. (2017). The size of juveniles was too small by day 30 to be sexed without stressing them by significant manipulation under the stereomicroscope. For this reason, they were sexed only at day 60 and a distinction between males and females were done when analyzing length gain and weight gain between days 30 and 60 (see below).

2.3. Statistical analyses

The response variables *total number of eggs*, *percentage of fertilized eggs*, *number of newly hatched juveniles* and *postorbital cephalothorax length of newly hatched juveniles* were compared between breeding treatments using generalised linear mixed

models (GLMM), with “breeding treatment” as the fixed factor (2 levels: inbreeding and outbreeding) and “B1” (13 levels) and “aquarium” (26 levels) as random factors (aquarium nested in B1). Female weight was included as a covariate for the total number of eggs, the number of fertilized eggs and number of newly hatched juveniles, since *N. davidi* fecundity correlates with female size (Tropea and López Greco, 2015). The response variables recorded in juveniles subjected to the food restriction test were analyzed as follows: the *wet weight at day 20* was compared between treatments using GLMM, with “breeding treatment” and “food treatment” (three levels: DF, S10 and CS) as the fixed factors, and “B1” (13 levels), “aquarium” (26 levels) and “brood” (2 levels) as the random factors (brood nested in aquarium and aquarium nested in B1). Repeated measures ANOVA was used to evaluate the *percentage of surviving juveniles at days 10 and 20*, along with *length gain between days 0 and 10 and between days 10 and 20* of the experimental period, considering “time” (2 levels) as an additional fixed factor. The response variables considered to evaluate juvenile growth under optimal laboratory conditions were analyzed as follows: the *wet weight at day 30* and *length gain between days 0 and 30*, were compared between treatments using GLMM, with “breeding treatment” as the fixed factor, and “B1” and “aquarium” as random factors (aquarium nested in B1). For the *percentage of surviving juveniles at day 30 and day 60* of the 60-day experimental period an additional fixed factor was considered: “time”, with 2 levels. For juvenile *length gain* and *weight gain between days 30 and 60* another additional fixed factor was considered: “sex”, with 2 levels.

A Poisson distribution of data was assumed for the total number of eggs and number of newly hatched juveniles. A Binomial distribution of data was assumed for the percentage of fertilized eggs, percentage of surviving juveniles subjected to the food restriction test, and percentage of surviving juveniles reared under optimal laboratory

conditions. A Normal distribution was assumed for the remaining variables after checking data for normality with the Shapiro–Wilk test. Models with the variance structure showing the lowest Akaike information criterion were chosen. Multiple pairwise comparisons were made with Fisher’s least significant difference (LSD) method. All tests were carried out at the 95% significance level with packages included in the R software (Venables and Ripley, 2002; R Core Team, 2019). Results are presented as means \pm SE.

3. Results

3.1. Egg number and fertilization success

All surviving females from the inbreeding and outbreeding treatments mated and spawned. The total number of eggs ($F_{1,68} = 0.21$, $p = 0.645$) and number of fertilized eggs ($F_{1,66} = 0.58$, $p = 0.451$) were similar in inbred and outbred clutches. These variables averaged 33.44 ± 0.55 eggs *per* female, 94.50 ± 0.01 % of which were fertilized (Fig. 2).

3.2. Juvenile quality

The number of newly hatched juveniles was significantly higher ($F_{1,52} = 9.61$, $p = 0.003$) in outbred clutches (31.43 ± 1.24 juveniles *per* female) as compared to inbred clutches (24.67 ± 1.93 juveniles *per* female) (Fig. 3A). The postorbital cephalothorax length of newly hatched juveniles was similar ($F_{1,9} = 0.43$, $p = 0.528$) between both breeding treatments and averaged 0.83 ± 0.01 mm (Fig. 3B).

3.2.1. Juvenile quality: food restriction test

The interaction among fixed factors (breeding treatment, food treatment, time) was not significant for the percentage of surviving juveniles ($F_{2,576} = 2.53$, $p = 0.081$). Hence, each factor was analyzed alone, averaging levels of the remaining factors. No differences were found in survival between inbred and outbred juveniles ($F_{1,276} = 1.17$, $p = 0.281$), reaching a mean value of 72.32 ± 4.05 % (Fig. 4A). This variable differed among food treatments ($F_{2,276} = 40.57$, $p = 0.001$; LSD Fisher test, $p < 0.05$), with highest values in DF (89.25 ± 2.81 %), intermediate values in S10 (65.05 ± 5.06 %) and lowest values in CS (55.51 ± 5.90 %) (Fig. 4B). These results were similar ($F_{1,276} = 0.08$, $p = 0.783$) both between days 0 and 10 and between days 10 and 20 of the test period (Fig. 4C).

Significant interactions were found between the fixed factors “time” and “breeding treatment” ($F_{1,617} = 12.34$, $p = 0.005$), and “time” and “food treatment” ($F_{2,617} = 16.42$, $p = 0.001$) for juvenile length gain. Hence, these factors were analyzed together. Length increment up to day 10 was significantly higher in outbred juveniles (54.84 ± 2.47 %) than in inbred ones (46.60 ± 2.56 %) for all the food treatments tested (LSD Fisher test, $p < 0.05$). This difference was no longer evident during the following 10 days of the test (LSD Fisher test, $p > 0.05$), length increment averaging 22.94 ± 2.38 % (Fig. 5A). Daily fed juveniles showed the highest length gain, both for the first half (64.85 ± 2.47 %) and second half (31.43 ± 2.08 %) of the test period (LSD Fisher test, $p < 0.05$). S10 juveniles showed a similar length gain (44.44 ± 2.46 %) as CS juveniles (42.87 ± 2.37 %) during the first 10 days of starvation (LSD Fisher test, $p < 0.05$). After feeding for the subsequent 10 days, S10 juveniles showed a significantly higher length gain (26.73 ± 2.45 %) than CS juveniles (10.54 ± 2.07 %) (LSD Fisher test, $p < 0.05$) (Fig 5B).

The interaction between fixed factors (breeding treatment and food treatment) was not significant for juvenile wet weight at day 20 of the 20-day test period ($F_{2,50} = 1.50$, $p =$

0.232). No significant differences in this variable were evident between inbred and outbred juveniles ($F_{1,50} = 2.53$, $p = 0.118$) (Fig. 5C). Highest values (4.08 ± 0.26 mg) were observed in daily fed juveniles, intermediate values (2.05 ± 0.20 mg) for S10 treatment and lowest values (1.08 ± 0.18 mg) for CS treatment ($F_{2,50} = 70.33$, $p = 0.001$; LSD Fisher test, $p < 0.05$) (Fig. 5D).

3.2.2. Juvenile quality: growth under optimal laboratory conditions

The interaction between fixed factors (breeding treatment and time) was not significant for juvenile survival ($F_{1,41} = 2.08$, $p = 0.157$). Both at day 30 and day 60 of the experimental period, survival was significantly lower ($F_{1,41} = 8.65$, $p = 0.005$) in inbred juveniles than in outbred ones (Fig. 6A). This variable showed no change over time ($F_{1,41} = 1.01$, $p = 0.320$). Length gain up to day 30 was similar in juveniles from both breeding treatments ($F_{1,9} = 0.02$, $p = 0.903$), reaching a mean value of 200.56 ± 0.82 % (Fig. 6B). Wet weight at day 30 was also similar in inbred and outbred juveniles ($F_{1,9} = 1.21$, $p = 0.299$), reaching a mean value of 15.20 ± 0.56 mg.

The interaction between fixed factors was not significant for length gain ($F_{1,118} = 0.26$, $p = 0.612$) and weight gain ($F_{1,118} = 0.20$, $p = 0.653$) of juveniles between days 30 and 60 of the experimental period. Length gain was slightly higher in progenies from non-siblings (33.73 ± 3.16 %) than in those from full-siblings (27.82 ± 3.63 %), although this difference was not statistically significant ($F_{1,6} = 1.51$, $p = 0.265$). Females showed a significantly higher ($F_{1,118} = 33.04$, $p = 0.001$) length gain than males (37.00 ± 2.63 % and 24.56 ± 2.64 %, respectively) (Fig. 7A). A similar pattern was observed for weight gain: it was slightly but not significantly higher ($F_{1,6} = 2.33$, $p = 0.178$) in outbred than in inbred juveniles (161.53 ± 18.73 % and 123.61 ± 20.84 %, respectively) and was

significantly higher ($F_{1,118} = 21.71$, $p = 0.001$) in females than in males (172.07 ± 16.60 % and 113.07 ± 16.78 %, respectively) (Fig. 7B).

The final weight was 28.57 ± 2.63 mg in inbred males, 30.23 ± 2.03 mg in outbred males, 36.90 ± 3.73 mg in inbred females, and 39.06 ± 4.50 mg in outbred females. The testes and vas deferens were visible in most inbred and outbred males at day 60, while most females were at stage I (62%) and stage II (32%) of ovarian development. Mature females (i.e. with stage IV ovaries) were detected only in outbred clutches, although in a low percentage (4%).

4. Discussion

The effects of inbreeding on life-history and morphological traits were addressed for the first time in a freshwater decapod crustacean, under optimal and stressful laboratory conditions. Variable degrees of inbreeding depression were detected depending on the evaluated trait, rearing conditions, and developmental stage of *N. davidi*. In particular, fewer hatched juveniles and fewer surviving juveniles under optimal laboratory conditions were recorded for inbred clutches, with no apparent deficiencies on their growth. These results were expected considering that inbreeding depression tends to be more acute for traits closely related to fitness, such as survival, and milder for traits indirectly related to fitness, such as adult body size (DeRose and Roff, 1999; Keller and Waller, 2002). The greater influence of inbreeding on survival may be a consequence of the cumulative effect on each of the many underlying features that determine this life-history trait (Houle, 1992; Roff, 1997).

Among all the evaluated variables, survival and growth after one generation of inbreeding seemed to behave differently in *N. davidi* than in some marine crustaceans with indirect development. For example, the swimming crab *Portunus trituberculatus*

and the penaeid shrimps *Fenneropenaeus chinensis* and *Penaeus vannamei* showed a decline in growth, but no changes in survival, due to inbreeding (Moss et al., 2007; Luo et al., 2014; Gao et al., 2015). However, inbred and outbred postlarvae of other penaeid shrimps, such as *P. japonicus*, showed no differences in growth (Keys et al., 2004), which agrees with our results.

4.1. Offspring production

Females paired with their brothers laid a similar number of eggs as those paired with non-brothers, with no detectable consequences on fertilization success. By contrast, the number of hatched juveniles was lower in inbred clutches. Our results agree with those on high-fecundity marine crustaceans with indirect development (i.e. penaeid shrimps) (Moss et al., 2008; De los Ríos-Pérez et al., 2017), and low-fecundity crustaceans with direct development (i.e. terrestrial isopods) (Fortin et al., 2018). Unlike these data, fecundity usually declines in inbred clutches of diverse invertebrate species, including the cockroach *Blattella germanica* (Lihoreau et al., 2007), the bulb mite *Rhizoglyphus robini* (Radwan, 2003) and the wolf spider *Pardosa astrigera* (Chen et al., 2017). Hatching success, on the other hand, seems to be more consistently affected by inbreeding across invertebrate taxa, with *N. davidi* (present results), the butterfly *Melitaea cinxia* (Nieminen et al., 2001), the bay scallop *Argopecten irradians irradians* (Zheng et al., 2012) and the cabbage beetle *Colaphellus bowringi* (Liu et al., 2014) as clear examples of lower hatching rates of inbred embryos.

Even though the number of eggs and hatched juveniles were recorded in different spawn numbers (first and second brood of each female, respectively), the fewer hatched juveniles may reflect a higher egg loss in inbred clutches. Parental effort is modified in

some vertebrate species when individuals are paired with relatives (Margulis, 1997; Thünken et al., 2007). However, as no such pattern has been reported for crustaceans, we assume that maternal care for the eggs sired by full-siblings was as effective as that for the eggs sired by non-siblings. In this context, the higher egg loss in inbred clutches may reflect inherent deficiencies in embryonic development and higher embryo mortality rather than lower maternal care for the eggs.

4.2. Embryonic and juvenile growth

The growth of the embryos that completed development was not affected by inbreeding, as evidenced by the similar size of inbred and outbred juveniles at hatching. Under optimal laboratory conditions, juvenile growth seemed to be slightly higher in outbred shrimps, although this result was not statistically significant. It is possible that juvenile mortality was size-dependent, with a higher incidence on the smallest individuals (Gjerde et al., 1983). In such case, the higher mortality in inbred broods may have eliminated a greater proportion of small juveniles than in outbred broods, with surviving shrimps growing equally well in both treatments. This could have led to an underestimation of inbreeding depression for growth.

Alternatively, stable and optimal rearing conditions could have prevented deleterious effects from being revealed. In fact, a significant negative effect of inbreeding on growth was only detected when juveniles were exposed to food deprivation for 10 consecutive days. Such food restriction was an effectively stressful condition for the early developmental stages of *N. davidi* because it led to a lower survival and growth of starved juveniles compared to daily fed juveniles. The potentially severe nutrient deficiencies provoked by starvation has been addressed by Anger (2001), who

highlighted the importance of the physiological stress triggered by starvation in crustacean larvae.

Interestingly, the length gain of S10 juveniles partially recovered after they started feeding (i.e. from day 10 onwards), getting closer to that of DF juveniles. This relatively rapid growth is known as compensatory growth, a phenomenon rarely studied in crustaceans. Those marine and freshwater species evaluated so far show a variety of responses to starvation, including no compensation at all, partial compensation, and full compensation (Wu and Dong, 2001; Zheng et al., 2008; Li et al., 2009; Stumpf et al., 2014). Our results are compatible with partial compensatory growth, since S10 juveniles failed to achieve the same size as DF juveniles during the feeding period (Ali et al., 2003). An improvement in food conversion ratio and/or hyperphagia may explain compensatory growth of *N. davidi* juveniles when food became available, as previously proposed by Stumpf et al. (2014) for the freshwater crayfish *Cherax quadricarinatus*. Although this physiological response is reported in the study species for the first time, its potential extent and underlying mechanisms clearly deserves further research.

4.3. Juvenile survival

Contrary to the results obtained in 30-day-old (S11) and 60-day-old (S>16) shrimps reared under optimal laboratory conditions, inbreeding had no apparent effect on the survival of 10-day-old (S5) and 20-day-old (S8) daily fed juveniles from the nutritional stress test. The lack of effects on early juvenile stages disagrees with previous studies on marine shrimps and oysters reporting significant inbreeding depression for the survival of 8-day-old and 3-month-old larvae, respectively (Launey and Hedgecock, 2001; Moss et al., 2008). In some invertebrate species, the decrease in offspring fitness has been shown to appear after two or more generations of inbreeding (Radwan, 2003;

Moss et al., 2007), while in other species inbreeding depression has been reported to increase with the inbreeding coefficient (Luo et al., 2014; Gao et al., 2015). Hence, it is possible that the magnitude of inbreeding depression was too mild in early *N. davidi* juveniles from the first inbred generation or the variability among individuals was too high to allow the statistical detection of any effect on survival. In this sense, large sample sizes (i.e. more than 20 full-sib matings) have been necessary to detect inbreeding depression in vertebrates (Keller and Waller, 2002).

The survival of starved juveniles (S10 and CS treatments) was also unaffected by inbreeding. Fox and Reed (2010) have demonstrated that inbreeding depression increases very little or does not increase at all under mild forms of stress, while it shows a positive relationship with the magnitude of stress when this gets stronger. It is worth noting that although lower than in daily fed juveniles, survival was still high (50-60 %) for juveniles starved for 10 and 20 consecutive days. This outcome agrees with the high starvation resistance reported by Pantaleão et al. (2015) for the first and third juvenile stages of *N. davidi*, which is probably related to the freshwater habitats where the species occurs. In fact, the low nutritional vulnerability of *N. davidi* is comparable to that of another freshwater decapod with abbreviated development, the redclaw crayfish *C. quadricarinatus* (Calvo et al., 2012), and it is far lower than that of many marine species with indirect development (e.g. Gebauer et al., 2010).

Agrawal and Whitlock (2010) have suggested that inbreeding depression tend to be higher in stressful environments that are also novel environments. This is because past selection will not have purged sites that are neutral or weakly selected in the former, representing potential deleterious sites in the latter. In this context, it is likely that the food deprivation protocol used in our study resemble a situation commonly faced by juveniles in freshwater habitats, preventing it from being strong or novel enough to

enhance inbreeding effects on mortality of early juvenile stages. Future work would benefit from the evaluation of juvenile performance under different combinations of starvation with other biotic and abiotic factors to determine the actual magnitude of inbreeding depression.

4.4. Incestuous mating

The proportion of ovigerous females was similar and maximal (100%) in the inbreeding and outbreeding treatments. This result may indicate that mating with brothers was as likely as mating with non-brothers in our no-choice experiment. The probability of females rejecting siblings as mates when no other males are available is variable across invertebrate taxa. For example, the proportion of females mating with related males was lower than that mating with unrelated males in the hermaphroditic snail *Physa acuta* (Facon et al., 2006), the parasitoid wasp *Venturia canescens* (Metzger et al., 2010), the sawfly *Neodiprion lecontei* (Harper et al., 2016) and the wolf spider *P. astrigera* (Chen et al., 2017). However, no kin discriminatory behavior has been observed in other species evaluated under no-choice conditions, such as the parasitic wasp *Aphidius matricariae* (Bourdais and Hance, 2009), the fruit fly *Drosophila melanogaster* (Tan et al., 2012) and the leaf beetle, *Phaedon cochleariae* (Müller and Müller, 2016).

Inbreeding may be more costly for *N. davidi* females than males due to their higher reproductive investment (i.e. vitellogenesis and egg maternal care) and fewer breeding opportunities. The energy invested in reproduction could be alternatively derived to somatic growth, as demonstrated by Tropea and López Greco (2015), who observed a higher growth rate in adult females with no reproductive activity than in reproductive ones. Hence, females rejecting unattractive males could eventually enhance their reproductive output in the future by achieving larger sizes and, consequently, higher

fecundity (Tropea and López Greco, 2015). Male rejection is feasible in this species, since males are smaller than females and lack cheliped weaponry, allowing for females to exert some degree of control over which male will sire their offspring and when. In this context, we assumed as possible that females prioritize somatic growth over reproduction when brothers were the only available males, by recognizing and rejecting them as mates. However, it was clear from our results that in case any control mechanism exists, it was not an effective mean to avoid copulation and egg fertilization as all females from the inbreeding treatment spawned and incubated fertile eggs. Whether *N. davidi* females choose their mates based on male relatedness and whether they use pre- or post-copulatory mechanisms to avoid inbreeding warrants further investigation.

In conclusion, the fitness of the freshwater shrimp *N. davidi* decreases with inbreeding in magnitude that varies with the evaluated trait, rearing condition and developmental stage. Incestuous mating negatively affected survival of embryos and late juvenile stages (from S8 to S>16), but not of early juvenile stages (from hatching to S8). Juvenile growth was only slightly influenced by inbreeding under optimal laboratory conditions, with nutritional stress evidencing a higher impact of inbreeding on this variable. Other traits, such as the number of eggs laid and juvenile size at hatching, were similar in inbred and outbred clutches. The evaluation of only one generation of full-sibling mating may explain the absence or subtle effects of inbreeding for some traits and developmental stages. Moreover, the fact that nearly 50% of juveniles survived after 20 days of food deprivation may suggest that the protocol used resembled a natural situation that was not adverse enough to evidence a more acute effect of inbreeding on juvenile fitness. The comparison of our results with those previously reported in other

crustaceans with direct and indirect development shows that the effect of inbreeding on fecundity, survival and growth is variable across species, with no evident pattern relating inbreeding consequences with life-history traits. More comparative studies are needed at this point to further investigate the effects of inbreeding at equivalent developmental stages of freshwater and marine species under similar rearing conditions.

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Figure captions

Fig. 1. Scheme of the experimental design. Each first brood (B1) of females from the reproductive stock was raised with a physical separation between male and female siblings, until shrimps attained sexual maturity. At that moment, B1 females were randomly selected and reared in quartets either with their brothers (Inbreeding treatment) or non-brothers (Outbreeding treatment). Each aquarium was a replicate. The first brood of all B1 females were used to evaluate egg production, while the second brood of three out of the four females from each aquarium were used to evaluate juvenile quality. Two of these three second clutches *per* replicate were selected to analyze juvenile survival and growth under food deprivation conditions. The remaining second clutch was selected to analyze those variables under optimal laboratory conditions.

Figure 1

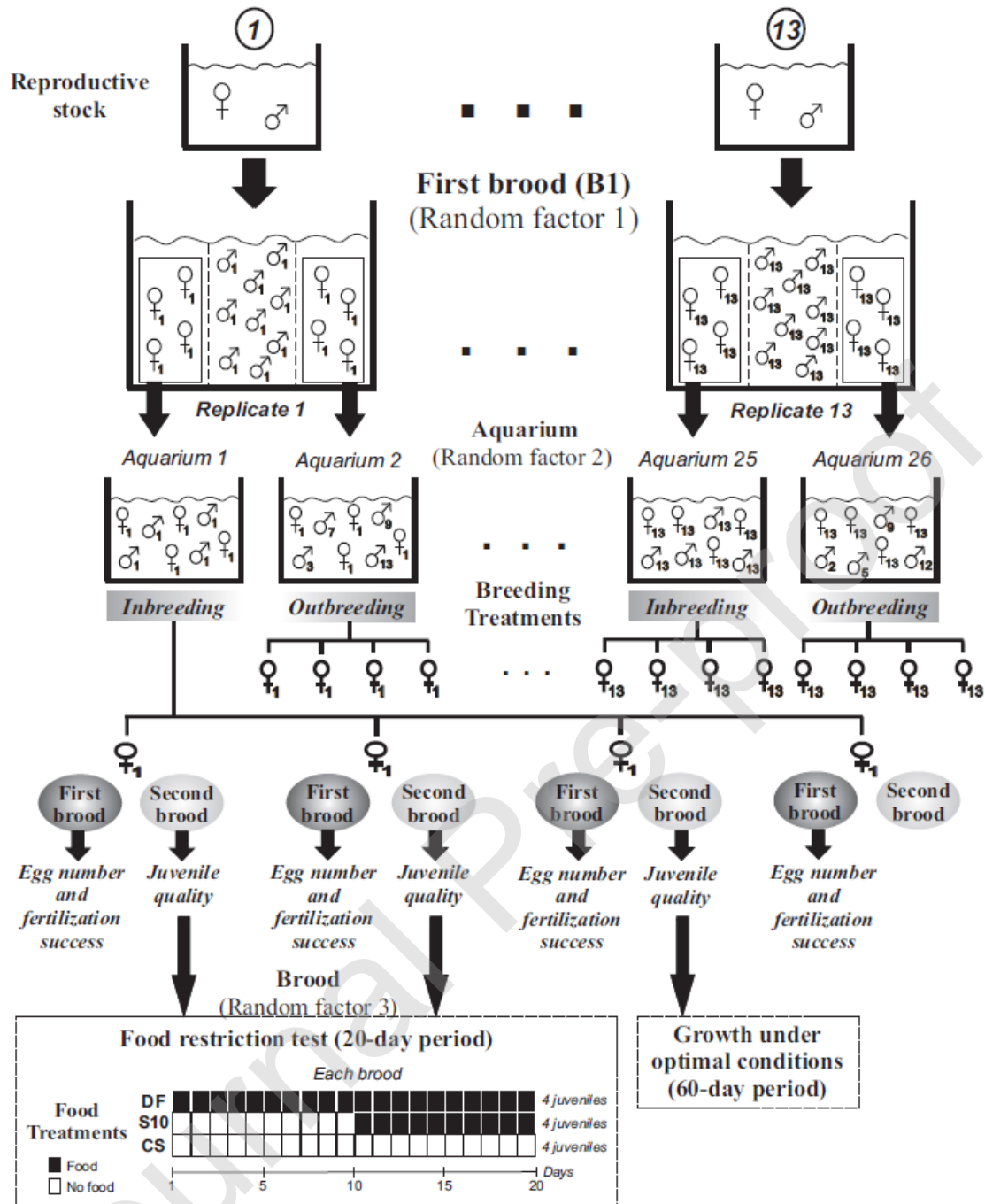
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Fig. 2. Egg production in the caridean shrimp *Neocaridina davidi*. The number of total eggs (A) and fertilized eggs (B) *per* female were analyzed in the first brood of females that had mated with brothers (Inbreeding treatment) and females that had mated with non-brothers (Outbreeding treatment). Results are presented as mean ± SE. Same letters

indicate the absence of statistically significant differences between treatments ($p > 0.05$).

Figure 2

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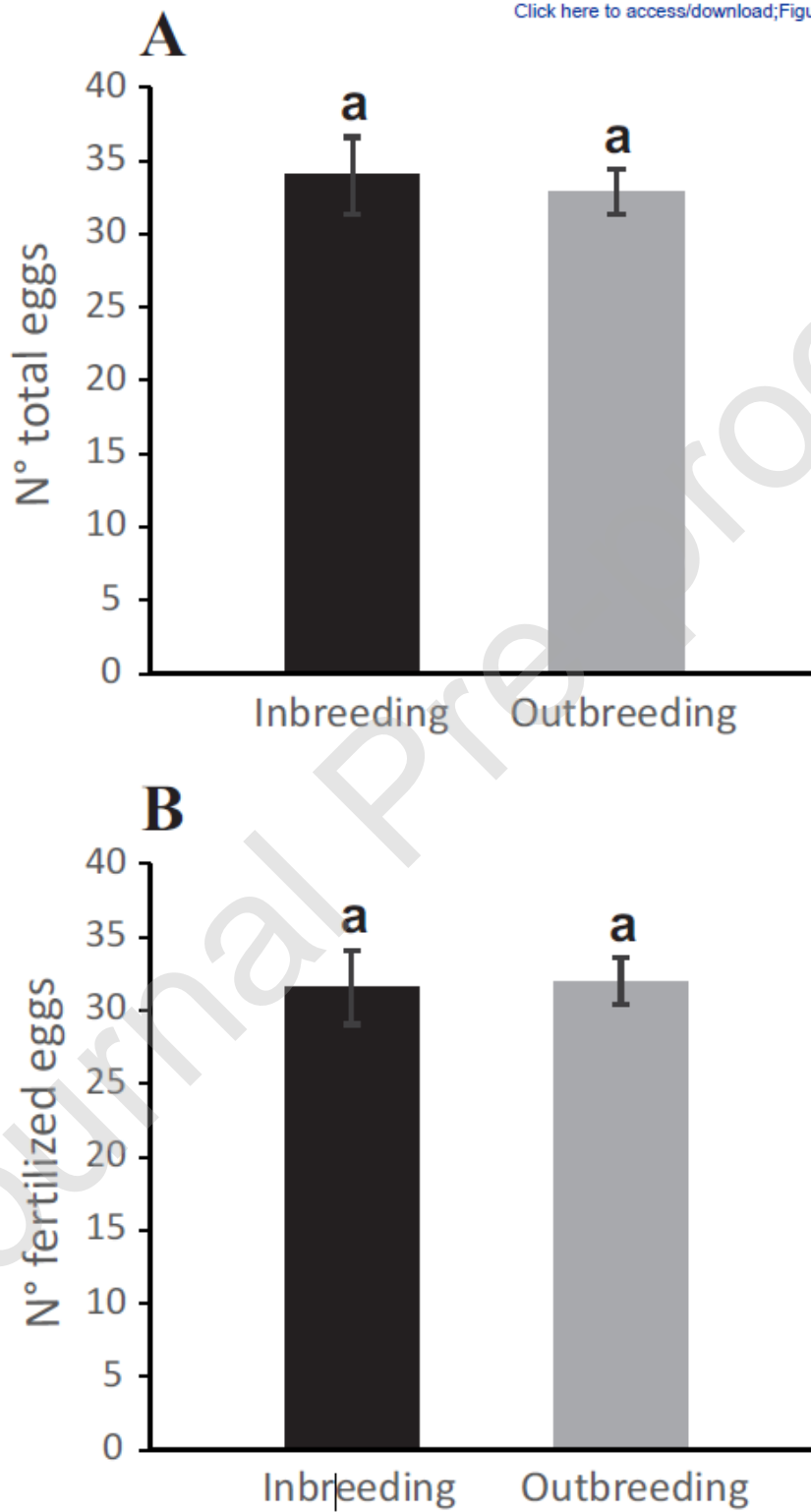


Fig. 3. Juvenile quality in the caridean shrimp *Neocaridina davidi*. The number of newly hatched juveniles *per* female (A) and postorbital cephalothorax length of newly hatched juveniles (B) were analyzed in the second brood of females that had mated with brothers (Inbreeding treatment) and females that had mated with non-brothers (Outbreeding treatment). Results are presented as mean \pm SE. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

Figure 3

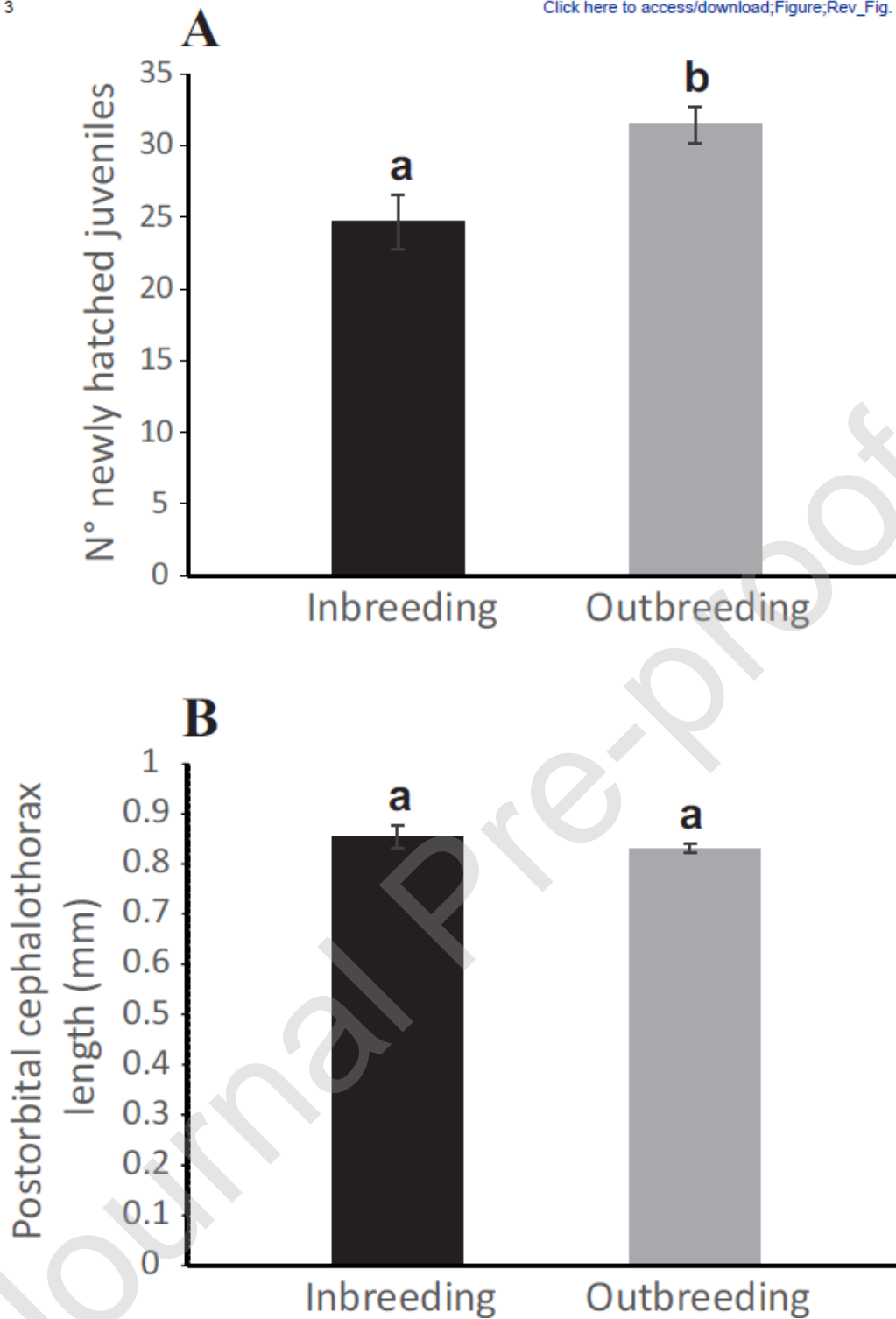
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Fig. 4. Survival of juveniles of the caridean shrimp *Neocaridina davidi* under food deprivation conditions. The percentage of surviving juveniles was compared between clutches produced by full-siblings (Inbreeding treatment) and clutches produced by non-siblings (Outbreeding treatment) (A), and among the food treatments DF (daily feeding

for 20 days following hatching), S10 (food deprivation for 10 days following hatching and daily feeding for the subsequent 10 days) and CS (food deprivation for 20 days following hatching) (B). The temporal variation in juvenile survival was also analyzed (C). Results are presented as mean \pm SE. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$).

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Figure 4

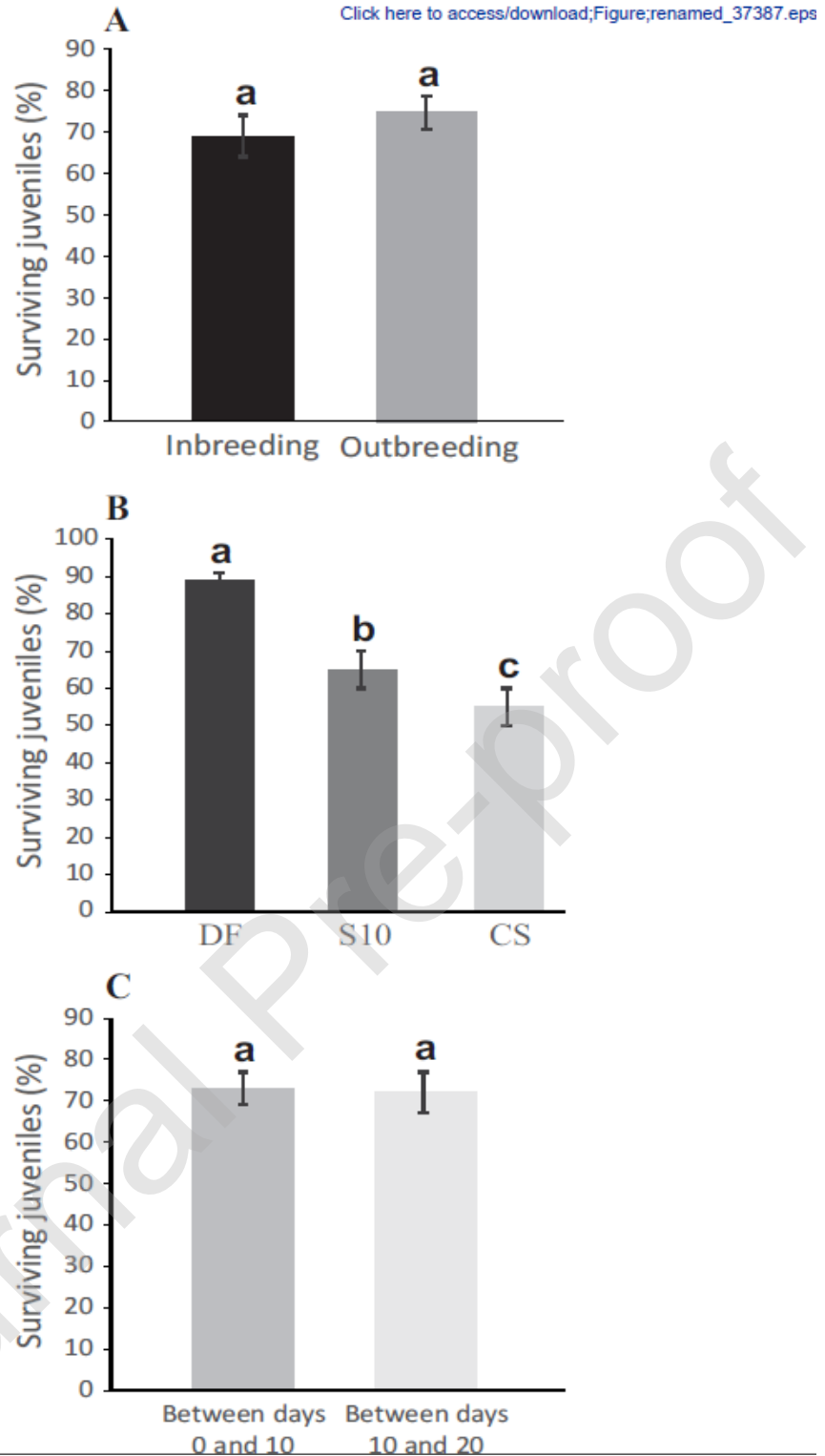


Fig. 5. Growth of juveniles of the caridean shrimp *Neocaridina davidi* under food deprivation conditions. Juvenile length gain between days 0 and 10 and between days 10 and 20, and wet weight at day 20 of the 20-day test period were compared between clutches produced by full-siblings (Inbreeding treatment) and clutches produced by non-

siblings (Outbreeding treatment) (A, C), and among the food treatments DF (daily feeding for 20 days following hatching), S10 (food deprivation for 10 days following hatching and daily feeding for the subsequent 10 days) and CS (food deprivation for 20 days following hatching) (B, D). Results are presented as mean \pm SE. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$).

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Figure 5

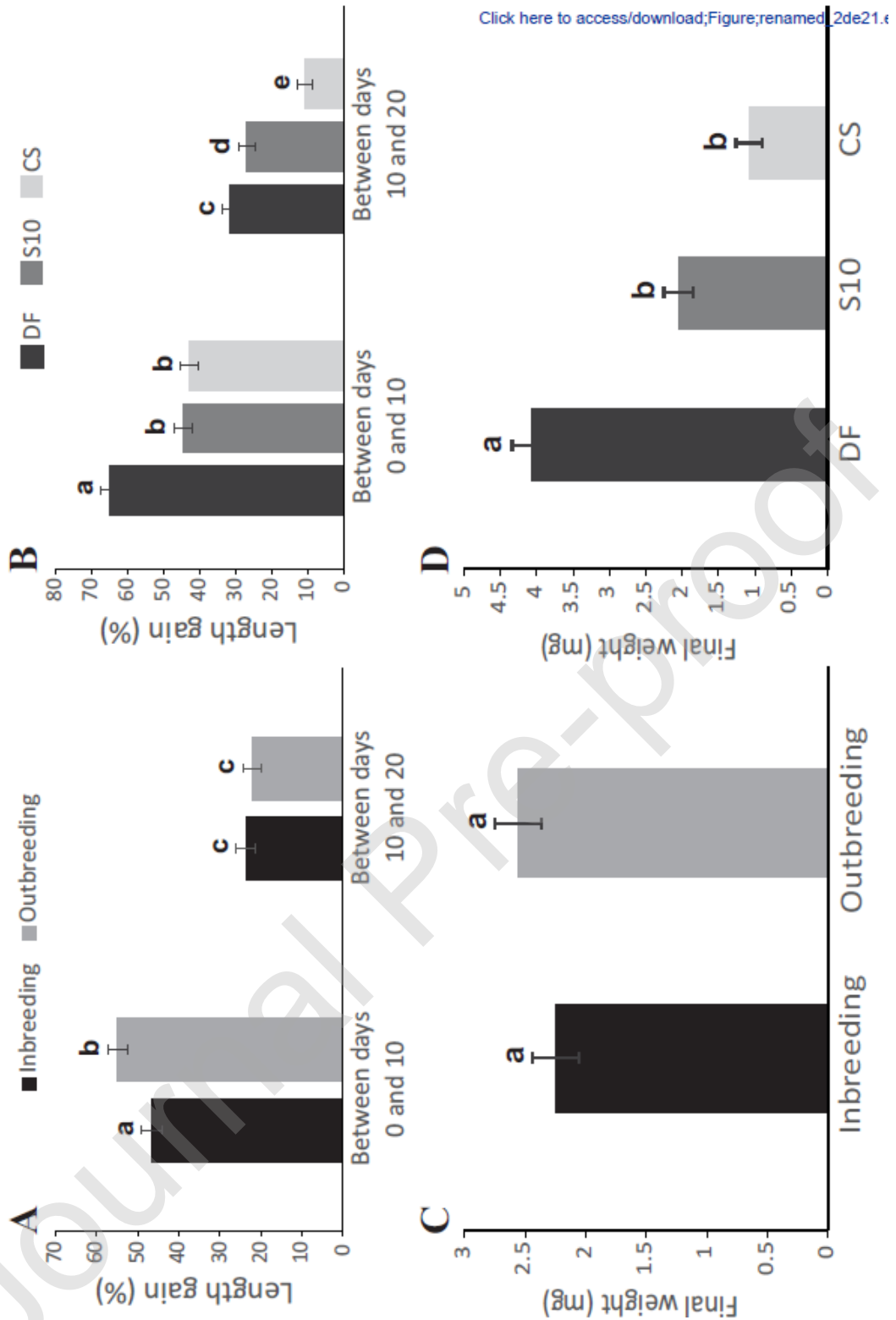


Fig. 6. Performance of juveniles of the caridean shrimp *Neocaridina davidi* under optimal laboratory conditions. The survival (A) and growth (B) of inbred and outbred juveniles were analyzed at days 30 and 60 (survival) and up to day 30 (length gain) of a

60-day period. Results are presented as mean \pm SE. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

Figure 6

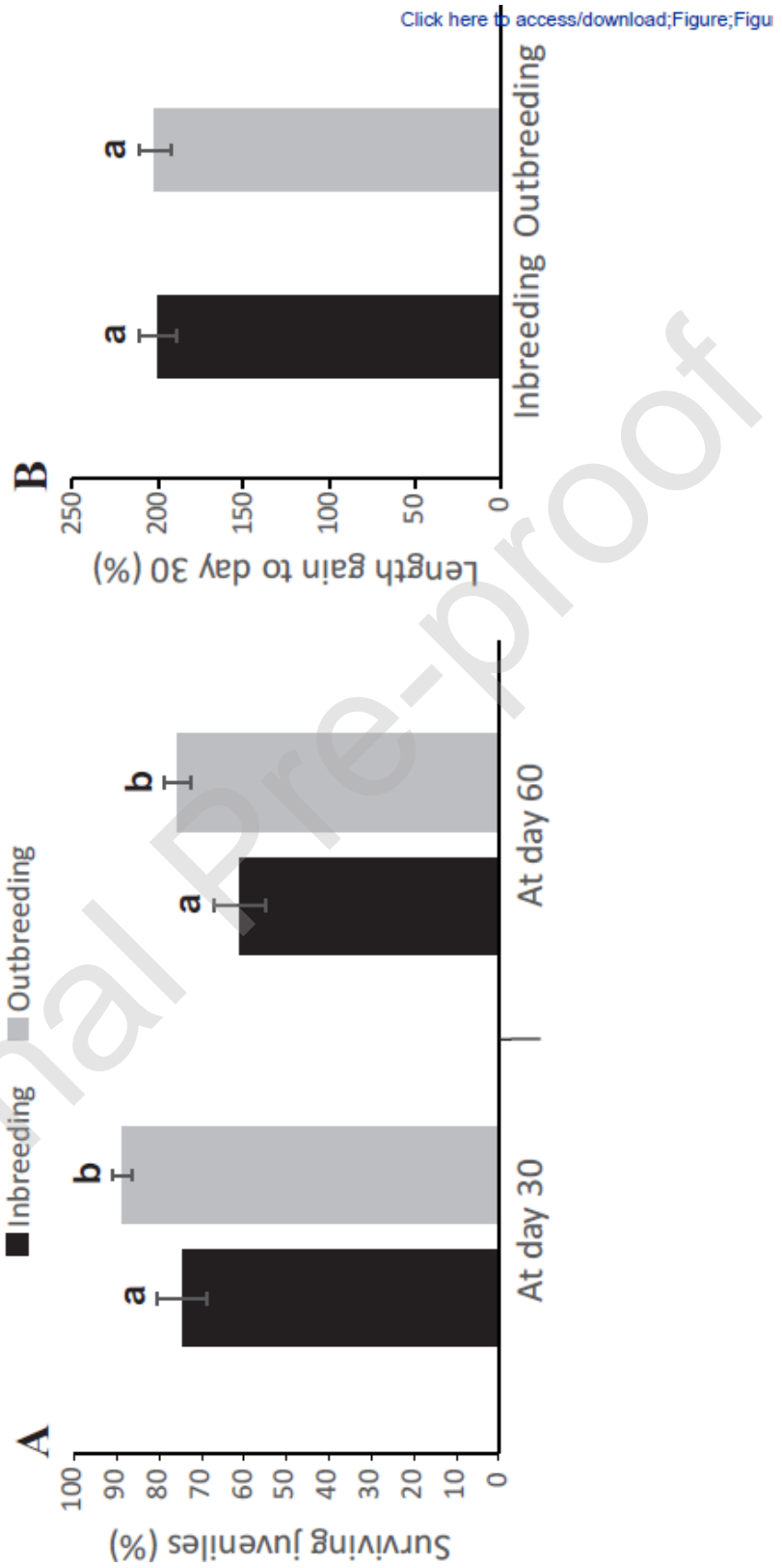


Fig. 7. Growth of juveniles of the caridean shrimp *Neocaridina davidi* under optimal laboratory conditions. Length gain (A) and wet weight gain (B) of female and male shrimps sired by full-siblings (Inbreeding treatment) and those sired by non-siblings (Outbreeding treatment) were analyzed between days 30 and 60 of a 60-day period. Results are presented as mean \pm SE. Different letters indicate statistically significant differences between experimental groups ($p < 0.05$).

Figure 7

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