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Feasibility of compensatory growth in early juveniles of "red claw" crayfish *Cherax quadricarinatus* under high density conditions

Running title: High density culture and compensatory growth response in *Cherax quadricarinatus* crayfish

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Abstract

The aim of this work is to study the feasibility to induce compensatory growth in *Cherax quaricarinatus* crayfish at an early stage of development under high density, the typical conditions of nursery phase. An advantageous characteristic of this species is the capacity to face temporary starvation, especially at early stages of development. This would help to design feeding strategies avoiding overfeeding, and diminishing operating costs in aquaculture. In this sense, during the last 8 years it was analyzed in this species the application of intermittent feeding, known as *unfavorable feeding condition* followed by daily feeding (*favorable feeding condition*). This alternative feeding protocol was used in the present study to trigger compensatory growth. Juveniles weighing 0.07±0.01g were distributed in 2 feeding regimes: C (control): juveniles fed daily during 60 days, and IF (intermittent feeding): juveniles deprived of food for 4 days and then fed for the following

4 days, these 4 days' cycles were repeated during the first 20 days, on day 21 they were daily fed until day 60. Juveniles were stocked in each tank under 0.0096 crayfish/cm^2 density, and zootechnical and biochemical parameters were evaluated throughout 60 days. A very suitable and similar survival (~ 65%) was maintained between feeding regimes, and the previously *unfavorable feeding condition* did not promote greater aggression among juveniles. There was a small compensatory response, but no recovery occurred probably because the *favorable feeding condition* was too short to trigger a strong compensatory response. Hyperphagia and improvement of feed conversion were not observed in juveniles of IF, suggesting that the high density was the key for these primary compensatory mechanisms to be absent. The competition for food, could have affected and changed the priority in allocating energy resources for accelerated growth. Lipids and glycogen content from body mass were strongly depleted after *unfavorable feeding condition*, but there was almost a 100% recovery during *favorable feeding condition*. We suggest that this response was detrimental to body mass as a priority and as a strategy for juveniles to extend survival during the 'double' nutritional stress caused by intermittent feeding and high density. The applicability of this alternative feeding strategy during an intensive production system can be viable, however, some changes must be considered in order to trigger compensatory growth. We suggest that a long-term of the *favorable feeding condition* could trigger a strong compensatory response if the high density tested in the study is maintained. We believe that juveniles of the current study had to face two nutritionally stressful factors: food restriction and high density. This could change the priority in allocation of energetic reserves and then the other suggestion would be to reduce the density if the same alternative feeding protocol is maintained.

Keywords

nutritional resistance; intermittent feeding; compensatory growth; nursery; high density; *Cherax quadricarinatus*

1.Introduction

Compensatory growth is an accelerated somatic growth after an unfavorable feeding condition, e.g., feeding restriction. This unusual response temporarily induces a greater growth rate than cohorts not previously exposed to adverse conditions. This response may result in a catch-up growth, i.e., restricted-fed animals reach the same size than control-fed animals (Jobling, 2010;

Hector and Nakagawa, 2012; Won and Borski, 2013). A suitable favorable feeding condition is essential to achieve major advantages in aquaculture after nutritional restriction. Similarly, the applied unfavorable feeding condition must be appropriate, i.e., it must not be excessive or insufficient (Won and Borski, 2013; Stumpf et al., 2014a; Stumpf and López Greco, 2015).

Among compensatory growth in aquatic organisms, fish is the most studied group due to its great importance in aquaculture (FAO, 2018). There are at least 42 papers published in the last 11 years covering 23 cultivated species or with aquaculture potential (Gabriel et al., 2017, Morshedi et al., 2017; Torfi Mozanzadeh et al., 2017; Peng et al., 2017; Reyes and Baker, 2017; Savoie et al., 2017; Delgadin et al., 2018; Yılmaz et al., 2018, and many others). In crustaceans, there are 23 papers published in the last 18 years (bibliographical citations below), and a few papers were published in other aquatic groups such as molluscs (Vidal et al., 2006) and echinoderms (James and Siikavuopio, 2012; Zhao et al., 2013; Cárcamo, 2015). These studies showed that an adequate feeding strategy to trigger a compensatory response contributes to save the amount of food supplied (Oh et al., 2013; Stumpf et al., 2014a), and the animal protein content in feed (Dong et al., 2012; Sevgili et al., 2012), as well as maintaining better water quality indexes (Turano et al., 2008; Stumpf et al., 2014b; Zhu et al., 2014). All these responses are advantages improving the sustainability of aquaculture practices, since conventional farming methods deploy a constant feeding regime causing waste (Won and Borki, 2013; Limbu and Jumanne, 2014; Adaklı and Taşbozan, 2015).

The first compensatory growth study in decapod crustaceans, was made in the marine shrimp *Fenneropenaues chinensis* in the early 2000s (Wu et al., 2000; Wu et al., 2001; Wu and Dong, 2002a, 2002b, 2002c) using different unfavorable conditions. In the late 2000s, five more studies were published, once more in the marine shrimp *F. chinensis* (Wei et al., 2008), and the first record in the freshwater shrimp *Macrobrachium rosenbergii* (Singh and Balange, 2007) and *M. nipponense* (Li et al., 2009); also, the first record in the marine shrimp *Litopenaeus vannamei* (Zheng et al., 2008), and finally in the freshwater crayfish *Cherax quadricarinatus* (Stumpf et al., 2010). In the past 8 years (2010-2018), the number of papers about this response increased 40% and 14 scientific papers were published. The marine species studied were the shrimp *L. vannamei* by Wasielesky et al. (2013), Fóes et al. (2016), Lara et al. (2017), and Yildirim and Aktaş (2018), *Farfantepenaeus brasiliensis* by Hostins et al. (2015), and *Penaeus monodon* by Mohanty and Mohapatra (2017). In turn, the freshwater species were represented by the shrimp *M. rosenbergii*

(Marques and Lombardi, 2011), and the crayfish *Astacus leptodactylus* (Mazlum et al., 2011) and *C. quadricarinatus* (Stumpf et al., 2011, 2014a, 2014b, Stumpf and Lopez Greco, 2015). This brief benchmarking analysis of compensatory growth in decapods crustacean showed several important issues: 1) the studies in the freshwater crayfish *C. quaricarinatus* were carried with juveniles of nursery phase in a culture system. However, the feasibility to induce compensatory growth was not analyzed at an early stage of development under high density (a real condition in a farm production); 2) the stage of development studied in all decapods crustaceans cited above was the post-larvae or juveniles, which is the nursery phase in a culture system. This shows the importance of this phase in farming; 3) half of these studies were made in group conditions showing the need to evaluate the compensation in a real scale of culture for a future applicability; and 4) more than 60% of the unfavorable conditions assayed were starvation or intermittent feeding, therefore showing the need to look for protocols that imply savings in production costs.

An important characteristic that makes a species relevant for aquaculture is having a low nutritional vulnerability, meaning a high resistance to temporary starvation or feeding restriction, especially in the early stages of development (Anger, 2001, Gebauer et al., 2010). This characteristic helps to design feeding protocols or strategies that avoid overfeeding and diminish operating costs in aquaculture (Calvo et al., 2018). In this sense, early juveniles of *C. quadricarinatus* crayfish have been characterized as highly resistant to endure longer periods of starvation (Calvo, et al., 2011, 2012, 2013, 2018), longer intermittent feeding periods (Stumpf et al., 2011, 2014a, 2014b; Stumpf and López Greco, 2015), and mechanical stress (Castillo Díaz, 2014). A low nutritional vulnerability is very relevant when the organism starts to eat exogenous food, because it is a phase usually related to a bottle neck survival feature in aquaculture (Anger, 2001; Calvo et al., 2012, 2018). Therefore, to expose early juveniles to a physiologically stressful situation, such as starvation or restriction feeding, is also a commonly used tool to evaluate offspring quality in crustaceans (Anger, 2001; Racotta et al., 2003; Marciano et al., 2018).

This study is focused on assessing the capability of juveniles of *C. quadricarinatus* to resist and compensate for intermittent feeding, named as *unfavorable feeding condition,* when they are exposed under high-density conditions (typical conditions of nursery phase). The zootechnical and biochemical parameters were evaluated during 60 days. For this reason, we measured throughout the experiment the survival, the growth as wet and dry body mass, and the variables associated with compensatory growth during the return of *favorable feeding condition,* such as specific

growth rate, feeding intake, and feed conversion ratio. We also assessed the dynamics of energy reserves (total proteins, total lipids, and glycogen) in dry body mass after *unfavorable feeding condition*, and during the return to *favorable feeding condition*. This paper presents new information and discusses the applicability of this alternative feeding strategy in a conventional intensive production system.

2. Materials and Methods

2.1. Animals and acclimatization

Cherax quadricarinatus juveniles used in the present study were the first brood of 5 ovigerous females under laboratory conditions. These females were supplied from a reproductive stock of Centro Nacional de Desarrollo Acuícola (CENADAC), Corrientes, Argentina. Each ovigerous female was placed in an individual glass aquarium ($60 \times 40 \times 30$ cm) containing 30 L of dechlorinated water (pH 7–8, hardness 70–100 mg/L as $CaCO_3$ equivalents) under continuous aeration to maintain a dissolved oxygen concentration of > 5 mg/L. Water temperature was held constant at 27 ± 1 °C and the photoperiod was 14 L:10 D according to Jones (1997). These females were fed daily *ad libitum* with *Elodea* sp. and commercial balanced food for tropical fish Tetracolor, TETRA® (containing 47.5 % crude protein; 6.5 % crude fat; 2.0 % crude fiber; and 6.0 % moisture). This diet is adequate for growth and reproduction of the species under laboratory conditions (Vázquez et al., 2008; Sánchez De Bock and López Greco, 2010; Stumpf et al., 2010; Tropea et al., 2010). The incubation period was around 30 days with 27 ± 1 °C, and once hatched juveniles remained with their mother until complete independence at juvenile stage III (nearly 15 days post hatching). As soon as the hatchlings turned into free juveniles, they were separated from each female and were maintained in glass aquaria ($60 \times 40 \times 30$ cm) containing 30 L of dechlorinated water, termed *growth aquaria*. In these *growth aquaria* juveniles from each female were maintained during 30 days under the same feeding, water quality, temperature, and photoperiod conditions as described above. After 30 days in the *growth aquaria*, a pool of these juveniles weighting around 0.07 g were selected and transferred to experimental units (see below in the section *Experimental procedure and culture conditions*). According to Stumpf et al. (2011), after 30 days of independence from the mother, the stage of development is at the minimum juvenile

VI, because they molt at least three times after juvenile III and weigh between 0.065 g and 0.120 g.

2.2. Feeding regimes

The feeding regimes (treatments) were:

- **Intermittent Feeding (IF)**: juveniles deprived of food for 4 days and then fed for the following 4 days, these 4 days' cycles were repeated during the first 20 days. This condition was termed *unfavorable feeding*. On day 21 they were daily fed until day 60, termed as the *favorable feeding condition*. Thus, the unfavorable condition represents 33 % and the favorable condition represents 67 % of the total experimental period.

- **Control (C)**: juveniles were daily fed throughout the experimental period (days 1 to 60). The daily feeding is the usual feeding condition in a culture, also considered the *favorable condition*. The control was always fed in both conditions: *unfavorable and favorable feeding conditions*.

These feeding regimes were selected based on previous results in this species obtained by Stumpf et al. (2010), Stumpf et al. (2011), Stumpf et al. (2014a), Stumpf and López Greco (2015). As shown in these papers, there was a quick effect in growth after 15 days of intermittent feeding, furthermore compensatory growth was observed when a favorable feeding condition returned.

2.3. Experimental procedure and culture conditions

A random block design with repetition was performed: the brood of each female was a random factor with five levels (offspring 1, 2, 3, 4 and 5), and feeding regimes were a fixed factor with two levels (C and IF). At the beginning of the experiment, a total 240 juveniles weighing 0.07 ± 0.01 g (mean ± standard deviation) from the 5 females were distributed among 30 experimental units (*u.e.*) (Table 1). Each *u.e.* was a rectangular plastic tank (33.5 × 25 × 17 cm) filled with 12 L of freshwater and eight juveniles stocked in each tank, in an equivalent density of 0.0096 crayfish/cm^2 (96 crayfish/m^2). Fifteen initial replicates were used for each feeding regime (Table 1), and during the experimental time (days 20 and 40) five replicates from each feeding regime was sacrificed for biochemical analysis. At the end of the experiment (day 60) five replicates for each feeding regime remained.

In order to reduce cannibalism each tank was provided with 16 PVC tubes (5 cm long and 1 cm in diameter) and 1 piece of synthetic net (52 × 30 cm) as shelters. The experiment was performed under the same conditions of photoperiod, temperature, and water aeration as described above in the subsection *Animals and acclimatization*. The water of the tank was partially replaced (80 %) once a week to remove uneaten food and accumulated excretions. Meanwhile, at days 20 and 40 it was completely replaced (100 %). On feeding days, juveniles were fed at a rate of 10 % of body mass during days 1–20, and at a rate of 7 % of body mass on subsequent days 21–60. The amount of food was adjusted based on the crayfish body mass registered after days 20 and 40. The diet (TETRA®) was the same one used for feeding ovigerous females and during the phase of *growth aquaria* as described in the subsection *Animals and acclimatization*.

2.4. Sample collection and analysis

Juveniles were weighed (on wet basis) at days 1, 20, 40, and 60. Five replicates for each feeding regime were randomly selected at days 20 and 40 and sacrificed (all the juveniles from this plastic tank) after being cold-anesthetized in ice for 15 min. Immediately afterwards, the samples were frozen in a freezer at -70 °C for biochemical analysis. At initial time (day 1), these juveniles and a pool of 10 juveniles from each *growth aquaria,* were lyophilized, in order to analyze the body mass on a dry basis. For an adequate statistical analysis of feeding intake during the *favorable feeding condition*, three replicates for each feeding regime were randomly chosen at the end of the *unfavorable feeding condition,* and their feeding intake was daily registered. Food supplied was weighed and recorded. Uneaten food was collected after 2 h, dried in an oven at 60 °C up to constant mass, weighed and recorded again based on Stumpf and López Greco (2015). Feeding intake was estimated through the difference between the amount of food supplied and uneaten food.

2.5. Biochemical analysis of the energy reserves

To evaluate the dynamics of storage of energy reserves throughout the experiment, the contents of total protein, total lipids, and glycogen were evaluated in the whole body mass. For that, juveniles freeze-dried were pulverized to ensure a homogeneous sample. For total protein determination, a homogenate of each tissue weighting ~ 50 mg was prepared with 800 µL of 50 mM Tris–HCl buffer pH 7.5, and centrifuged at 10,000 rpm for 30 min, at 4 °C. The protein content

of the supernatant was measured colorimetrically by the Coomassie blue dye method (Coomassie blue-distilled water-phosphoric acid mixture), in a spectrophotometer at 595 nm (Bradford, 1976). Serum bovine albumin (Fracc.V, Standard®) was used (1mg/ml) to build the standard curve. For total lipids determination, a modified protocol from Folch et al. (1957) was used. Tissues weighting ~ 50 mg were homogenized with a chloroform-methanol-water mixture (4:2:3 volume) during 3 minutes. After that, this homogenate was filtered, washed with NaCl 0.9 % (20 % of the filtering volume was added), and centrifuged to obtain a lipid phase (chloroform). The sulfo-phospho-vanillin method (sulfuric acid-vanillin- distilled water-phosphoric acid mixture) was used for colorimetric measurement in a spectrophotometer at 530 nm (procedures modified from Frings et al., 1972). Olive oil diluted with absolute ethanol (5 mg/ml) was used to build the standard curve. For glycogen determination, a modified protocol from Van Handel (1965) was used. Tissues weighting ~ 50 mg were digested by boiling with alkaline solution (KOH 30%). Saturated Na_2SO_4 and absolute alcohol were added as precipitants and after that, centrifugation was used to achieve complete glycogen precipitation. The resultant pellet was dissolved with 700 μl of distilled water. Afterwards, the Anthrone reagent (0.2 % anthrone-sulfuric acid mixture) was used for colorimetric measurement of glycogen, in a spectrophotometer at 620 nm. Glycogen of liver rabbit (Fluka®) was used (1 mg/ml) to build the standard curve.

All concentrations of biochemical determinations were expressed as mg/g of tissue. All determinations were performed in triplicate (three sub-samples for each sample) using the Thermo – UV/VIS spectrophotometer.

2.6. Data calculation

For each *u.e.*(tank) the following variables were calculated: Survival, % = (final number of crayfish/initial number of crayfish) ∗ 100; Injuries (%) = (number of crayfish with missing chelae (one or both) / total number of crayfish) * 100; Specific growth rate, %/day = (ln final wet body mass−ln initial wet body mass/days) ∗ 100; Feeding intake, % wet body mass/day = (total dry food consumed/ (final wet body mass initial + wet body mass/2)/days); Feed conversion = (total dry food consumed/wet total mass gain) *100.

2.7. Statistical analyses

As described above in the subsection *Experimental procedure and culture conditions,* a random block design with repetition was performed: the brood of each female was a random factor with five levels (offspring 1, 2, 3, 4 and 5), and feeding regime was a fixed factor with two levels (C and IF). The experimental unit was the tank with the eight juveniles. All analyses were done according to Zuur et al. (2010) and were carried out using R Studio version 3.5.1.

Survival and injuries was tested during the experiment with a generalized linear mixed-effects model (*glmer*) with a repeated measures design. A general linear mixed-effects model (*lme*) with a repeated measures design was used to analyze the wet and dry body mass during *unfavorable and favorable feeding conditions.* The specific growth rate during *favorable feeding condition* was also analyzed using *lme*. For analysis of these models in a repeated measures design, the *u.e* and brood were included as a random effect, and the fixed effect factors were feeding regime and time. The biochemical analysis of crayfish at days 1, 20, 40, and 60 were compared separately using *lme* with brood as a random effect. Feeding regime and time as fixed factors.

When the homogeneity of variance assumption was violated, variance was corrected according to Zuur et al. (2010). The level of significance was set at 0.05. *A posteriori* comparisons were made using Tukey's test in all cases.

3. Results

3.1. *Effect of unfavorable feeding condition and return to favorable feeding condition on zootechnical parameters*

Juvenile survival during the experiment was similar between feeding regimes (*P*=0.557) with a mean value of 65 % at day 60 (Fig.1). The density was reduced from 0.0096 juveniles/cm^2 at the beginning of the experiment to ~0.0058 juveniles/cm^2 at day 60. However, there were more individuals injured in C with an average of 45 %, than in IF with an average of 18 % (Wald Chi-square test: $\chi^2 = 4.130$; degree of freedom=1; *P*=0.042).

At the end of *unfavorable feeding condition*, the juveniles fed intermittently were 36 % smaller (in terms of wet body mass) than those juveniles fed daily (*P*<0.0001, Fig.2a). When the growth was measured without the water content, this difference increased to 53 % (*P*<0.0001, Fig.2c). Once returned to the *favorable feeding condition*, the difference in terms of wet body mass was reduced over time (36 % to 21 % at day 40, *P*=0.020). However, the juveniles fed

intermittently for an additional 20 days, did not achieve the same wet body mass than those juveniles fed daily. Juveniles from IF treatment were smaller than juveniles from C (19 % lower) (Fig.2b). When body mass was measured in terms of organic compounds plus mineral fraction (dry basis), this difference was reduced to 10 % (Fig.2d).

There was a slight compensatory growth in juveniles exposed to intermittent feeding during the first 20 days after returning to the *favorable feeding condition* (P=0.001). However, Tukey's test did not recognize this 20 % higher growth than C, during this period. The percentage of this growth was weak, as it did not lead to a later recovery or catch-up (Fig.3a). None of the physiological mechanisms involved in compensatory growth were observed in the juveniles of IF, such as a higher feeding intake (P=0.434), and a lower feed conversion ratio (P=0.782). the absence of these primary compensatory mechanisms, might explain the weakness of the compensation in this experiment (Fig.3b, c).

3.2. Effect of unfavorable feeding condition and return to favorable feeding condition on energy reserves

A significant effect of time over protein content (P=0.302) was observed, and it was higher in the juveniles at the end of the experiment (Fig.4a). In regard to the lipid content, only the effect of time was significant (P= 0.028). However, a trend was observed, showing an impact of the *unfavorable feeding condition* in the amount of lipids for juveniles exposed to IF. These reserves were in average 50% lower than in C at day 20 (Fig.4b). Considering glycogen, an effect of time ($P = 0.020$) and of feeding regime ($P = 0.002$) were observed. In this case, at day 40, IF juveniles showed a 30 % lower than in C (Fig.4c).

The feeding regime did not have a significant effect on some components of the biomass of juvenile crayfish (Table 2). The exception was the percentage of glycogen in relation to dry mass, where the Anova of the feeding regime was significant (P=0.002), but Tukey's contrasts did not make any difference between feeding regimes. However, taking into account the difference between means at the end of both conditions (unfavorable and favorable), some interesting biological trends were observed. The content of water did not change at days 20 and 60, but the content of protein was ~ 8 % lower in IF, and this difference increased to 16 % over time. In the case of lipids and glycogen, both followed the trend already described in Figure 4, where these

reserves were 50 % and 30 % lower, respectively, than C at day 20. However, at the end of the experiment this difference decreased to 5 % and 11 %, respectively.

4. Discussion

The analysis about the applicability of a temporary unfavorable feeding condition, as an alternative feeding strategy in the nursery phase of *C. quadricarinatus,* must start with a discussion about survival. In this study, a very suitable and similar survival (~ 65 %) was maintained in either intermittently or daily fed juveniles, growing in an initial high-density (0.0096 juveniles/cm^2). The nursery phase in crustaceans must be cultured with an appropriate stocking density to avoid the problems caused when there is an excess of individuals in a small area. The problems caused by a critical reduction of the space available are exacerbated cannibalism and injuries, affecting the survival and growth of the juveniles (Yuan et al., 2018). This agonistic behavior of conspecifics attacking or cannibalizing each other are found especially in the early phase of growth in crustaceans, due a high frequency of molting (Romano and Zeng, 2017). Then for the nursery phase to be efficient, a survival higher than 70 % is desirable (Moss and Moss, 2004; Mishra et al., 2008; Foes et al., 2016). In this sense, the current study showed that the survival obtained was close to the value considered efficient. However, the experiment was carried out in laboratory conditions, and this is not a minor issue, because there was no contribution of a primary and secondary production. In real farming conditions, a natural food source provides benefits to survival and growth, especially during nursery phase (Wasielesky et al., 2013). Therefore, we hypothesized that in semi-intensive culture, where natural productivity in the pond may partly satisfy the protein requirements of the red claw crayfish, the use of this kind of an intermittent feeding protocol, can be a good strategy to reduce the use of formulated feeds (Steinberg, 2018). Our study showed that the type of *unfavorable feeding condition*, did not promote greater aggression among juveniles intermittently fed, when compared with juveniles daily fed. In fact, as was displayed in the lower percentages of injured juveniles in IF, a lower agonistic behavior was exhibited.

Regarding the applicability of this strategy to trigger compensatory growth, in our study there was a small response and certainly no recovery was displayed. We suggest that a long-term of the *favorable feeding condition* could trigger a strong compensatory response. This assumption is based on the fact that crayfish in early stages of development (0.02 g – 1 g) present a high

frequency of molting with a high growth increment (Stumpf et al. 2011, 2014a). Hence, two situations could have taken place: that growth increase after molting of those juveniles previously fed intermittently was insufficient, or that they did not achieve the same number of molts as the control. In both situations, they would need a longer recovery time. The relation between favorable feeding and unfavorable feeding conditions must be adequate to induce a strong compensatory response as was observed in a polyculture of fingerlings of three species of Indian major carps: *Catla catla, Labeo rohita,* and *Cirrhinus mrigala* by Mohanta et al. (2017).

None of the physiological mechanisms, such as hyperphagia and improvement of feed conversion, were triggered in juveniles of IF for the promotion of the compensatory response during the experimental time. According to Won and Borski (2013) there is a catabolic phase that is necessary to trigger the compensatory growth potential. In this catabolism, endogenous energy reserves are depleting and endocrine profiles are altered, modulating the inhibitors and stimulants of appetite. However, a moderate catabolism is necessary because brief periods of unfavorable feeding conditions do not exhaust sufficiently the stored energy, and excessively long periods of this condition can lead to an irrecoverable lapse in growth that prevents full catch-up (Dar, 2018). In addition, the fuel for compensatory growth is the hyperphagic influx of exogenous energy and the food, is as well assimilated with heightened efficiency as the result of modifications to metabolic substrate absorption (Won and Borski, 2013). In this sense, *C. quadricarinatus* juveniles were able to compensate the delayed growth in previous studies as shown by Stumpf et al. (2014a), and Stumpf and López Greco (2015), because they had a hyperphagic response and improved their feeding conversion ratio. In the first study, the same intermittent feeding protocol was used here: 1/3 unfavorable condition + 2/3 favorable condition, but in an experimental time period of 120 days, and 1 gram juveniles under a density of $0.0021/cm^2$. In the latter study, the unfavorable and favorable feeding conditions were 50 % each, but the juveniles (1 gram) were under individual conditions of growth. Therefore, we wondered, why in the present study the mechanisms of hyperphagia and improvement of feed conversion were not activated to compensate for the slow growth? Our assumption was that the high density was the key. This could have affected the competition for food, which may have caused an unequal food intake, and also changed the priority in allocating energy resources for accelerated growth. In this sense, Farhadi and Jensen (2015) observed a reduction in the growth of crayfish *A. leptodactylus* cultured in a stress situation (longer photophase). They argued that this stress could have suppressed the appetite and the reallocation

of energy to activities such as restoring homoeostatic processes and tissue repair. On the other hand, when the stress factor was a predation risk for a short-term exposure, the lobster *Jasus edwardsii* exhibited immobility as a response (Briceño et al., 2018). These authors argued that if this behavior persists for a long term, it could have significant consequences on foraging time and foraging area, with an overall impact on the lobster performance. As described above, we believe that juveniles of the current study had to face two nutritionally stressful factors: food restriction and high density. This could change the priority in allocation of energetic reserves.

In regards to the dynamics of energetic reserves, lipids, and glycogen of body mass were strongly depleted after the *unfavorable feeding condition*. The same reserves were depleted in crayfish early juveniles when they were starved during 8 and 9 days, as observed by Calvo et al. (2018), and after 15 days of starvation in advanced juveniles (Sacristán et al., 2016). The saving of protein in both cases can be related to the high growth taking place in this stage (Anger, 2001). In our study, the recovery of reserves depleted was almost 100 % during *favorable feeding condition*. This response might be detrimental to body mass as a priority and a strategy for *C. quadricarinatus* juveniles to extend survival during the 'double' nutritional stress. Finally, the applicability of this alternative feeding strategy can be viable in a usual condition in an intensive production system, because highly satisfactory results were obtained in this study. However, some changes must be taken into account to trigger a strong compensatory growth and to improve the protocol.

5. Conclusion

Early juveniles of red claw crayfish fed intermittently and cultured under high-density conditions, have the capability of compensation and the information bellow is complementary for this species:

- The early juveniles of crayfish (depending exclusively on exogenous balanced food) were resistant to intermittent feeding under high density, showing sound survival values.
- Intermittent feeding for 20 days had a strong impact in dry body mass of juveniles, showing that lipids, and glycogen were depleted to face this *unfavorable feeding condition.*
- There was a priority in allocating energy resources during the *favorable feeding condition* to recover these reserves, instead of allocating them to accelerated growth.

- At least for this size (~0.07 g), and density (0.0096 juveniles/cm^2), these juveniles would need a longer time than 40 days of *favorable feeding condition* after 20 days of intermittent feeding. The grounds for this assumption might be, that the juvenile's growth was insufficient after molts, or that they did not achieve the same number of molts as the control.

- Another use of this feeding strategy: 1/3 *unfavorable feeding + 2/3 favorable feeding conditions* may give responses about progeny quality (stress test), since the objective of the nursery phase is to obtain healthy, larger and more resilient organisms for the subsequent grow out phase in a commercial production.

Acknowledgments

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Figure captions

Figure 1. Survival of *Cherax quadricarinatus* juveniles reared under high density over a 60-day period. Feeding regimes: **C** (juveniles fed daily during *unfavorable* and *favorable feeding condition*); **IF** (juveniles starved for 4 days followed by 4 days of feeding in repeated cycles during the *unfavorable feeding condition* (days 1-20), and fed daily during the *favorable feeding condition* (days 21-60). Data are displayed as mean and standard deviation. Numbers inside parenthesis indicate replicates *per* treatment. **Wald Chi-square test results in a generalized linear mixed-effect model** for feeding regime: χ^2-value = 0.344; degrees of freedom = 1; *P*-value = 0.557. For time: χ^2-value = 10.352; degrees of freedom = 2; *P*-value = 0.005. For feeding regime*time: χ^2-value = 0.0128; degrees of freedom = 2; *P*-value = 0.993.

Figure 2. Wet and dry body mass (g) *Cherax quadricarinatus* juveniles reared under high density over a 60-day period. (**a, c**) During the unfavorable condition and (**b, d**) during the favorable con dition. Feeding regimes: Feeding regimes: **C** (juveniles fed daily during *unfavorable* and *favorab le feeding condition*); **IF** (juveniles starved for 4 days followed by 4 days of feeding in repeated c ycles during the *unfavorable feeding condition* (days 1-20), and fed daily during the *favorable fee ding condition* (days 21-60). Data are displayed as Box-plot: cross = mean, line = median, box = 25–75%, whiskers = min–max. Number inside parenthesis indicates replicates *per* treatment. Let ters indicate when feeding regime x time interaction has *P*< 0.05; ** indicates when time has *P*<

0.05; * indicates when feeding regime has $P < 0.05$. (a) **ANOVA results in a linear mixed effects model** for feeding regime: F-value = 0.956; degrees of freedom = 1; P-value = 0.338. For time: F-value = 0.956; degrees of freedom = 1,24; P-value = 0.338. For feeding regime*time: F-value = 75.23; degrees of freedom:1,28; P-value = <.0001. (b) **ANOVA results in a linear mixed effects model** for feeding regime: F-value = 5.737; degrees of freedom = 1;23 P-value = 0.025. For time : F-value = 64.807; degrees of freedom = 1,23; P-value = 4.436e-08. For feeding regime*time: F-value = 0.416; degrees of freedom:1,23; P-value = 0.525. (c) **ANOVA results in a linear mixed effects model** for feeding regime: F-value = 0.956; degrees of freedom = 1;24 P-value = 0.338. For time: F-value = 742.786; degrees of freedom = 1,28; P-value = <.0001. For feeding regime*time: F-value = 75.222; degrees of freedom:1,28; P-value = <.0001 . (d) **ANOVA results in a linear mixed effects model** for feeding regime: F-value = 7.622; degrees of freedom = 1;10 P-value = 0.020. For time: F-value = 14.218; degrees of freedom = 1,10; P-value = 0.004. For feeding regime*time: F-value = 0.633; degrees of freedom:1,10; P-value = 0.444.

Figure 3. Compensatory response and primary physiological mechanisms of *Cherax quadricarinatus* juveniles reared under high density over a favorable feeding condition. (**a**) Growth as specific growth rate, (**b**) Food consumption as feeding intake and, (**c**) Feed efficiency as feed conversion ratio. Feeding regimes: Feeding regimes: **C** (juveniles fed daily during *unfavorable* and *favorable feeding condition*); **IF** (juveniles starved for 4 days followed by 4 days of feeding in repeated cycles during the *unfavorable feeding condition* (days 1-20), and fed daily during the *favorable feeding condition* (days 21-60). Data are displayed as mean and standard deviation. Numbers inside parenthesis indicate replicates *per* treatment. (a) **ANOVA results in a linear mixed effects model** for feeding regime: F-value = 2; degrees of freedom = 1;14 P-value = 0.136. For time: F-value = 1069055; degrees of freedom = 1,8; P-value = <.0001. For feeding regime*time: F-value = 3108023; degrees of freedom:1,8; P-value = <.0001. (b) **ANOVA results in a linear mixed effects model** for feeding regime: F-value = 0.9446; degrees of freedom = 1;2 P-value = 0.434. For time: F-value = 4.654; degrees of freedom = 1,4; P-value = 0.09. For feeding regime*time: F-value = 1.818; degrees of freedom:1,4; P-value = 0.249. (c) **ANOVA results in a linear mixed effects model** for feeding regime: F-value = 0.100; degrees of freedom = 1;2 P-value = 0.782. For time: F-value = 2.212; degrees of freedom = 1,4; P-value = 0.211. For feeding regime*time: F-value = 0.249; degrees of freedom:1,4; P-value = 0.065.

Figure 4. Energy reserves (in terms of mg/g dry body mass) of *Cherax quadricarinatus* juveniles reared under high density over a 60-day period. (**a**) Total proteins, (**b**) Total lipids, and (**c**) Glycogen. Feeding regimes: Feeding regimes: **C** (juveniles fed daily during *unfavorable* and *favorable feeding condition*); **IF** (juveniles starved for 4 days followed by 4 days of feeding in repeated cycles during the *unfavorable feeding condition* (days 1-20), and fed daily during the *favorable feeding condition* (days 21-60). Data are displayed as Box-plot: cross = mean, line = median, box = 25–75%, whiskers = min–max. Numbers inside parenthesis indicate replicates *per* treatment. (a) **ANOVA results in a linear mixed effects model** for <u>feeding regime</u>: F-value = 3.574; degrees of freedom = 1,20; *P*-value = 0.073. For <u>time</u>: F-value = 3.594; degrees of freedom = 3,20; *P*-value = 0.032. For <u>feeding regime*time</u>: F-value =0.467; degrees of freedom:3,20; *P*-value = 0.708. (b) **ANOVA results in a linear mixed effects model** for <u>feeding regime</u>: F-value = 1.123; degrees of freedom = 1,19; *P*-value = 0.302. For <u>time</u>: F-value = 3.773; degrees of freedom = 3,19; *P*-value = 0.028. For <u>feeding regime*time</u>: F-value =1.895; degrees of freedom:3,19; *P*-value = 0.164. (c) **ANOVA results in a linear mixed effects model** for <u>feeding regime</u>: F-value = 6.736; degrees of freedom = 1;18 *P*-value = 0.020. For <u>time</u>: F-value = 7.457; degrees of freedom = 3,18; *P*-value = 0.002. For <u>feeding regime*time</u>: F-value =0.631; degrees of freedom:3,18; *P*-value = 0.604.

Table 1. Experimental design used in the current experiment. Feeding regimes: **C** (juveniles fed daily during *unfavorable* and *favorable feeding condition*); **IF** (juveniles starved for 4 days followed by 4 days of feeding in repeated cycles during the *unfavorable feeding condition* (days 1-20), and fed daily during the *favorable feeding condition* (days 21-60). Data about initial body mass (in wet basis) are displayed as mean and standard deviation.

Number of brood (block)	Feeding regime (fixed)	Initial number of replicate (tank)	Initial number of juveniles	Initial body mass (g)
1	C	1	8	0.056±0.008
1	C	2	8	0.059±0.009
1	C	3	8	0.056±0.007
2	C	4	8	0.060±0.005
2	C	5	8	0.061±0.005
2	C	6	8	0.061±0.004
3	C	7	8	0.075±0.012
3	C	8	8	0.076±0.018
3	C	9	8	0.076±0.014
4	C	10	8	0.068±0.023
4	C	11	8	0.069±0.020
4	C	12	8	0.072±0.022
5	C	13	8	0.077±0.015
5	C	14	8	0.078±0.015
5	C	15	8	0.080±0.020
1	IF	1	8	0.056±0.009
1	IF	2	8	0.055±0.009
1	IF	3	8	0.059±0.006
2	IF	4	8	0.062±0.004
2	IF	5	8	0.061±0.004
2	IF	6	8	0.062±0.004
3	IF	7	8	0.076±0.014
3	IF	8	8	0.076±0.017
3	IF	9	8	0.075±0.015
4	IF	10	8	0.076±0.019
4	IF	11	8	0.064±0.016
4	IF	12	8	0.068±0.015

5	IF	13	8	0.076±0.018
5	IF	14	8	0.076±0.017
5	IF	15	8	0.081±0.024

Table 2. Changes in the content of composition of biomass (water and energy reserves, in terms of dry mass percentage -%DM) of *Cherax quadricarinatus* juveniles reared under high density over a 60-day period. Feeding regimes: Feeding regimes: **C** (juveniles fed daily during *unfavorable* and *favorable feeding condition*); **IF** (juveniles starved for 4 days followed by 4 days of feeding in repeated cycles during the *unfavorable feeding condition* (days 1-20), and fed daily during the *favorable feeding condition* (days 21-60). At each time 3 replicates *per* treatment were analyzed.

Composition of biomass	Feeding regimes	Time of experiment (days)			
		1	20	40	60
Water (%DM)[1]	C_{60}	77.96 ± 5.14 **	88.97 ± 6.31	86.20 ± 7.84	87.10 ± 5.16
	$IF_{20}+DF_{40}$		90.95 ± 4.73	90.97 ± 2.75	84.58 ± 1.94
Protein (%DM)[2]	C_{60}	6.72 ± 0.56	6.38 ± 1.94	7.47 ± 0.90	8.85 ± 1.20 **
	$IF_{20}+DF_{40}$		5.86 ± 1.98	6.01 ± 1.75	7.47 ± 1.01
Lipids (%DM)[3]	C_{60}	1.60 ± 0.22	1.59 ± 0.42	0.76 ± 0.53 **	1.17 ± 0.49
	$IF_{20}+DF_{40}$		0.80 ± 0.46	0.90 ± 0.15	1.11 ± 0.65
Glycogen (%DM)[4]	C_{60}	0.50 ± 0.15	0.49 ± 0.09	0.43 ± 0.05	0.97 ± 0.25 **
	$IF_{20}+DF_{40}$		0.35 ± 0.04 *	0.36 ± 0.02 *	1.08 ± 0.56*

[1] **ANOVA results in a linear mixed effects model** for <u>feeding regime</u>: F-value = 0.250; degrees of freedom = 1,20; *p*-value = 0.622. For <u>time</u>: F-value = 7.295; degrees of freedom = 3,20; *p*-value = 0.02. For <u>feeding regime*time</u>: F-value = 0.466; degrees of freedom:3,20; *P*-value = 0.809.

[2] **ANOVA results in a linear mixed effects model** for <u>feeding regime</u>: F-value = 3.571; degrees of freedom = 1,20; *P*-value = 0.073. For <u>time</u>: F-value = 3.601; degrees of freedom = 3,20; *P*-value = 0.031. For <u>feeding regime*time</u>: F-value = 0.466; degrees of freedom:3,20; *P*-value = 0.709.

[3] **ANOVA results in a linear mixed effects model** for <u>feeding regime</u>: F-value = 1.118; degrees of freedom = 1,19; *P*-value = 0.304. For <u>time</u>: F-value = 3.795; degrees of freedom = 3,19; *P*-value = 0.027. For <u>feeding regime*time</u>: F-value = 1.888; degrees of freedom:3,19; *P*-value = 0.166.

[4] **ANOVA results in a linear mixed effects model** for <u>feeding regime</u>: F-value = 8.986; degrees of freedom = 1,18; *P*-value = 0.008. For <u>time</u>: F-value = 7.246; degrees of freedom = 3,18; *P*-value = 0.002. For <u>feeding regime*time</u>: F-value = 0.856; degrees of freedom:3,18; *P*-value = 0.481.

 ** indicates when time has *P*< 0.05; * indicates when feeding regime has *P*< 0.05.

Highlights:

- Early juvenile crayfish was resistant to intermittent feeding under high density, showing suitable survival values.

- Early juvenile crayfish fed intermittently for 20 days had a strong impact in dry body mass, showing that lipids and glycogen were depleted to face this *unfavorable feeding condition.*

- Early juvenile crayfish at size ~0.07 g and under density of 97 juveniles/m^2 would need a longer time than 40 days of *favorable feeding condition* after 20 days of intermittent feeding.