

ARTICLE

Acute hyperosmotic stress test for vigor assessment of first-feeding larvae of spotted sand bass *Paralabrax maculatofasciatus* and spotted rose snapper *Lutjanus guttatus*

Prueba de estrés hiperosmótico agudo para evaluar el vigor de larvas a la primera alimentación de la cabrilla arenera *Paralabrax maculatofasciatus* y del pargo flamenco *Lutjanus guttatus*

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Resumen.- En este estudio se investigó la tolerancia de larvas a la primera alimentación de la cabrilla arenera *Paralabrax maculatofasciatus* y del pargo flamenco *Lutjanus guttatus* a un estrés hiperosmótico agudo. Embriones de *P. maculatofasciatus* y *L. guttatus* fueron incubados en microplacas de 48 celdas a 23 y 28°C, respectivamente. Larvas de primera alimentación fueron expuestas por triplicado a salinidades de 35 a 95 g L⁻¹ ó 35 a 85 g L⁻¹, y la supervivencia fue monitoreada de 0,5 a 3,5 h ó 0,5 a 53 h, para *P. maculatofasciatus* y *L. guttatus*, respectivamente. Los resultados mostraron que la mortalidad aumentó de manera diferente entre los tratamientos desde el primer tiempo de exposición en cada especie. Salinidades extremas fueron letales en 30 min. Salinidades cercanas al control fueron sub-letales. En salinidades intermedias, la mortalidad se ajustó a un modelo potencial o lineal. Para *P. maculatofasciatus*, los valores de LC₅₀ obtenidos entre 0,5 y 3,5 h fueron de 73,4 a 65,4 g L⁻¹. Para *L. guttatus* los valores de LC₅₀ obtenidos entre 2 y 5 h fueron de 57,3 a 54,5 g L⁻¹. La comparación de LC₅₀ entre especies mostró que las larvas de *P. maculatofasciatus* tuvieron una mayor tolerancia al estrés hiperosmótico. Con base en los resultados obtenidos en cada especie, se recomienda la exposición a 70 g L⁻¹ por 1 h para *P. maculatofasciatus* y 57 g L⁻¹ por 2 h para *L. guttatus*, cuando se realicen pruebas de estrés hiperosmótico. Esta prueba de estrés es objetiva, sencilla y puede utilizarse tanto en estudios sobre nutrición y manejo de reproductores como en plantas de producción de juveniles como método de control de calidad.

Palabras clave: Calidad de larvas, estrés osmótico, prueba de estrés, tolerancia a la salinidad, análisis Probit

Abstract.- This study investigated the tolerance of first-feeding larvae of spotted sand bass *Paralabrax maculatofasciatus* and spotted rose snapper *Lutjanus guttatus* to acute hyperosmotic stress. *P. maculatofasciatus* and *L. guttatus* embryos were incubated in 48-well microplates at 23 and 28°C, respectively. The first-feeding larvae were exposed in triplicate to salinities of 35 to 95 g L⁻¹ or 35 to 85 g L⁻¹, and survival was monitored from 0.5-3.5 h or 0.5-53 h, for *P. maculatofasciatus* and *L. guttatus*, respectively. The results showed that mortality increased differently among treatments from the first exposure time in each species. Extreme salinities were lethal in 30 min. Salinities close to the control were sub lethal. At intermediate salinities, mortality was fitted to a potential or linear model. For *P. maculatofasciatus*, the LC₅₀ values obtained between 0.5 and 3.5 h were 73.4 to 65.4 g L⁻¹. For *L. guttatus* the LC₅₀ values obtained between 2 and 5 h were 57.3 to 54.5 g L⁻¹. Comparison of LC₅₀ between species showed that *P. maculatofasciatus* larvae had a higher tolerance to hyperosmotic stress. Based on the results for each species, it is recommended to expose to 70 g L⁻¹ for 1 h for *P. maculatofasciatus* and 57 g L⁻¹ for 2 h for *L. guttatus* when conducting hyperosmotic stress tests. This stress test is objective, simple, and can be used in both studies on broodstock nutrition and management and in hatcheries as a quality control method.

Key words: Larvae quality, osmotic stress, stress test, salinity tolerance, Probit analysis

INTRODUCTION

In larviculture of marine fishes high mortalities occur during the endogenous to exogenous feeding transition (Yúfera & Darías 2007). These high mortalities can occur in larvae from embryo batches characterized as high quality (e.g., high hatching rates and high survival of larvae at first-feeding stage) (Duncan 2013). Therefore, it is necessary to develop practical and reliable criteria for the assessment of the first-feeding larvae quality in terms of physiological condition or vigor. Vigor tests based on stress tolerance have been developed (Lavens *et al.* 1995). Such tests are based on increases in the metabolic demand of energy caused by the stressor to maintain homeostasis. Therefore, mortality caused by acute exposure to a stressor for a specific period of time shows the vigor of the organism in real time (Dhert *et al.* 1992). Several stressors have been used in acute stress tests (e.g., starvation, air exposition, and temperature), which acute change in salinity has proven to be the most simple, controllable and reliable (Dhert *et al.* 1992). Saline stress tests have been used to test the quality of larvae or juveniles of mollusks (Maguire *et al.* 1999), crustaceans (Samocha *et al.* 1998), and fishes (Kjørsvik *et al.* 2003, Jelkix *et al.* 2014). Saline stress tests have not yet been conducted on first-feeding larvae of marine fishes because they are very susceptible to mechanical stress caused by handling during stress tests. However, quality assessment at this stage is important because structural and energetic resources from the yolk have been depleted and incorporated into the larval body. Therefore, hyperosmotic (high salinity) stress tolerance is a reliable indicator of the first-feeding larvae vigor.

An alternative incubation method that avoids mechanical stress and allows fast, easy and accurate monitoring of many first-feeding larvae is stocking embryos individually in multiwell culture microplate (Panini *et al.* 2001). This method of incubation presents an opportunity to develop a simple method to assess the vigor of first-feeding larvae by the determination of tolerance to acute hyperosmotic stress using robust statistics, such as the median lethal concentration (LC_{50}) and median lethal time (LT_{50}) from Probit analysis. Spotted sand bass (*Paralabrax maculatofasciatus*) and spotted rose snapper (*Lutjanus guttatus*) are important species for artisanal and sport fisheries in the Eastern Central Pacific (Froese & Pauly 2016). Due to their high demand in Latin America and to over-exploitation of wild populations (Arreguín-Sánchez & Arcos-Huitrón 2011), research has focused on the development of technology

for juvenile production of both species in captivity (Civera-Cerecedo *et al.* 2002, Alvarez-Lajonchère & Puello-Cruz 2011).

Therefore, the goal of this study was to investigate the tolerance of first-feeding larvae of these species to acute hyperosmotic stress, to select a level of salinity and exposure time that can be used for a simple vigor test. For this purpose, the LC_{50} at different exposure times to acute hyperosmotic stress, and LT_{50} at different salinities were determined. This quality criteria will be useful for studies aimed at optimizing the processes that affect larvae quality at the onset of first feeding (e.g., broodstock management and nutrition), or in experimental larviculture of both species.

MATERIALS AND METHODS

BROODSTOCK HUSBANDRY

Wild broodstock of *P. maculatofasciatus* (24 males: 172 ± 20 g; 36 females: 173 ± 17 g) were caught in Bahía de La Paz, Baja California Sur, Mexico and were kept in six 1,100-L cylindrical tanks connected to a recirculation system with environmental control at the Laboratory of Experimental Biology, CICIMAR-IPN, La Paz, Mexico. Environmental conditions were (mean \pm SD): flow, 20.1 L min^{-1} ; temperature, $21.3 \pm 0.6^\circ\text{C}$; $\text{NH}_3 + \text{NH}_4^+$, $0.2 \pm 0.1 \text{ mg L}^{-1}$; NO_2 , $0.02 \pm 0.02 \text{ mg L}^{-1}$; salinity, $35 \pm 0.7 \text{ g L}^{-1}$; dissolved oxygen, $6 \pm 1 \text{ mg L}^{-1}$; photoperiod, 13L:11D; sex rate, 1.5 female: 1 male; stoking density, 2.7 kg m^{-3} . The fish were hand-fed once per day to apparent satiation with frozen whole juvenile fish (*Eucinostomus* spp.). Under these conditions, reproduction was induced.

Wild broodstock of *L. guttatus* (20 males: 1668 ± 269 g; 11 females: 1803 ± 368 g) were caught in Sayulita, Nayarit, Mexico and were kept in 18,000-L cylindrical tanks at the Pilot Hatchery of CIAD, Mazatlan, Mexico. The tank was supplied with a seawater flow-through system (75 L min^{-1}) and aeration (60 L min^{-1}) (Ibarra-Castro & Álvarez-Lajonchère 2011) and the natural environmental conditions were as follows (mean \pm SD): temperature, $30 \pm 0.9^\circ\text{C}$; salinity, 35 g L^{-1} ; O_2 , 6 mg L^{-1} ; photoperiod, 12L:11D; sex rate, 1 female: 1.8 male; stoking density, 2.9 kg m^{-3} . The broodstock were hand-fed on alternate days to apparent satiation with squid (*Loligo* sp.), skipjack tuna (*Katsuwonus pelamis*), and shrimp (*Xiphopenaeus* sp.) in equal proportions. In these fish, reproduction occurred without hormonal treatment.

INCUBATION AND ASSESSMENT OF EMBRYO QUALITY

Embryos from voluntary spawning of both species were collected, cleaned and separated by buoyancy in a graduated cylinder and gently rinsed with UV-sterilized seawater. Subsequently, embryos were randomly selected and transferred into flat-bottom 48-well microplate (one embryo per well) fitted with a low evaporation lid (Cellstar®, Greiner Bio-One, Monroe, USA). Each well was filled with 0.5 mL UV-sterilized seawater (35 g L⁻¹). Microplates were immediately placed in an incubator with a controlled temperature at 23 ± 0.5°C and 28 ± 0.5°C for *P. maculatofasciatus* and *L. guttatus*, respectively. During incubation, the photoperiod (13L:11D) was maintained by cool white fluorescent lamps (500 lx). To monitor the embryonic development and survival, microplates were examined every day using a stereomicroscope (SZ40, Olympus®, Tokyo, Japan), until pigmentation of eyes and complete yolk absorption were observed (first-feeding stage: 76 h for *P. maculatofasciatus* and 68 h for *L. guttatus*). For each microplate, hatching and survival to first-feeding stage were calculated as percentage of the initial number of eggs.

EXPERIMENTAL DESIGN

Once the first-feeding stage was reached, acute hyperosmotic stress tests were initiated. The *P. maculatofasciatus* first-feeding larvae were exposed to salinities of 35 (control), 50, 65, 80, and 95 g L⁻¹. The *L. guttatus* first-feeding larvae were exposed to salinities of 35 (control), 45, 55, 65, 75, and 85 g L⁻¹. Each saline concentration was achieved by adding 0.5 mL of brine (specifically adjusted by the dissolution of synthetic salt [Instant Ocean®, Vancouver, USA] in UV-sterilized seawater) into each well of three microplates for each saline treatment. Each microplate was one experimental unit; therefore, each treatment had an average of 135 first-feeding larvae (subtracting mortality at bioassay onset). The control treatment (35 g L⁻¹) was prepared by adding UV-sterilized seawater. To avoid thermal shock, brine solutions were placed in an incubator for 30 min prior to exposure at 23 and 28°C, for *P. maculatofasciatus* and *L. guttatus*, respectively. Microplates were again placed in the incubator immediately after the addition of brine. The larvae survival of *P. maculatofasciatus* from each microplate was monitored at 0.5; 1; 1.5; 2; 2.5; 3; and 3.5 h, whereas the larvae survival of *L. guttatus* was monitored at 0.5; 1.3; 1.5; 1.7; 2; 3; 4; 11.8; 17.7; 41.5; and 53 h. Larvae were considered dead when they displayed no movement in response to a slight mechanical stimulus.

At the end of each experiment, salinity was measured in each of the 48 wells by a temperature-compensated refractometer (SR5-AQ, China) to verify the salinity.

STATISTICAL METHODS

Results are reported as the mean ± SD (standard deviation) from three replicates. Cumulative mortality from each salinity treatment throughout the exposure time was fitted to regression models. Differences among salinity treatments for each exposure time were tested by a one-way ANOVA, followed by a Tukey's post-hoc test using arcsine-square-root-transformed data. The significance level was fixed at 0.05. These analyses were performed using SigmaPlot 11® (Systat Software, San Jose, USA). LC₅₀ for each exposure time and LT₅₀ for each salinity, and their respective fiducial limits were determined with Probit analyses using PASW® Statistics 18 (SPSS, IBM, New York, USA).

RESULTS

The mean coefficient of variation of salinity among wells from each microplate in all treatments for both species was 1.1 ± 0.2%, which represents a negligible variation from the nominal salinity in each treatment. Overall, the quality of embryos and larvae of both species was high. The hatching rate, survival to hatching, and survival to first-feeding stage for *P. maculatofasciatus* were 99 ± 0.9%, 93 ± 3.6%, and 91 ± 3.4%, respectively; similar values were observed for *L. guttatus*, at 99 ± 1%, 98 ± 2%, and 87 ± 14%, respectively. These results demonstrate that the incubation technique in microplates has no adverse effect on the quality determination or embryonic development in either species.

PARALABRAX MACULATOFASCIATUS

In the control group mortality throughout the experiment was insignificant (2.3%; Fig. 1). At 50 g L⁻¹ mortality gradually increased to 20% at 3.5 h after fitting a linear model ($y = -1.4241 + 5.8783x$, $r^2 = 0.55$); however, there were no significant differences from the control at any time ($P > 0.05$). At 65 g L⁻¹ mortality increased linearly ($y = 2.8319 + 16.1516x$, $r^2 = 0.92$) to 58.3% at 3.5 h and showed a significant difference from the control at 1 h of exposure ($P < 0.05$). At 80 g L⁻¹, mortality increased exponentially ($y = 92.4186x^{0.1983}$, $r^2 = 0.96$), reaching total mortality at 1.5 h and only did not show significant differences with the 95 g L⁻¹ treatment at any time ($P < 0.05$). At 95 g L⁻¹, rapid mortality occurred (100% in 0.5 h).

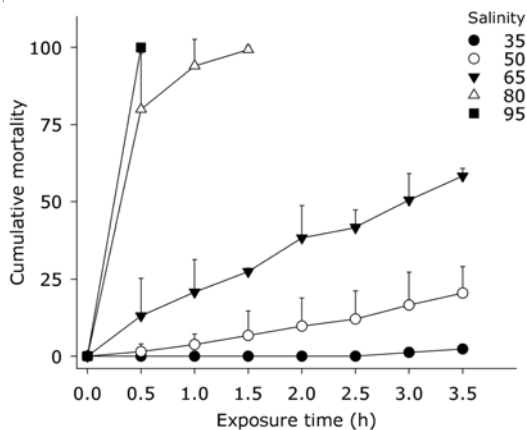


Figure 1. Cumulative mortality (mean \pm SD) of *Paralabrax maculatofasciatus* first-feeding larvae subjected to hyperosmotic stress using different salinity (g L^{-1}) concentrations and exposure times (h) at 23°C / Mortalidad acumulada (promedio \pm desviación estándar) de larvas de *Paralabrax maculatofasciatus* a la primera alimentación sujetas a estrés hiperosmótico usando diferentes concentraciones de salinidad (g L^{-1}) y tiempos de exposición (h) a 23°C

The LC_{50} values decreased as the exposure time increased, from 73.4 g L^{-1} at 0.5 h to 65.4 g L^{-1} at 3.5 h; however, it is possible that there were no differences in LC_{50} values after 1 h, given the overlap in the 95% fiducial limits (Table 1). The LT_{50} values were estimated only for salinities of 50, 65, and 80 g L^{-1} (Table 1). The Probit model had a good fit to these data (Chi-square test: 0.192, 4 for 50 g L^{-1} ; 0.529, 4 for 60 g L^{-1} ; 3.91, 4 for 80 g L^{-1}).

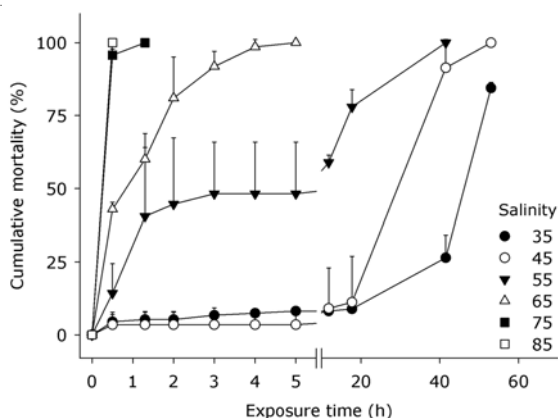


Figure 2. Cumulative mortality (mean \pm SD) of *Lutjanus guttatus* first-feeding larvae subjected to hyperosmotic stress using different salinity (g L^{-1}) concentrations and exposure times (h) at 28°C / Mortalidad acumulada (promedio \pm desviación estándar) de larvas de *Lutjanus guttatus* a la primera alimentación sujetas a estrés hiperosmótico usando diferentes concentraciones de salinidad (g L^{-1}) y tiempos de exposición (h) a 28°C

Table 1. Parameters of Probit regressions, LC_{50} and LT_{50} of *Paralabrax maculatofasciatus* first-feeding larvae subjected to acute hyperosmotic stress at 23°C / Parámetros de regresión Probit, LC_{50} y LT_{50} de larvas a la primera alimentación de *Paralabrax maculatofasciatus* sujetas a estrés hiperosmótico agudo a 23°C

	a^a	b^a	$\text{LC}_{50}(\text{g L}^{-1})$ (95% FL ^b)	P^c
Time (h)				
0.5	-43.7180	23.4353	73.4 (71.7-74.9)	0.4
1	-50.7065	27.4597	70.2 (68.6-71.7)	0.9
1.5	-64.0783	34.9084	68.5 (67.0-70.0)	0.8
2	-58.1548	31.8124	67.3 (65.8-68.8)	0.9
2.5	-57.0846	31.2558	67.0 (65.4-68.6)	0.9
3	-53.1588	29.1970	66.1 (64.3-67.8)	0.9
3.5	-49.3091	27.1608	65.4 (63.1-67.1)	0.9
Salinity (g L^{-1})				
50	-1.9216	1.9325	9.9 (6.2-80.2)	0.9
65	-1.3229	2.4800	3.4 (2.7-4.3)	0.9
80	1.5778	2.3136	0.2 (0.0-0.8)	0.4

^a Probit (p) = $a + b(\log_{10} X)$; $P = Z_{(0,1)} \rightarrow P(0.5) = Z(0)$

^b Fiducial limits

^c P -value of Chi-square test for Probit model

LUTJANUS GUTTATUS

In the control group, mortality increased exponentially ($y = 1.9287e^{0.0706x}$, $r^2 = 0.95$), from 9% within 17.6 h, to 26.4% at 41.5 h, and 84.4% at 53 h (Fig. 2). At 45 g L^{-1} mortality increased exponentially ($y = 7.5335e^{0.0509x}$, $r^2 = 0.88$) and was significantly higher than the control at 41.5 h, reaching 91.3%, and 100% at 53 h ($P < 0.05$). At 55 g L^{-1} , mortality followed a sigmoid curve with a stable phase from 40.4 to 48.2% within 1.3 and 5 h, with the highest rate (100%) after 41.5 h. In this treatment, mortality was significantly higher than in the lower salinities at any time between 1.5 h and 17.7 h ($P < 0.05$). At 65 g L^{-1} , mortality increased exponentially ($y = 57.1349x^{0.4124}$, $r^2 = 0.88$), reaching 100% at 5 h and was significantly different from the other treatments at any time ($P < 0.05$), except between 1.3 and 2 h for the 55 g L^{-1} treatment. At 75 and 85 g L^{-1} , the effect was extreme with abrupt mortality (100% at 0.5 h).

The LC_{50} values of the acute hyperosmotic stress tests were determined only for the first eight exposure times. The highest LC_{50} values were 63.7 and 59.6 g L^{-1} for 0.5 and 1.3 h, respectively; however, the fiducial limits for these LC_{50} values were wide and the model had a poor fit (Chi-square test: 36.8, 3 for 0.5 h; 22.7, 3 for 1.3 h) (Table 2). After 2 h, with small fiducial limits and a well-fitting model (Chi-square test: 5, 3 for 2 h; 0.57, 3 for 3 h; 2.9, 3 for 4 h; 6.8, 3 for 5 h; 4.4, 3 for 11.8 h; 0.6, 3 for 17.6 h), the LC_{50} values showed a gradual decrease from 57.3 g L^{-1} at 2 h to 51.2 g L^{-1} at 17.6 h. The LT_{50} were determined only for 55 and 65 g L^{-1} (3.9 and 0.7 h, respectively), but they showed a poor fit to the model (Table 2).

Table 2. Parameters of Probit regressions, LC₅₀ and LT₅₀ of *Lutjanus guttatus* first-feeding larvae subjected to acute hyperosmotic stress at 28°C / Parámetros de regresión Probit, LC₅₀ y LT₅₀ de larvas a la primera alimentación de *Lutjanus guttatus* sujetas a estrés hiperosmótico agudo a 28°C

	<i>a</i> ^a	<i>b</i> ^a	LC ₅₀ (g L ⁻¹) (95% FL ^b)	<i>P</i> ^c
Time (h)				
0.5	-33.0124	18.2995	63.7 (49.0-73.2)	0.0
1.3	-29.9756	16.8830	59.6 (51.0-66.0)	0.0
2	-32.5960	18.5377	57.3 (56.0-58.6)	0.2
3	-37.0154	21.2011	55.7 (54.5-56.9)	0.9
4	-44.4888	25.5938	54.7 (53.6-55.9)	0.4
5	-47.9985	27.6476	54.5 (51.4-57.4)	0.1
11.8	-41.9325	24.3360	52.9 (51.7-54.0)	0.2
17.6	-44.4620	26.0143	51.2 (50.1-52.3)	0.9
Salinity (g L ⁻¹)				
55	-0.7235	1.2075	3.9 (2.5-6.2)	0.0
65	0.2941	2.2877	0.7 (0.3-1.1)	0.0

^a Probit (*P*)= *a* + *b*(log₁₀ X); *P*= Z_(0,1) → *P* (0.5)= Z (0)

^b Fiducial limits

^c *P*-value of Chi-square test for Probit model

DISCUSSION

This study showed a species-specific differential mortality response of first-feeding larvae at varying stressor intensities (salinity level and exposure time). The results indicated a gradient of individual homeostatic capabilities within the examined population of each species. Extreme salinities were lethal in 30 min, while salinities closest to the control (50 g L⁻¹ for *P. maculatofasciatus*; 45 g L⁻¹ for *L. guttatus*) were sub-lethal over the full exposure time. Moreover, intermediate salinities (65 and 80 g L⁻¹ for *P. maculatofasciatus*; 55 and 65 g L⁻¹ for *L. guttatus*) showed progressive mortality patterns. Particularly, *L. guttatus* larvae exposed at 55 g L⁻¹ showed a sigmoidal response with three phases: 1) short lethal phase; 2) mid-duration stable sub-lethal phase; and 3) large exhaustion phase. Mortality in each phase is likely due to a deficit in the capacity of homeostasis and energy depletion caused by osmotic stress. Studies on the early ontogeny of marine fish confirm that high levels of salinity increase the energy cost of osmoregulation (Tseng & Hwang 2008). In contrast to other levels of salinity, the 55 g L⁻¹ level allows us to distinguish clearly between two groups of physiological conditions (vigor) in the *L. guttatus* larvae experimental population. In *P. maculatofasciatus*, the amplitude of salinity levels tested in this study did not allow for the observation of a sigmoidal curve as in *L. guttatus*; however, the LC₅₀ values resolve this difficulty. The LC₅₀ values provide a convenient way to compare tolerance between various groups (Van Veld & Nacci 2008).

Comparison of LC₅₀ and LT₅₀ (particularly at 65 g L⁻¹) between both species showed that *P. maculatofasciatus* larvae tended to have a higher tolerance to hyperosmotic stress. In contrast, LC₅₀ of *L. guttatus* at 17.6 h was higher than that reported for first-feeding larvae of *Cynoscion nebulosus* (18 h-LD₅₀ = 42.5 g L⁻¹; Banks *et al.* 1991) and *Rachycentron canadum* (18 h-LD₅₀ = 41.9 g L⁻¹; Faulk & Holt 2006).

The LC₅₀ and LT₅₀ values are conventionally used in toxicology studies as indicators of acute toxicity of a substance (Rand 2008). However, due to the occurrence of values below 50% of the response corresponding to the sensitive portion of the population, these criteria can also be used as quality indicators. Samocha *et al.* (1998) proposed a simple method for evaluating the quality of white shrimp larvae *Litopenaeus vannamei*, based on the 2 h-LC₅₀ observed from osmotic or formalin shock. These authors note the utility and statistical reliability of Probit analysis to determine LC₅₀. In this analysis, the optimization algorithm of the parameters of the linear regression model determines that fiducial limits are narrower in the middle region (values approximately 50% of response). Given that the amplitude of fiducial limits is a criterion for selecting the best model (Debanne & Haller 1985), the LC₅₀ and LT₅₀ estimated in this way are statistically more accurate and reliable. As a result, it was considered the shortest exposure time with a best fit of the Probit regression model in our experiment (1 h-LC₅₀ is 70 g L⁻¹ for *P. maculatofasciatus* and 2 h-LC₅₀ was 57 g L⁻¹ for *L. guttatus*) as the appropriate benchmark in our hyperosmotic stress tests. However, the LT₅₀ values for both species were inadequate as benchmarks due to a poor fit of the model and wide fiducial limits.

Based on the above, this study proposed the following hyperosmotic stress test to assess the first-feeding larvae vigor of *P. maculatofasciatus* and *L. guttatus*: 1) Incubate embryos in three 48-well microplates and evaluate the quality as described in Materials and methods section for each species; this sample size is sufficient to give statistical power to the test, if considering a population of 1 × 10⁶, an error margin of 10% and a confidence level of 95% (sample size estimated to population proportion is 96 specimens). 2) Add 0.5 mL of brine at 105 g L⁻¹ in each well to achieve 70 g L⁻¹ for *P. maculatofasciatus* or 0.38 mL to achieve 57 g L⁻¹ for *L. guttatus*. 3) Count dead larvae after 1 h for *P. maculatofasciatus* or 2 h for *L. guttatus*. If larval survival is equal to or greater than 50%, the quality is comparable or better than that obtained in the present study.

Other osmotic stress tests have been proposed for marine fish (e.g., Dhert *et al.* 1992, Kjørsvik *et al.* 2003). Typically, these tests are conducted by placing 10 specimens in 50 mL (frequently per triplicate), which are subjected to 70 g L⁻¹ (salinity arbitrarily selected) and survival assessment is carried out in short periods (5-15 min). The result is reported as a stress index (sum of cumulative mortalities at each time period) or LT₅₀. Under these operating conditions, the stress test is not suitable for the vigor assessment of first-feeding larvae due to handling, statistically inappropriate sample sizes, salinity levels not selected relative to the tolerance of the target species and impractical frequency survival assessment. These disadvantages are solved by the stress test proposed in this work. This stress test has been used in the routine evaluation of the quality of different batches of first-feeding larvae of *L. guttatus* (Martínez-Brown *et al.* unpublished data) and used to determine fatty acids related to first-feeding larvae quality in nutrition studies of *P. maculatofasciatus* broodstock (Rodríguez-Trejo *et al.* 2009)¹.

In conclusion, this study recommends a simple method to objectively determine the vigor of first-feeding larvae of *P. maculatofasciatus* and *L. guttatus*, based on tolerance to acute hyperosmotic stress. Application of this method to other species is necessary to determine the LC₅₀ at different exposure times for each species, using Probit analysis. However, the salinity of spawning should be considered when determining the LC₅₀, as it affects the larvae tolerance to variation in salinity (Kucera *et al.* 2002). This test can be used together with other quality criteria of embryos and larvae (biochemical, morphometric, transcriptomics, etc.) to establish interactive relationships and elucidate the maternal contribution in studies of nutrition and management of broodstock. Further studies are required to validate the predictive value of this stress test on zootechnical performance achieved at the end of the larviculture process.

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