Aquaculture 422–423 (2014) 253–260

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Factorial experimental designs as tools to optimize rearing conditions of fish larvae

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ARTICLE INFO

Article history:
Received 26 April 2013
Received in revised form 5 December 2013
Accepted 10 December 2013
Available online 22 December 2013

Keywords:
Cod larvae
Light
Microalgae
Tank colour
Rotifer density
First feeding

ABSTRACT

The objective of this study was to test short-term factorial designs to generate detailed knowledge about environmental demands of marine fish larvae, in order to optimize rearing conditions. The joint effects of the factors light intensity, tank bottom colour, microalgae addition and prey density were tested on foraging success in Atlantic cod (*Gadus morhua*) larvae at 5, 10, 15 and 20 days post hatch (dph), using independent 2^4 factorial short-term screening designs. The larval response to environmental factors changed with age. White tank bottoms negatively affected foraging at all ages, as compared to black and grey bottoms. Additional microalgae affected foraging at 5 dph, but then this effect vanished until day 20 dph. At 15 dph both light, bottom colour and prey density jointly affected foraging, and at 20 dph, an effect from prey density as well as an interaction between light intensity and algal density was observed. The results indicate that grey tank bottom colour is advantageous for cod larvae, and that microalgae addition may not be necessary beyond the first week of feeding. The factorial design approach was discussed in relation to the traditional one-variable-at-a-time (OVAT) approach commonly applied in studies of larval rearing. Our approach identified both interaction structure between experimental factors and stage-dependency of responses to rearing environment, not generally highlighted in OVAT designs. This suggests that short-term factorial designs are useful tools for future optimization of production of fish larvae.

1. Introduction

In marine aquaculture, juvenile production is a biological and economical bottleneck due to high mortality and frequent deformities at early stages related to suboptimal husbandry conditions (Chatain, 1997; Rosenlund and Skretting, 2006). In tanks, marine fish larvae face a complex range of physical, chemical and biotic factors as well as nutritional issues that may affect production (Kjesbu et al., 2006; Rosenlund and Halldorsson, 2007), so efficient ways to improve and optimize the multivariate tank environment are needed.

Studies of domestication of new species traditionally follow a one-variable-at-a-time (OVAT) approach (Fontaine et al., 2012), studying factors one by one, and keeping other factors controlled. In contrast, factorial designs consider effects from multiple factors simultaneously (Box et al., 2005; Fisher, 1926), and are capable to identify both interactions and optimal combinations of multiple factors. They also have an advantage over OVAT to reduce replication and costs needed for a certain level of precision in effect estimates.

Traditional experiments often last for several weeks from hatching to metamorphosis, a time period associated with rapid ontogenetic changes (Blaxter, 1986; Hunt von Herbing, 2001) and thus changed environmental demands. Treatment effects are typically assessed by repeated post-hoc tests at defined points of time. An issue arising from this approach is that once between-group differences are established, they accumulate and cause temporal dependency that may compromise validity of later comparisons. An alternative approach includes series of independent short-term experiments at chosen larval sizes, using appropriate short-term response variables. Each separate experiment then includes larvae at similar size and developmental stage, reared under identical conditions, and thus provides information unique to the specific larval size range studied.

We wanted to examine if factorial short-term experiments constitute a suitable alternative to the traditional long-term studies applied in larval fish rearing. A factorial approach was applied to examine simultaneously effects from four factors that shape the visual environment of tanks using larval cod *(Gadus morhua)* as a model species. As most marine larvae are obligate visual feeders/hunters (Blaxter, 1986) with poorly developed vision at hatch, visual environment is of key importance for prey visibility and foraging, and subsequently affects growth and survival. Factors affecting visual environment in tanks have been studied both in general (Naas et al., 1996) and specifically for various marine fish species (Downing and Litvak, 2000; Naas et al., 1992; Ostrowski, 1989; Rotllant et al., 2003). Effects from factors of visual environment on cod larvae in intensive aquaculture production have predominantly been studied by traditional OVAT long-term studies, e.g. light intensity (Monk et al., 2006; Puvanendran and Brown, 2002),

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http://dx.doi.org/10.1016/j.aquaculture.2013.12.018

tank colour (Monk et al., 2008), prey density (Puvanendran and Brown, 1999; Puvanendran et al., 2002) and added microalgae (van der Meeren, 1991; van der Meeren et al., 2007). Though, the latter was a 2-way factorial long-term study.

The objective of this study was to assess the potential of short-term factorial designs to generate detailed knowledge about environmental demands of marine fish larvae, in order to later optimize rearing conditions. We studied joint effects of the following factors: light intensity, tank bottom colour, microalgae addition and prey density, by applying factorial short-term screening experiments at larval ages 5, 10, 15 and 20 days post hatch (dph). Cod was the model species and rotifer ingestion was the short-term response variable.

2. Material and methods

2.1. Larval rearing

Fertilized eggs from batch spawning brood stock were collected at Norwegian Cod Breeding Centre, Tromsø, Norway and incubated at 4.4 ± 0.1 °C. At 35.2 °D, eggs were transported by plane to the University of Nordland, disinfected (400 ppm glutar aldehyde in seawater for 5 minutes) and incubated until hatch in black, conical bottom 270-L incubators at 6.3 ± 0.4 °C, with gentle aeration and 5 water exchanges per day. Hatching was defined as the day when 50% of larvae had hatched (0 dph). At 1 dph larvae were transferred to a 100-L cylindrical black holding tank at a density of 100/L. Sea water filtered to 5 μm was supplied at rates of 4.5 exchanges per day from 1 to 5 dph, and 8 exchanges from 6 to 20 DPH. Larvae were fed rotifers cultivated on Super fresh Chlorella SV12 (Pacific Trading- Aquaculture Ltd, Ireland) and enriched with 0.4 mg L^{-1} Multigain/PhosphoNorse at a 70:30 weight ratio. Larvae were fed three times a day at a density of 10 rotifers mL^{-1}. For green water, algal paste (Instant Algae Nanno 3600 ®, Reed Mariculture Inc.), was used at a density of 1 million cells mL^{-1}. Gentle aeration was applied centrally in the tank, and light intensity at the surface was set at 600 lx with photoperiod 24:0 L:D. Water temperature was raised from 6.7 to 10 °C over the first 5 days, and then maintained at 10.6 ± 0.6 °C over the remaining 14 days. The tank was daily cleaned.

2.2. Execution of larval trials

Independent short-term experiments were carried out at 5, 10, 15 and 20 dph to evenly span a rotifer feeding period commonly used for cod larvae. All four experiments were executed in a temperature controlled room (10 °C). Experimental units were black, approximately cylindrical shaped PVC tanks with total volume 12 L, depth 0.25 m, upper diameter 0.29 m and lower diameter 0.20 m. Tanks were arranged in two rows of ten, and shielded from light from neighbouring units by black partitions. Two days before trials, 10 L of aerated and filtered sea water was added to each of the gently aerated tanks, allowing water temperature to adjust to 10 °C. Distribution of treatment combinations was randomized, and bottom colour and intensity of light sources (lx at the surface) adjusted accordingly. The day before each trial, ≈800 larvae were sampled from the holding tank. 30 larvae were transferred 10 at a time to each of 20 seawater filled beakers, and then distributed at random to the tanks. Larvae were kept unfed in darkness over night (18–20 h) to empty the gut before onset of the trial on the next morning.

Preset light sources were turned on at onset of trials, and algal paste and rotifers distributed to tanks according to the experimental design. Each trial lasted for 5 h, based on a pilot study performed on the extreme settings of the experimental domain, indicating that this time span is suited to reveal short-term difference in foraging (Nicolaisen, unpublished). At termination light was turned off to prevent further foraging. Tanks were sampled one by one in a random sequence. All larvae from each tank were gently poured into a wide, light bottomed container, and 10 larvae transferred to a small beaker with a pipette. Excess water was removed and larvae killed by an overdose of MS 222, fixated in 4% buffered formalin and stored in 1.5 ml Eppendorf tubes at 4 °C for maximum four weeks. At examination, larvae were photographed and dissected under an Olympus SZX 12 stereo microscope equipped with Cell ^{A} software (Soft Imaging system GmbH). The guts were dissected and the number of rotifers counted. Standard length (SL) to the nearest 0.1 mm was obtained on fixated larvae from photographs using Cell A and was used for statistical analyses. Estimates of fresh standard length at the different ages was obtained by correcting for fixation effects based on SL measured on fresh larvae from three replicate tanks ($n = 45$), produced simultaneously and with identical protocol (Lanes et al., 2012).

2.3. Experimental design of larval trials

The general design principle compared to OVAT designs is illustrated in Fig. 1. All four experiments were identically designed as 2^4 factorial screening designs, replicated ($n_0 = 4$) in the added centre point (Table 1). In full factorial designs, all levels of each factor are combined with all levels of every other factor included in the experiment (Hicks and Turner, 1999). This allows assessing both main effects and interactions. Estimates of error variance are model dependent and achievable by assuming a model less than the full factorial model prior to analysis (Mee, 2009). As this was a screening study, searching for influential factors for more elaborate studies, 2nd Order and higher interactions were kept out from the model. This gave four main effects and six 1st Order interactions to be estimated, and left sufficient degrees of freedom to perform F-tests on model terms. The inclusion of centre points applies to cases where replication is costly or work demanding, and their main purpose is to increase overall replication and check for curvature (non-linearity) in responses (Esbensen, 2006). Our strategy left us with 20 tanks ($2^4 + 4$), as compared to 36 ($2\times (2^4) + 4$) if the whole 2^4 design was to be duplicated. The experimental domain (Table 1) was set to closely resemble factor levels as suggested from recent research (Brown et al., 2003; Puvanendran and Brown, 2002) and established production protocols. The commercial algae paste Instant Algae Nanno 3600 ®, Reed Mariculture Inc., USA, was used for green water. The tank bottom colour —originally dark —was adjusted to grey and white by circular plastic plates placed on the bottom. Grey bottom was estimated as the mean of averaged CMYK colour readings from black and white bottoms, obtained from 10 randomly chosen 5 × 5 pixel areas on photographs of tank bottoms using Adobe Photoshop CS4. Mean CMYK readings were 34, 35, 46 and 21, respectively.

2.4. Effects of surface light intensity, algae and bottom colour on illumination in tanks

As a follow-up experiment to the larval trials, the combined effects of light, bottom colour and algae on illumination in tanks were examined, using a duplicated 2^3 factorial design with $n_0 = 4$ additional centre points. The experiment was run under exactly the same conditions as in the larval trial. A LI-193 Spherical Quantum Sensor measuring photosynthetically active radiation (PAR) in the range 400–700 nm wavebands (μmol s^{-1} m^{-2}) was mounted through the tank bottom, and connected to a LI-1400 Data Logger (LI-COR Biosciences). Tanks were filled with filtered sea water, and experimental runs assigned at completely randomized order.

2.5. Data analysis

2.5.1. Larval trials

The response variable was the average number of prey eaten by feeding larvae in each tank, excluding non-feeding larvae. To assess overall effect from age and experimental factors, pooled data over all ages was analyzed by stepwise analysis of covariance (ANCOVA), allowing for 2-way interactions. At this point there was no a priori