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

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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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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

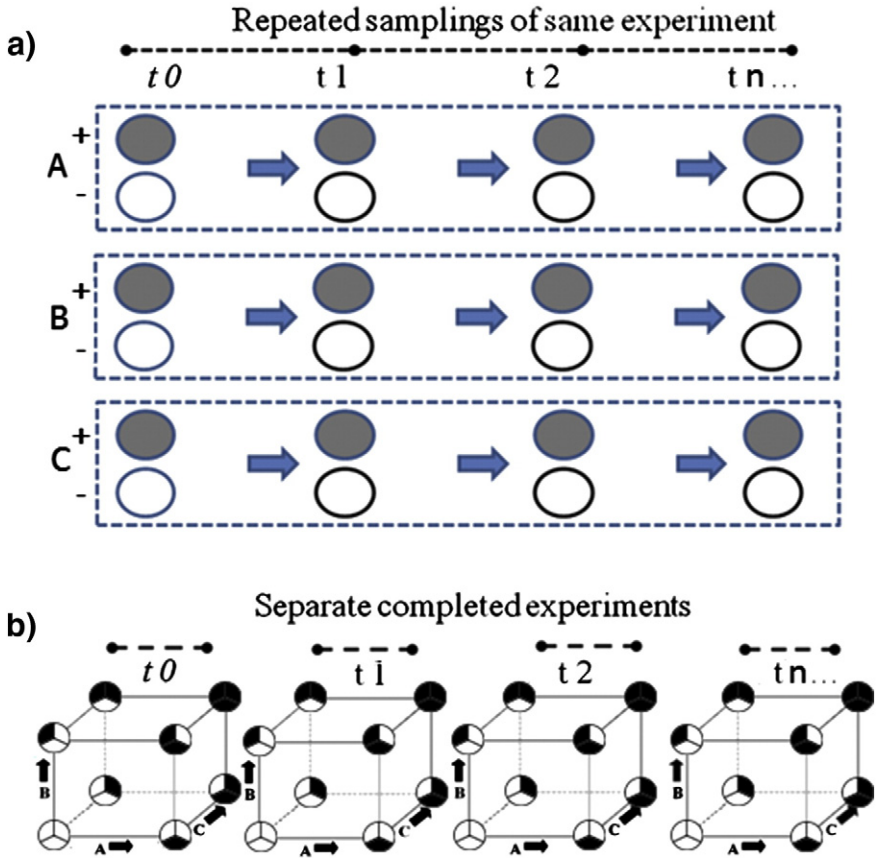

Fig. 1. Design principles of a) a traditional one-variable-at-a-time (OVAT) approach and b) short term factorial designs. a) Each experiment includes only one of the factors A-C, and experimental units are repeatedly sampled at defined time intervals. b) Shows separate complete 2^3 factorial experiments at different times, each including all factors A–C. Factor level combinations are indicated by the fill pattern of circles: The lower, left and right circle sectors correspond to levels of factors A, B and C respectively. Empty sectors indicate low factor level, while filled sectors indicate high level.

information about the quantitative aspects of bottom colour, so this factor was included as a categorical variable, with age, prey density, light and algal concentration as continuous covariates. Probabilities for entry and removal were set at P = 0.2 and P = 0.25, respectively. Model assumptions were checked by graphical examination of residuals. Effects at specific ages were analyzed by ANCOVA, allowing for two-way interactions. As this was a first screening, the usually accepted levels of significance ($\alpha = 0.05$) were relaxed, and an upper α-level of 0.15 accepted. ANCOVA was run with XLSTAT 2010, Addinsoft (Paris, France), data analysis and statistical software for Microsoft Excel.

Table 1
The structure of experimental design in the larval experiments, showing the design matrix coded in standard order and the factor levels in original units.

Coded standard order					Factor levels in original units		
Light	Algae	Prey	Bottom	Light (lx)	Algae (10^6 cells mL^{-1})	Prey mL^{-1}	Bottom
−	−	−	−	100	0.5	5	Black
+	−	−	−	1200	0.5	5	Black
−	+	−	−	100	2	5	Black
+	+	−	−	1200	2	5	Black
−	−	+	−	100	0.5	20	Black
+	−	+	−	1200	0.5	20	Black
−	+	+	−	100	2	20	Black
+	+	+	−	1200	2	20	Black
−	−	−	+	100	0.5	5	White
+	−	−	+	1200	0.5	5	White
−	+	−	+	100	2	5	White
+	+	−	+	1200	2	5	White
−	−	+	+	100	0.5	20	White
+	−	+	+	1200	0.5	20	White
−	+	+	+	100	2	20	White
+	+	+	+	1200	2	20	White
0	0	0	0	650	1.25	12.5	Grey

Plus and minus signs indicate the high and low factor levels respectively, following "Yates order" (Box et al., 2005). Additionally, the replicated center point (n = 4) is indicated by 0´es, indicating intermediate values of all factors. For analytical purposes in these particular experiments, bottom colour was considered a categorical variable, and thus does not strictly fit into the Yates coding order, but is included to visualize the design structure.

Table 2
Factorial analysis summary table: Effect from light intensity, bottom colour and algae on light illumination by the tank floor.

Source	Type III SS	df	MS	F	P	Parameter Estimates	
						Slope	SE
Intercept						4.00	0.11
Light	127.07	1	127.07	618.03	<0.001	2.81	0.11
Algae	1.04	1	1.04	5.04	0.046	−0.25	0.11
Bottom colour	5.12	1	5.12	24.90	<0.001	0.57	0.11
Light × Bottom colour	2.62	1	2.62	12.73	0.004	0.40	0.11
Light × Algae	0.551	1	0.55	2.68	0.130	−0.19	0.11
Bottom colour × Algae	1.11	1	1.11	5.39	0.040	−0.26	0.11
Bottom colour × Algae × Light	0.01	1	0.03	0.876	0.02	0.02	0.11
Curvature	2.625	1	2.625	12.76	0.004	0.91	0.25
Error	2.26	11	0.206				

Response variable: Light (μmol s^{-1} m^{-2}), $R^2 = 0.97$.

Shapiro-Wilks tests and Levene-tests for testing assumptions of normality and homogeneity of variance, as well as correlation analyses, were carried out using the statistical package SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Figures were produced using Minitab 16 ©2010 Minitab Inc., Adobe Photoshop CS4 and the Microsoft software Paint and Office PowerPoint 2007.

2.5.2. Effect of surface light intensity, algae and bottom colour on illumination in tanks

Prior to this follow-up experiment, preliminary measurements (Hanna HI 97500 luxmeter) showed no significant difference in light reflected to the tank surface between the average value of white and black bottoms pooled (0.016 lx) and values from grey bottoms (0.015 lx, SD = 0.003, n = 15, one sample *t*-test, two-tailed, t = −1.769, df = 14, P = 0.099), indicating that grey could be considered a quantitative midpoint. Thus, effect of light, algae and bottom colour on light intensity at the tank bottom was analysed as a full factorial duplicated 2^3 design with centre points (n = 4). Analysis was performed on the coded design matrix, assuming continuous variables and equal spacing between colour values. Factors were coded in ascending order (−1, 0, 1) from low to high values of light and algae, and from dark to white bottom, respectively. The significance level was set at

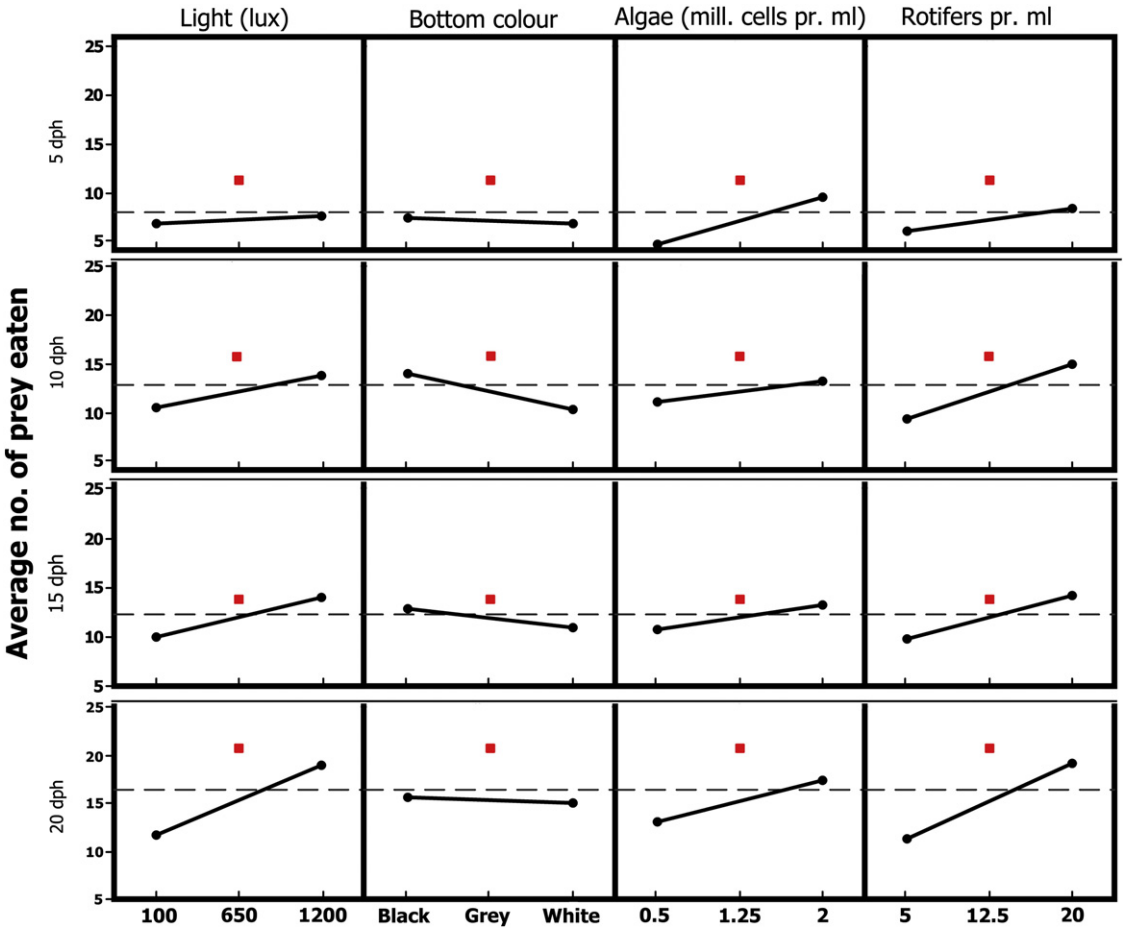

Fig. 2. Main effect plots for the experimental factors light, bottom colour, algal density and rotifer density at the larval ages 5, 10, 15 and 20 dph.

Table 3
Model summary from stepwise ANCOVA at the different larval ages.

			ANCOVA table			
Age	Model formula	Source	SS_{III}	df	F	p
5dph	$Y = 3.9 + 3.2\ A$					
		A	94.25	1, 18	2.75	**0.114**
10 dph	$Y = 10.9 + 0.0065\ LB$					
		LB	204.01	3, 18	1.23	0.327
15 dph	$Y = 3.8 - 3.4 \times 10^{-3}\ L + 9.9 \times 10^{-3}\ LB + 16.1\ AB + 1.2\ FB$					
		L	11.79	1, 15	0.66	0.482
		LB	160.02	1, 15	9.01	0.009*
		AB	28.04	2, 15	0.79	0.472
		FB	812.17	2, 15	22.87	<<0.001*
20 dph	$Y = 4.3 + 0.5\ F + 5.4 \times 10^{-3}\ LA + 4.4\ AB$					
		F	252.08	1, 16	5.64	0.030*
		LA	330.32	1, 16	7.40	0.015*
		AB	102.24		0.76	0.5311

Model formulas are coded as follows: Y = mean no. of prey eaten, A = algal concentration (million cells/ml), L = light intensity (lx), B = bottom colour (black/grey/white) and F = feed concentration (rotifers/ml). Significance at $P < 0.15$ is indicated in bold. Significance at the $P < 0.05$ is indicated by asterisk (*).

$\alpha = 0.05$ and analysis was carried out using the DoE module of the statistical package Minitab 16.0 (Minitab Inc.).

3. Results

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

Both increased light intensity ($P < 0.001$) and changed bottom colour from dark to lighter ($P < 0.001$) increased the illumination in the tanks, while increased amount of algae reduced light ($P = 0.046$) (Table 2). As analysis was performed on the design matrix, parameter estimates show change from one factor level to another, while the intercept estimates the overall mean. Thus, elevated light intensity from medium to high increased illumination in the water by around 70% (slope = 2.81), while changing the bottom colour from grey to white gave about one fifth of this effect (slope = 0.57). The interaction between bottom colour and light intensity ($P = 0.004$), showed that elevated light source intensity more strongly increased light levels by the tank bottom in white than in black bottomed tanks. In white bottomed tanks light increased from 1.34 ± 0.3 to 7.79 ± 0.5 μmol s^{-1} m^{-2} (mean ± se) while in black bottomed tanks the increase was 1.02 ± 0.2 to 5.85 ± 0.2. The interaction between algae and bottom colour ($P = 0.04$) indicated reduced bottom light due to elevated algal density in white bottomed tanks (-1.03 μmol s^{-1} m^{-2}), while in black bottomed tanks no such change was observed ($+0.02$ μmol s^{-1} m^{-2}). A positive curvature ($P = 0.004$) indicates that light measured at the design midpoint exceeds the value of the overall mean.

3.2. Larval trials —age groups pooled

Analysis of overall data revealed two significant interactions: A highly significant interaction between larval age and prey density (stepwise ANCOVA, $F_{1,75} = 18.27$, $P < 0.001$) indicated increasingly positive effect from high prey density on foraging with age. An interaction between prey density and bottom colour ($F_{1,75} = 5.04$, $P = 0.009$) was due to a generally lower beneficial effect from prey density on feeding in white bottomed tanks, as compared to black and grey bottomed tanks. There was also a less clear-cut interaction between light and algal density ($F_{1,75} = 3.31$, $P = 0.073$).

3.3. Larval trials —analysis by age

For all factors at all ages, the response at the design midpoint (intermediate values of all factors) exceeded expectations from a simple linear relationship, indicating presence of interactions or curved responses. A consistent pattern of increased foraging efficiency from low to higher levels of light, algal concentration and prey density was seen at all ages, while white tank bottoms generally lowered feeding compared to black ones (Fig. 2). At ages 5, 10, 15 and 20 average standard length (mm) ± SD was 5.0 ± 0.3, 5.7 ± 0.3, 6.1 ± 0.6 and 7.0 ± 0.6

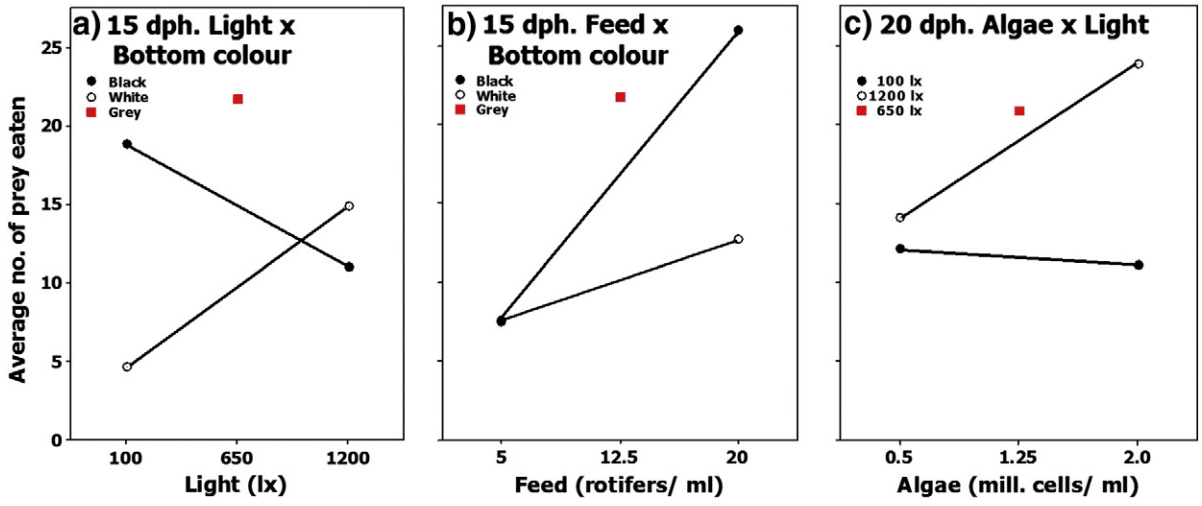

Fig. 3. Interaction plots showing significant interactions from the stepwise ANCOVA at different larval ages.

(n = 200), and overall fraction of larvae with feed in guts was 61%, 57%, 52% and 70%, respectively. The number of feed items (mean/max) in guts of feeding larvae at the respective ages was 8/40, 12/70, 16/110 and 18/101. The fraction of feeding larvae among tanks showed no important deviation from assumptions of normality or equal variance between factor levels at any of the ages. There was no correlation between standard length of feeding larvae and no. of prey in guts at any of the ages.

3.3.1. Five days post hatch

Enhanced algal density positively affected feeding ($P = 0.114$) (Table 3). All factors showed higher response at the design midpoint than at both the low and high factor settings (Fig. 2).

3.3.2. Ten days post hatch

Highly variable data resulted in uncertain interpretation at this age, and no significant effects from experimental factors appeared (Table 3). The main effect plots, however, still showed consistence with the overall pattern of effects; at the design midpoint the response was generally higher than values at low and high factor settings (Fig. 2).

3.3.3. Fifteen days post hatch

There were two significant 2-way interactions (Table 3). The light × bottom colour interaction ($P = 0.009$) indicates opposite effects from increased light on feeding depending on the bottom colour (Fig. 3a): Enhanced light intensity reduced feeding in black bottomed tanks, while in white bottomed tanks feeding increased. The design midpoint had the highest response. The feed × bottom colour interaction ($P < 0.001$) showed increased feeding as feed density increased, both for black and white bottoms, with the highest rate of change for black bottoms (Fig. 3b). Here, the overall highest response was at the combination black bottom – high feed. In sum, light intensity, feed density and bottom colour jointly affected feeding.

3.3.4. Twenty days post hatch

There was a significant interaction between algal density and light intensity (Table 3, Fig. 3c): Increased algal density had no effect at low light intensity, while at high intensity feeding was enhanced. There was a significant main effect from prey density on feeding, and the main effect plots indicate enhanced feeding due to increased light, algal density and feed density (Fig. 2). In sum, at 20 dph light, feed density and algae affected feeding.

4. Discussion

The present study demonstrates different influence of the environmental factors light intensity, microalgae density, bottom colour and prey density on larval feeding over the first 20 days post hatch in Atlantic cod. The short-term factorial screening approach allowed independent assessment of influence from the different factors at specific larval ages/sizes. The presence of interactions between experimental factors pinpoints the multi-factorial nature of tank environment on larval behaviour, and thus a need to simultaneously consider multiple factors when aiming to fine-tune or optimize environmental settings. When interactions are present, OVAT approaches fall short in identifying joint effects of co-acting factors, and are thus not well suited to identify optimal factor settings. The overall enhanced response at intermediate factor levels (the design center point) suggests that the present experimental domain represents suitable delimitation to carry out more thorough optimization studies.

The generally negative effect from white and positive effect from grey bottom colour on feeding suggests darker colours as better choices than white. Compared to black bottoms, lighter bottoms facilitate day-to-day visual control within tanks (Naas et al., 1996). Thus, for cod a grey bottom colour should be chosen, and further optimization studies should focus more thoroughly on the remaining factors; light, algae and prey density.

High variability in data was observed at 10 dph, past the point of no return in cod larvae (Ellertsen et al., 1980; Overton et al., 2010). At this age the population includes successfully feeding, growing larvae as well as larvae suffering from malnutrition, and discrepancies in nutritional status of larvae could thus subdue effects from environmental factors.

It is well known that fixation may significantly affect morphology of marine fish larvae (e.g. Hay, 1984). For cod larvae Yin and Blaxter (1986) estimated a formalin induced shrinkage of 8–12%. Though, within all four single experiments in this study, larvae were of similar size and fixated for the same time span prior to examination, so fixation effects would not bias experiment-wise results.

Larval density was kept low to reduce potential social interactions between larvae like aggression or competition for food. For instance, Puvanendran et al. (2008) demonstrated aggressive behaviour in cod already at 6 mm SL. In commercial situations larval densities are high, so optimization of light conditions for foraging in full scale production is one next step that has to be examined.

The response variable applied in this study measured short-term feed intake. It is well known that factors that affect feed intake consequentially will affect condition, growth and survival of fishes (Nunn et al., 2012). Similar measures of feed intake have proven capable to separate effects between levels of environmental predictor variables in cod, e.g. light intensity (Puvanendran and Brown, 2002; van der Meeren et al., 2007), light regime (Monk et al., 2006) prey concentration (Puvanendran and Brown, 1999) and microalgae (van der Meeren et al., 2007) in long-term experiments. Except for in the latter study, there was a correspondence between observed differences in foraging and growth, supporting the relevance of feeding related response measures for assessing overall larval success.

The improved foraging due to age, seen from analysis of overall data, is likely caused by improved feeding skills due to larval growth and development: Larvae generally undergo comprehensive ontogenetic changes, which enhance the ability to detect, capture and ingest prey through improved vision, locomotion and gap functionality, and ultimately improves foraging (Nunn et al., 2012). In a study of the foraging behaviour in cod larvae, both attack frequency, swimming speed, success of capture, search efficiency and total search area increased over the size range 5–9 mm SL, and total prey catch increased strongly while time spent for foraging decreased (Hunt von Herbing and Gallager, 2000). This dependency on ontogeny for larval performance is a strong argument for applying an age/size specific focus when seeking to fine-tune environmental conditions throughout the larval period.

4.1. Effects from tank bottom colour

Effect plots indicate negative effects from white tank bottom on feeding activity, as compared to grey and black, supporting the suggestions from Naas et al. (1996) that very light bottom colours should be avoided. Overall, the grey bottom had the strongest positive effect. Our results are in accordance with studies for species like herring *Clupea harengus* L (Blaxter, 1968), turbot *Scophthalmus maximus* L (Howell, 1979), dolphin fish *Coryphaena hippurus* (Ostrowski, 1989) and striped bass *Morone saxatilis* Walbaum (Martin-Robichaud and Peterson, 1998), where higher growth and survival was obtained in all-black than in all-white tanks. However, in haddock *Melanogrammus aeglefinus* larvae Downing and Litvak (2000) found improved survival in white tanks and lowered length growth in black tanks at low light intensity. In contrast to our study, cod larvae reared at commercial scale did not differ in foraging, growth or survival in black walled tanks between black and beige tank bottoms (Monk et al., 2008). This may be due to design differences between studies for factors like tank size and shape, added algae, lighting etc. which make direct comparisons difficult. Nevertheless, based on practical benefits from lighter bottoms for tending of tanks, the grey bottom is a practically good choice.

The interaction between bottom colour and light intensity at 15 dph reveals contradictory effects due to increased light between black and white coloured bottoms. Light is a potentially important cue for vertical position (Blaxter, 1975), and it may be that at this stage, the combinations of light intensity and bottom reflection changed spatial distribution in ways that affect larval prey acquisition. Vollset et al. (2009) found that at 10 dph cod larva stay close to the surface. Though, marine fish larvae also show a tendency to collect near tank walls, possibly due to phototaxis (Naas et al., 1996), and in striped trumpeter *Latris lineata* such a behaviour increases with larval age (Cobcroft and Battaglene, 2009). Regarding prey, some rotifer species also demonstrate positive phototaxis (Cornillac et al., 1983). Thus, white bottoms may introduce dual light sources in tanks due to reflection that affect access to prey, either directly by affecting larval spatial position or indirectly by affecting prey distribution.

4.2. Effects from microalgae density

Although there was a general trend that increased amount of algae increased feeding, significant main effect on gut fill due to increased algal concentration was seen only at 5 dph.

Many studies report beneficial effect from added microalgae (Cahu et al., 2003; Naas et al., 1996; van der Meeren et al., 2007), though reasons are not clear. It has been suggested that algae change turbidity and thus prey contrast (Naas et al., 1992), which may enhance feeding. Also, filtration of microalgae prior to first feeding has been seen both in nature and aquaculture (Reitan et al., 1994, 1998; van der Meeren, 1991), and in sea bass (*Dicentrarchus labrax*) Cahu et al. (1998) found positive effects on intestinal enzyme activity from algae at early feeding.

In cod, Van der Meeren et al. (2007) reported increased gut fill at 3 dph due to presence of algae, but no subsequent effects on growth. Skiftesvik et al. (2003) found no behavioral changes due to algae at 5 dph, while Overton et al. (2010) on the other hand, using the copepod *Acartia tonsa* as feed, found significant difference in gut fullness index at 5 to 10 dph between clear water tanks and tanks added algae. Again, design differences between studies (light intensity, tank designs, amount and species of algae and prey types) make generalizations difficult.

We conclude, due to the short-term nature and chosen response variable of this study, that observed effects relate to prey detection or behaviour rather than nutritional effects. This agrees with results from Attramadal et al. (2012), who found no negative effects on survival or growth in cod when exchanging algae paste with inorganic clay.

At 20 dph enhanced algal density improved feeding only at high light levels, indicating that optimal light at this age exceeds 100 lx while 1200 lx without added algae is too high. This is in accordance with Monk et al. (2006) who found that reducing light from 1200 to 650 lx at 28 dph improved capture success and growth in cod larvae.

Results for algae suggest a need for follow-up studies. Reducing or ending the use of algae in favor of fine-tuning of light or replacement with other substances would substantially reduce work demand, costs and the amount of organic load introduced to fish tanks.

4.3. Effects from light intensity

Effects from light were initially vague, but became more pronounced at 15 and 20 dph, indicating that optimal light intensity for cod larvae changes over time. Also, foraging success depends on the combinations of light and the factors bottom colour and algae, as discussed above for bottom colour and algae. The increased importance of light is probably due to the generally improved vision with age in marine fish larvae (Blaxter, 1986). In agreement with our results, Van der Meeren et al. (2007) found no difference in feeding incidence between different light intensities in 5 dph cod larvae, while Puvanendran and Brown (2002) observed improved prey capture from 9 dph, gut fullness from 14 dph and orientation towards prey from 16 dph at 1200 lx, as compared to lower intensities. Though, in the latter study, discrepancies in larval size between treatments evolved over time due to different growth rates, so repeated comparisons of foraging behaviour after size differences start to establish are debatable. In comparison, the present study compares effects between similarly sized larvae, exposed to identical rearing conditions prior to experiments.

4.4. Effects from prey density

Due to optimal foraging theory, increased prey density is expected to enhance prey encounter rate, and thus improve foraging success up to a certain point. The interaction between prey density and bottom colour at 15 dph suggests that larvae respond more strongly to increased feed density in the grey and black bottomed tanks. As bottom colour is a main factor determining illumination in tanks this could reflect improved prey acquisition at beneficial levels of light at this specific developmental stage. It could also be due to some mechanism affecting distribution of larva or prey, thus changing the experienced prey availability (see: Effects from tank bottom colour). The beneficial effects from increased prey density at 15 and 20 dph contradicts with results from Puvanendran and Brown (1999) who observed no improvement in foraging activity at rotifer densities above 4 prey mL^{-1}. However, Hamre et al. (2008) reported elevated survival at rotifer densities of 25–65 mL^{-1}, and Tønnesen Bush (2010) observed lowered prey sinking rates and high survival of cod larvae at 10 rotifers mL^{-1}, and suggested that prey density may have been a limiting factor for cod larval survival in previous studies. State-of-the-art aquaculture practices in general are in support of our results, as prey density is normally increased during the early larval period (Brown et al., 2003), and transition to more energetic feed types occurs as early as possible. Our results suggest that effects from prey density from 10 to 15 dph onwards should be closer examined in order to further optimize feeding protocols.

4.5. Practical implications and follow-up strategies

The main purpose of screening experiments is to identify influential factors and eventual non-additive effects (interactions) between factors, to suggest directions for further optimization. Natural next steps are to fix categorical factors at their optimal level, exclude non influential factors from future experiments and then to perform follow-up sequential experiments to narrow the experimental domain to an area of optimum response, using the method of "steepest ascent" (Box et al., 2005; Debye, 1909). Ultimately, higher order optimization models may be applied to accurately estimate optimum factor values.

In our particular case, tank colour should be fixed at its overall best level, grey, and remaining factors studied further using e.g. a Central Composite Design (Box and Wilson, 1951; Box et al., 2005). With f factors and n_0 replicates in the centre point, total number of design points (tanks) would be $n = 2^f + 2f, + n_0$ (Khuri and Mukhopadhyay, 2010), so e.g. $f = 3$ factors and $n_0 = 6$ centre replicates would demand the same work load and number of tanks (20) as the initial screening. When increased replication is needed, this may be achieved without more tanks, more personnel or drastically increased work load by repeating each single experiment in e.g. randomized blocks at successive days.

4.6. Conclusion

This study demonstrates the usefulness of short-term factorial experiments at discrete larval age/size as tools to track ontogenetically changed environmental demands and accumulate knowledge to support further fine-tuning and optimization of aquaculture conditions. It points out the importance to set levels of environmental factors in accordance with the changing developmental status of larvae, and offers knowledge at a micro scale to guide further fine-tuning of husbandry conditions. Specifically, grey tank bottoms enhanced feeding, and the modest effect from algae suggests that the period of adding algae could be shortened, or algae substituted with non-biotic substances or

improved overall light settings in tanks. The approach is cost effective and easily refined through the use of well established experimental design techniques.

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References

Attramadal, K.J.K., Tøndel, B., Salvesen, I., Øie, G., Vadstein, O., Olsen, Y., 2012. Ceramic clay reduces the load of organic matter and bacteria in marine fish larval culture tanks. Aquacult. Eng. 49, 23–34.
Blaxter, J.H.S., 1968. Rearing herring larvae to metamorphosis and beyond. J. Mar. Biol. Assoc. UK 48, 17–28.
Blaxter, J.H.S., 1975. Do fish have an absolute sense of light intensity? In: Ali, M.A. (Ed.), Vision in fishes. New approaches in research. Plenum Press.
Blaxter, J.H.S., 1986. Development of Sense organs and Behaviour of Teleost Larvae with Special Reference to Feeding and Predator Avoidance. Trans. Am. Fish. Soc. 115, 98–114.
Box, G.E.P., Wilson, K.B., 1951. On the experimental attainment of optimum conditions. J. R. Stat. Soc. B. 13, 1–38.
Box, G.E.P., Hunter, J.S., Hunter, G.W., 2005. Statistics for Experimenters, Design, Innovation and Discoverysecond ed. John Wiley & Sons, Inc., Hoboken, New Jersey.
Brown, J.A., Minkoff, G., Puvanendran, V., 2003. Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems. Aquaculture 227, 357–372.
Cahu, C.L., Zambonino Infante, J.L., Péres, A., Quazuguel, P., Le Gall, M.M., 1998. Algal addition in sea bass *Dicentrarchus labrax* larvae rearing: effect on digestive enzymes. Aquaculture 161, 479–489.
Cahu, C., Zambonino Infante, J., Takeuchi, T., 2003. Nutritional components affecting skeletal development in fish larvae. Aquaculture 227, 245–258.
Chatain, B., 1997. Development and achievements of marine fish-rearing technology in France over the last 15 years. Hydrobiologia 358, 7–11.
Cobcroft, J.M., Battaglene, S.C., 2009. Jaw malformation in striped trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour. Aquaculture 289, 274–282.
Cornillac, A., Wurdak, E., Clément, P., 1983. Phototaxis in monochromatic light and microspectrophotometry of the cerebral eye of the rotifer *Brachionus calyciflorus*. Hydrobiologia 104, 191–196.
Debye, P., 1909. Näherungsformeln für die Zylinderfunktionen für große Werte des Arguments und unbeschränkt veränderliche Werte des Index. Math. Ann. 67, 535–558.
Downing, G., Litvak, M.K., 2000. The effect of photoperiod, tank colour and light intensity on growth of larval haddock. Aquacult. Int. 7, 369–382.
Ellertsen, B., Solemdal, P., Strømme, T., Tilseth, S., 1980. Some biological aspects of cod larvae (*Gadus morhua* L.). Fisk Dir. Skr. Ser. Havunder. 17, 29–47.
Esbensen, K.H., 2006. Multivariate data analysis – In practice, 5th edition. CAMO Software AS.
Fisher, R.A., 1926. The arrangement of field experiments. J. Min. Agric. G. B. 33, 503–513.
Fontaine, P., Teletchea, F., Gardeur, J.-N., 2012. Usefulness of multifactorial approaches for fish domestification. In: Wydawnicza, P. (Ed.), Domestication in Finfish Aquaculture. Olsztyn-Mragowo, Poland.
Hamre, K., Mollan, T.A., Sæle, Ø., Erstad, B., 2008. Rotifers enriched with iodine and selenium increase survival in Atlantic cod (*Gadus morhua*) larvae. Aquaculture 284, 190–195.
Hay, D.E., 1984. Weight loss and change of condition factor during fixation of Pacific herring, *Clupea Harengus pallasi*, eggs and larvae. J. Fish Biol. 25, 421–433.
Hicks, C.R., Turner, K.V., 1999. Fundamental concepts in the design of experiments, 5th edition. Oxford University Press.
Howell, B.R., 1979. Experiments of the rearing of larval turbot, *Scophthalmus maximus* L. Aquaculture 18, 215–225.
Hunt von Herbing, I., 2001. Development of feeding structures in larval fish with different life histories: winter flounder and Atlantic cod. J. Fish Biol. 59, 767–782.
Hunt von Herbing, I., Gallager, S.M., 2000. Foraging behavior in early Atlantic cod larvae (*Gadus morhua*) feeding on a protozoan (*Balanion sp.*) and a copepod nauplius (*pseudodiaptomus sp.*). Mar. Biol. 136, 591–602.
Khuri, A.I., Mukhopadhyay, S., 2010. Response surface methodology. Wiley Interdiscip. Rev. Comput. Stat. 2, 128–149.
Kjesbu, O.S., Taranger, G.L., Trippel, E.A., 2006. Gadoid Mariculture: Development and Future Challenges. ICES J. Mar. Sci. 63, 187–191.
Lanes, C.F.C., Bolla, S., Fernandes, J.M.O., Nicolaisen, O., Kiron, V., Babiak, I., 2012. Nucleotide Enrichment of Live Feed: A Promising Protocol for Rearing of Atlantic Cod *Gadus morhua* Larvae. Mar. Biotechnol. 14, 544–558.
Martin-Robichaud, D.J., Peterson, R.H., 1998. Effects of light intensity, tank colour and photoperiod on swimbladder inflation success in larval striped bass, *Morone saxatilis* (Walbaum). Aquac. Res. 29, 539–547.
Mee, R.W., 2009. A comprehensive guide to factorial two-level experimentation. Springer, Dordrecht.
Monk, J., Puvanendran, V., Brown, J.A., 2006. Do different light regimes affect the foraging behaviour, growth and survival of larval cod (*Gadus morhua* L.)? Aquaculture 257, 287–293.
Monk, J., Puvanendran, V., Brown, J.A., 2008. Does different tank bottom colour affect the growth, survival and foraging behaviour of Atlantic cod (*Gadus morhua*) larvae? Aquaculture 277, 197–202.
Naas, K.E., Naess, T., Harboe, T., 1992. Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L.) in green water. Aquaculture 105, 143–156.
Naas, K., Huse, I., Iglesias, J., 1996. Illumination in First Feeding Tanks for Marine Fish Larvae. Aquac. Eng. 15, 291–300.
Nunn, A.D., Tewson, L.H., Cowx, I.G., 2012. The foraging ecology of larval and juvenile fishes. Rev. Fish Biol. Fish. 22, 377–408.
Ostrowski, A.C., 1989. Effect of rearing tank background color on early survival of dolphin larvae. Prog. Fish Cult. 51, 161–163.
Overton, J., Meyer, S., Støttrup, J., Peck, M., 2010. Role of heterotrophic protists in first feeding by cod (*Gadus morhua*) larvae. Mar. Ecol. Prog. Ser. 410, 197–204.
Puvanendran, V., Brown, J.A., 1999. Foraging, growth and survival of Atlantic cod larvae in different prey concentrations. Aquaculture 75, 77–92.
Puvanendran, V., Brown, J.A., 2002. Foraging, growth and survival of Atlantic cod larvae reared in different light intensities and photoperiods. Aquaculture 214, 131–151.
Puvanendran, V., Leader, L.L., Brown, J.A., 2002. Foraging behaviour of Atlantic cod (*Gadus morhua*) larvae in relation to prey concentration. Can. J. Zool. 80, 689–699.
Puvanendran, V., Laurel, B.J., Brown, J.A., 2008. Cannibalism of Atlantic cod Gadus morhua larvae and juveniles on first-week larvae. Aquat. Biol. 2, 113–118.
Reitan, K.I., Bolla, S., Olsen, Y., 1994. A study of the mechanism of algal uptake in yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus*). J. Fish Biol. 44, 303–310.
Reitan, K.I., Natvik, C.M., Vadstein, O., 1998. Drinking rate, uptake of bacteria and microalgae in turbot larvae. J. Fish Biol. 53, 1145–1154.
Rosenlund, G., Halldorsson, O., 2007. Cod juvenile production: Research and commercial developments. Aquaculture 268, 188–194.
Rosenlund, G., Skretting, M., 2006. Worldwide status and perspective on gadoid culture. ICES J. Mar. Sci. 63, 194–197.
Rotllant, J., Tort, L., Montero, D., Pavlidis, M., Martinez, M., Wendelaar Bonga, S.E., Balm, P.H.M., 2003. Background colour influence on the stress response in cultured red porgy *Pagrus pagrus*. Aquaculture 223, 129–139.
Skiftesvik, A.B., Browman, H.I., St-Pierre, J.-F., 2003. Life in green water: the effect of microalgae on the behaviour of Atlantic cod (*Gadus morhua*) larvae. In: Browman, H.I., Skiftesvik, A.B. (Eds.), The 26. Annual Larval Fish Conference. Norwegian Institute of Marine Research, Bergen, Norway.
Tønnesen Busch, K.E., 2010. Optimal growth and survival of first-feeding cod (*Gadus morhua*) larvae in culture; challenges related to their small size. (PhD dissertation) Faculty of Biosciences, Fisheries and Economics, Department of Arctic and Marine Biology, University of Tromsø, Norway.
van der Meeren, T., 1991. Algae as first food for cod larvae, *Gadus morhua* L.: filter feeding or ingestion by accident? J. Fish Biol. 39, 225–237.
van der Meeren, T., Mangor-Jensen, A., Pickova, J., 2007. The effect of green water and light intensity on survival, growth and lipid composition in Atlantic cod (*Gadus morhua*) during intensive larval rearing. Aquaculture 265, 206–217.
Vollset, K.W., Fiksen, Ø., Folkvord, A., 2009. Vertical distribution of larval cod (*Gadus morhua*) in experimental temperature gradients. J. Exp. Mar. Biol. Ecol. 379, 16–22.
Yin, M.C., Blaxter, J.H.S., 1986. Morphological changes during growth and starvation of larval cod (*Gadus morhua* L.) and flounder (*Platichthys flesus* L.). J. Exp. Mar. Biol. Ecol. 104, 215–228.