



# Optimizing intensive culture protocols for Atlantic cod (*Gadus morhua*) larvae

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## Abstract

Larval cod require live prey as food, and prey concentration (PC) and feeding frequency (FF) often affect their growth and survival. Apart from this, water exchange rates/water flow (WER/WF) and water current (WC) also affect the prey resident time in the tank and larval/early juvenile behaviour, respectively. High water current is also known to induce stress in finfish larvae, and this stress response is believed to be dependent on larval developmental stage. Thus, we conducted a study to evaluate three larval rearing protocols varying in prey concentration, feeding frequency, and water current/exchange rate. Three protocols were used: low prey concentration (PC), low feeding frequency (FF), and low water flow (protocol 1); medium PC, medium FF, and medium WF (protocol 2); and high PC, high FF, and high WF (protocol 3) (see Table 1). Larvae were sampled periodically for growth and cortisol measurements. Cortisol extraction and radioimmunoassay (RIA) were conducted using methods previously validated for cod larvae. Larvae reared using protocols 2 and 3 were significantly bigger and heavier than larvae reared using protocol 1. Rearing protocol had a significant effect on the cortisol level in larval cod. Larvae showed a developmental stage-dependent stress response. Protocol 2 had significantly higher survival than protocols 1 and 3. Our results indicate that an intermediate PC, FF, and WF (protocol 2) is suitable during cod larval rearing.

**Keywords** Atlantic cod larvae · Prey concentration · Feeding frequency · Water exchange · Behaviour

## Introduction

Prey availability during first feeding is one of the most important factors that determine the growth and survival in the majority of marine fish larvae in natural and culture conditions (Houde and Schekter 1980; Puvanendran and Brown 1999). In culture conditions, prey concentration and feeding frequencies (number of feeding events per day) are shown to be important during larval stage, especially during first feeding (Rabe and Brown 2000). Increasing prey availability generally increases growth and

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**Table 1** Feeding protocol and water flow used during the experiment. The numbers in parenthesis are the prey concentration (number L<sup>-1</sup>), R, rotifer (in million feeding<sup>-1</sup>); A, *Artemia* (in million feeding<sup>-1</sup>); FF, feeding frequency (number day<sup>-1</sup>); WF, water flow (L min<sup>-1</sup>); WER, water exchange rate (day<sup>-1</sup>)

Age (days)	Protocol 1					Protocol 2					Protocol 3				
	R	A	FF	WF	WER	R	A	FF	WF	WER	R	A	FF	WF	WER
2–4	0.77 (4277)	–	5	0.8	6.4	1.06 (5888)	–	7	1.1	8.8	1.45 (8055)	–	10	1.5	12.0
5–10				0.9	7.2				1.3	10.4				1.8	14.4
11–24				1.1	8.8				1.5	12.0				2.0	16.0
5–24	1.31 (7277)	–	5			1.81 (10,055)	–	7			2.47 (13,722)	–	10		
25–29	0.9 (5000)	0.22 (1222)		1.3	10.4	1.26 (7000)	0.29 (1611)	7	1.8	14.4	1.7 (9444)	0.39 (2166)	10	2.5	20.0
30–37				1.6	12.8				2.2	17.6				3.0	24.0
30–44		0.67 (3722)	4				0.88 (4888)	5				1.18 (6555)	6		
38–44		Co-feeding		1.9	15.2		Co-feeding		2.6	20.8		Co-feeding		3.5	28.0
45–56		Weaning		2.4	19.2		Weaning		3.3	26.4		Weaning		4.5	36.0

survival of most marine finfish larvae until the maximum prey requirement is reached and growth and survival may decrease beyond the maximum likelihood prey concentration (Puvanendran et al. 2002; Puvanendran and Brown 1999). Holling (1965) described this relationship as functional response II where the feeding rates increased asymptotically at a continuously decreasing rate and plateaued after reaching a maximum prey concentration and the growth of the predator also follows a similar pattern. Further, increase in prey concentration (rotifers and *Artemia*) in intensive larval rearing systems will increase the microbial load, thus, could negatively affect the survival of the larvae beyond a certain prey concentration (Vadstein et al. 2018).

Despite the significant effects that water exchange and flow rates may have on the growth and physiological functions of marine finfish, little information is available on this subject. Continuous water exchange generally sustains the good quality of culture water, while low or zero water exchange may reduce the quality of the water (Tandler and Helps 1985). At very high-water flow rates, the fish spend a substantial amount of dietary energy for continuous swimming against the current, and leading to reduced growth and increased mortality (El-Sayed et al. 2005). At a low water flow rate, uneaten food, faeces, and other fish metabolites may accumulate in fish tanks and adversely affect the water quality (Tandler and Helps 1985). This means proper water exchange and flow rates should be adopted to obtain maximum fish performance. The effects of water exchange on growth and feed utilization of tilapia juveniles (19 g) reared in outdoor tanks have also been studied, and the best growth and FCR were achieved at a continuous flow rate of 0.5–1.0 L min<sup>-1</sup> kg<sup>-1</sup> (Siddiqui et al. 1991). Studies on the effects of water current in growth and survival of larval fish are limited. In Japanese eel, *Anguilla japonica*, larvae (leptocephali) higher water currents negatively affected the growth and resulted in higher deformity rates (Kuroki et al. 2016). Furthermore, water currents have been shown to affect the welfare of the fish through exercise to the body muscle and moderate water currents have been shown to be more effective than low or high currents (Leon 1986; Jørgensen and Jobling 1993; Davison 1997).

Atlantic cod *Gadus morhua* is an important commercial finfish species in the North Atlantic region. With the decline in the supply of Atlantic cod from capture fisheries, culture efforts have been intensified since early 2000 in Norway, Canada, Scotland, and Iceland. Several improvements have been made in the last decade in the early life stages, and the growth, survival, and quality of larvae and juveniles have been improved (Brown et al. 2003; Puvanendran et al. 2022). During this period, larval survival has been improved from 5 to 15–20%; however, variability still exists mainly due to gamete quality (Hansen et al. 2016). Larval growth, survival, and juvenile quality are dependent on husbandry techniques, environmental parameters, nutrition, and egg quality (Planas and Cunha 1999). Imbalance in any of these factors would result in poor growth, survival, and juvenile quality. Even with all the improvements made in the last decade, still commercialization of cod farming faced an uphill battle to survive and make a profit. In 2009/2010, several cod farms in Norway were shut down as the market prices dropped about 25%. Improvements in broodstock management, larval and juvenile growth, survival and quality, feed management in all life stages, delaying maturation, and reducing occurrence of fatty liver syndrome will help to reduce the production cost and improve the profit margin.

Thus, the main objective of this study was to see if efficiency of the production of cod juveniles can be improved by adjusting feeding protocol (prey concentration, feeding frequencies, and water flow rates) by using behavioural and physiological parameters of the cod larvae without affecting the growth and survival.

## Material and methods

### Egg collection and incubation

Egg and sperm were stripped from cultured Atlantic cod adults, originating from the 2005-year class kept at the Centre for Marine Aquaculture (CMA) in Tromsø, north Norway. In total, 2 L of eggs and 300 ml of sperm were stripped from six females and males and were artificially fertilized using dry fertilization method (Hansen and Puvanendran 2010). Fertilized eggs were incubated in 25 L conical incubators at 3.5–4 °C until hatch. At 50% hatch, 15 larvae were sampled for morphometric measurements, and this was taken as day 0 of the experiment.

### Larval rearing

Three experimental treatments (protocols 1–3 with protocol 3 as control which was the current larval rearing protocol used in our facility) with three replicates varying in prey amount added and feeding frequency per day and water flow were used (Table 1). At two dph, 26,000 larvae were transferred to 190 L circular fibreglass tanks providing a stocking density of 137 larvae L<sup>-1</sup>. Water was filtered through a 20-µm sand filter series and disinfected with UV. Water temperature was maintained at 4 °C from 2 to 5 days post hatch (dph) and gradually increased to 10 °C from 6 to 10 dph and remained at this temperature until the end of the experiment. Green water technique was used by adding *Nanochloropsis* sp. microalgae (Reed Mariculture, USA) from 2 to 12 dph. A flow-through water system was used, and the water entered the tanks through a spray bar and left by a perforated PVC centre pipe outlet. The centre pipe was covered with a 500-µm nitex net from 2 to 25 dph, 700 µm nitex net from 26 to 44 dph, and a 1000 µm nitex net from 44 dph onwards. Larvae were fed with enriched rotifers from 2 to 24 dph, with enriched *Artemia* from 25 to 45 dph and with dry diet from 45 to 55 dph. Each diet change had a mixed feeding period of the two diets. Rotifers were enriched with PhosphoNorse® and MicroNorse® (Trofi, Tromsø, Norway), and *Chlorella* and *Artemia* were enriched with enriched with Multigain® (BioMar, Norway), MicroNorse®, and PhosphoNorse®. From 38 to 44 dph, a co-feeding strategy was implemented where dry feed was added to the tanks 1 h before *Artemia* feeding. Weaning onto dry feed (Aglo-Norse®, Trofi, Tromsø, Norway) was achieved by gradually decreasing the amount and feeding frequency of *Artemia* and increasing the amount of dry feed in 5 steps. Weaning onto dry feed was started at 45 dph and was completed at 56 dph. During the rotifer and *Artemia* feeding stages, prey levels were counted in random days in random tanks after 1–2 h post feeding (data is not reported here). Pellet size at the start of the weaning was 200–300 µm and was gradually increased to 300–500 at 63 dph. Larvae were fed by a feeding robot (Storvik Aqua AS, Sunndalsøra, Norway) which has four separate silos and can carry live and dry feed at the same time and feed the larvae precisely. The robot moves on trails fitted on the roof and was controlled by a centralized computer system. Rotifers were fed every 5, 3.5, and 2.5 h for larvae reared in protocols 1, 2, and 3, respectively. *Artemia* were fed every 6, 5, and 4 h for larvae reared in protocols 1, 2, and 3, respectively. After weaning, the larvae were fed every hour in all three protocols. The number of larvae in each tank was counted at 70 dph.

## Data collection

The temperature was measured daily and was within the set range  $10 \pm 0.5$  °C. The oxygen levels were measured twice a week and ranged between 6 and 8 mg L<sup>-1</sup> in all tanks. The salinity of the sea water around Tromsø is very stable and stayed at 33.4‰. Water velocity speed was measured at mid-depth along the tank wall against the flow direction when the water flow was changed at 5, 11, 25, 30, 38, and 45 dph during the experiment using an HFA water velocity meter (Höntzsch GmbH, Waiblingen, Germany).

Fifteen larvae from each tank (45 per treatment) were sampled for length and dry weight measurements at 0, 13, 23, 33, 43, 53, and 63 dph. Larval standard lengths were measured using a stereoscope (LeicaMZ125, Oslo, Norway) fitted with an ocular micrometre. Larvae were washed in distilled water and placed in three aluminium foils (each foil contained a group of five larvae) and were dried at 92 °C for 24 h. Dried larval samples were weighed using an analytical weighing scale with a precision of 0.00001 g (Mettler Toledo GmbH, Switzerland).

The following five larval distribution patterns in the tank were also recorded at 11:00 a.m. from 11 dph onwards until the end of the experiment: (1) Homogenous distribution throughout the water column (HD), (2) larvae in the upper water column distribution (UD), (3) larvae on the bottom tank distribution (BD), (4) patchy (in clumps) distribution (PD), and (5) doughnut shape distribution (DD).

Solid waste (uneaten feed and faeces) settled at the bottom of the tank was collected by siphoning from 8 dph (6 days after transferring the larvae). Waste was collected every 5 days from 8 to 23 dph; every 4 days on 27 and 31 dph and every 3 days from 34 to 52 dph. Once collected in a bucket, the waste was concentrated in a graduated cylinder and the amount of waste was recorded.

Larvae (80–150 mg in total) were sampled for cortisol measurements at 14, 24, 28, 32, 37, 40, 44, 50, and 56 dph. Larvae were captured using a fine mesh dip net and were immobilized using tricaine methane sulphonate at a concentration of 400 mg L<sup>-1</sup>. Larvae were rinsed in sterilized sea water and placed in 2 mL cryovials. Another extra set of samples were also made on 14 dph for calibration. Samples were immediately frozen in liquid nitrogen and were stored at -80 °C until further analysis. All sampling procedures were carried out in accordance with the Institutional Animal Care and Use Committee at the Aquaculture Research Station in Tromsø.

## Cortisol analyses

The concentration of cortisol in cod larvae was quantified by means of radioimmunoassay (RIA), as described by Schulz (1985). Assay characteristics and antiserum cross-reactivities can be found in Tveiten et al. (2010). Prior to cortisol extraction, c. 125 mg of larvae (10–125 larvae depending on the age) was weighed to the nearest 0.1 mg; 700 µL RIA buffer was added, before being homogenized (Ultra Turrax) twice for 30 s on ice. In short, free (i.e., non-conjugated) steroids were extracted from the prepared homogenate with 4 mL diethyl ether under vigorous shaking for 4 min. Subsequently, after 2–3 min of phase separation, the aqueous phase was frozen in liquid nitrogen and the organic phase was transferred to a new glass tube and kept in a water bath at 45 °C until all ether was evaporated. The steroid was reconstituted by adding 300 µL

of RIA-buffer, and the extract was stored at  $-25\text{ }^{\circ}\text{C}$  until being assayed for cortisol. Cod larvae cortisol concentrations are presented as  $\text{ng/g}$  larvae wet weight.

To validate cortisol recovery after diethyl ether extraction, triplicates of larvae samples were spiked with a known amount of radiolabelled cortisol (20–30,000 cpm (counts per minute) and then underwent the extraction procedure described above. The cortisol extraction efficiency was  $85.5 \pm 2.1\%$ . The extraction efficiency was accounted for when calculating final sample concentration. Moreover, from selected samples with high cortisol concentrations, a dilution curve with four different concentrations was made, and found to be parallel to the standard assay curve.

## Data analysis

Two-way ANOVA was used to analyse the length, weight, survival, waste amount, larval distribution, and whole-body cortisol concentration with age as co-variable to determine the effect of the three rearing protocols. All data sets were tested for the assumptions of normality, heterogeneity, and independence of residuals by plotting a frequency distribution of the data and visually compared to a normal distribution. When larval dry weight and waste volume in the tanks were found to violate these assumptions and were log transformed to satisfy the normality assumptions. Significant differences between rearing protocols were identified using a Tukey multiple comparison test. A significance level of 5% ( $\alpha=0.05$ ) was used for all statistical tests. The SPSS statistical package software version 15.0 was used (SPSS inc. Chicago, IL, USA).

## Results

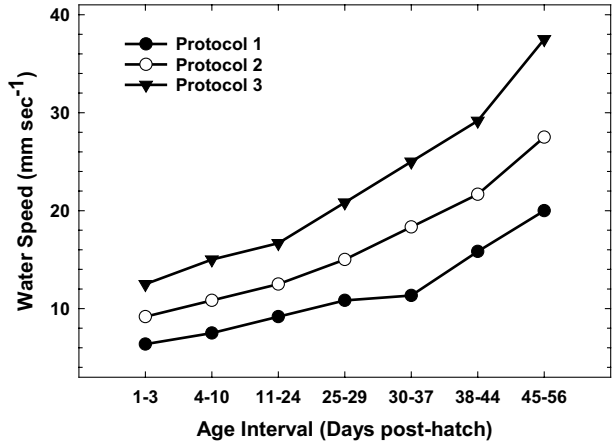
Protocol 1 used less live feed and water compared to protocols 2 and 3 (Table 2). Protocol 1 used one-quarter and one-half of rotifers than protocols 3 and 2, respectively. Similar trend is shown in *Artemia* and water usage too. The water current speed increased from 6.67, 9.2, and 12.5  $\text{cm s}^{-1}$  at 2 dph to 20, 27.5, and 37.5  $\text{cm s}^{-1}$  at 45 dph in protocols 1, 2, and 3, respectively (Fig. 1).

Rearing protocol had significant effect on the standard length ( $F=7.78$ ,  $\text{df}=2$ ,  $p<0.001$ ) dry weight ( $F=8.5$ ,  $\text{df}=2$ ,  $p<0.001$ ) and survival ( $F=6.75$ ,  $\text{df}=2$ ,  $p<0.038$ ) of the cod larvae. Larvae reared in protocol 1 were smaller in length and lighter in dry weight than the larvae reared in other two protocols since 43 dph (Fig. 2). Larvae reared in protocols 2 and 3 were significantly larger at 43 and 63 dph ( $p<0.05$ ) and heavier at 63 dph ( $p<0.028$ ) than larvae reared in protocol 1. Survival of larvae in protocol 2 was significantly higher ( $p<0.037$ ) than the other two protocols (Fig. 3).

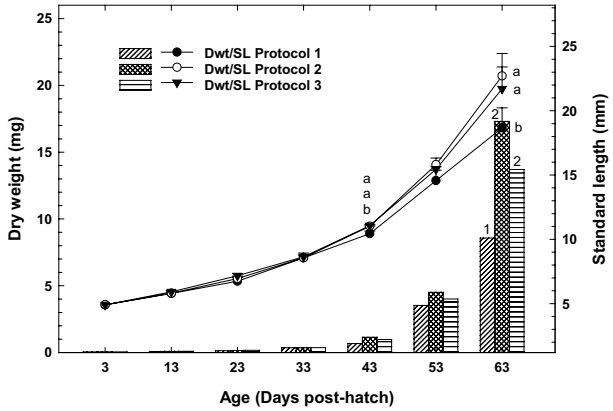
**Table 2** Number of rotifers and *Artemia* (in millions) and water (in L) used in the three treatments during the experiment

Treatment	Rotifer	<i>Artemia</i>	Water usage	Resources used relative to High (%)		
				Rotifer	<i>Artemia</i>	Water
Protocol 1	495	207	406,944	26.5	41.3	53.4
Protocol 2	957	321	560,304	51.2	64.0	73.5
Protocol 3	1867	501	761,616	100	100	100

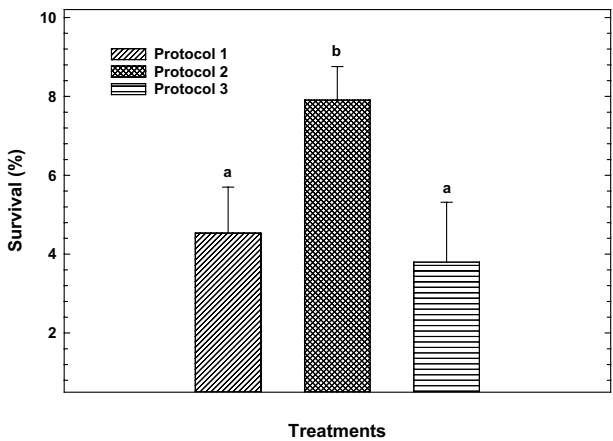
**Fig. 1** Water current speed in the tanks for the three larval rearing protocols used in this experiment



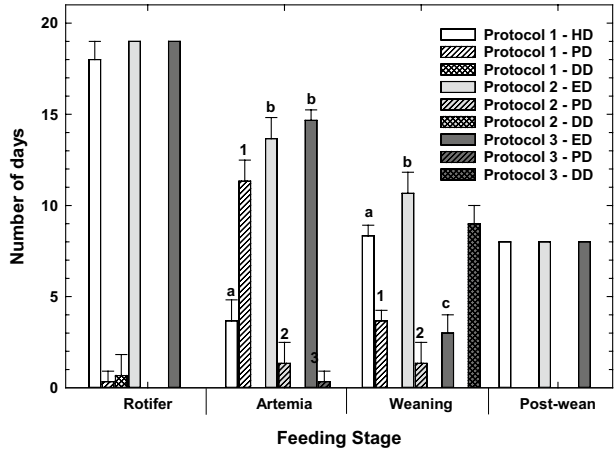
**Fig. 2** Dry weight and standard length of Atlantic cod larvae in the three larval rearing protocols from 3 to 63 dph. Values are mean  $\pm$  SD. Different letters (standard length) and numbers (dry weight) within each age indicate significant differences at  $p > 0.05$



**Fig. 3** Survival of Atlantic cod larvae in the three larval rearing protocols from 65 dph. Values are mean  $\pm$  SD. Different letters indicate significant differences at  $p > 0.05$



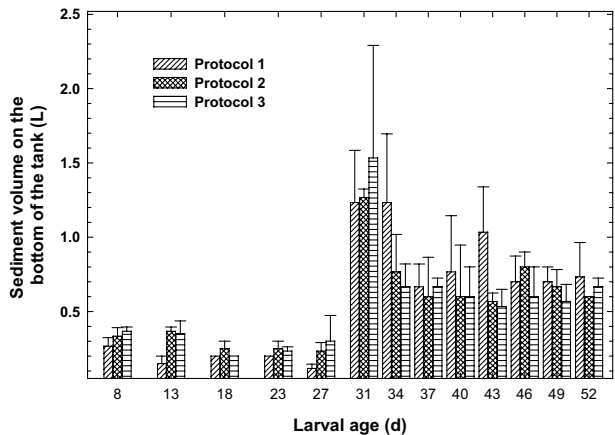
**Fig. 4** Larval distribution pattern in the tanks of three rearing protocols. Values are mean  $\pm$  SD. Different letters/numbers within each feeding stage indicates significant differences among the same distributional pattern at  $p > 0.05$



Cod larvae exhibited all the five distributional patterns during the experimental period at different feeding stages (Fig. 4). HD was the dominant distributional pattern in all feeding stages followed by the PD. DD was the dominant distribution pattern during the weaning phase in protocol 3. There were no significant effects of treatment on any of the behaviour of cod larvae during rotifer feeding stage ( $p > 0.05$ ). However, in the *Artemia* feeding and weaning phases, significant differences existed in HD and PD among the three treatments (Fig. 4). Only HD was present in the post-weaning phase, and there were no differences among the treatments.

Sediment volume in the tanks were not significantly different at any of the sampling dates except for 13 dph where protocol 1 had less sediment compared to protocols 2 and 3 ( $p < 0.026$  and  $0.046$ , respectively) and 43 dph where protocols 2 ( $p < 0.016$ ) and 3 ( $p < 0.007$ ) had significantly less sediment than protocol 1 (Fig. 5). There was a sharp increase in sediment volume in all treatments when the livefeed was switched from rotifers to *Artemia* but then declined and stayed at a stable level during the rest of the experimental period (Fig. 5).

**Fig. 5** Sediment volume collected from the bottom of the tanks in three rearing protocols. Values are mean  $\pm$  SD



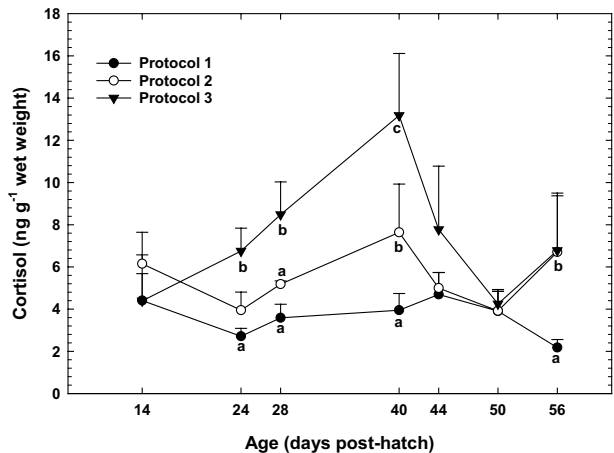


Treatment ( $F=27.81$ ,  $df=2$ ,  $p<0.000$ ) and age ( $F=6.66$ ,  $df=6$ ,  $p<0.000$ ) had significant effects on the cortisol levels in cod larvae. Throughout the experimental period, larvae reared in protocol 1 had the lowest cortisol level compared to the larvae reared in other two treatments (Fig. 6). Larvae reared in protocol 3 had significantly higher cortisol levels at 24, 28, 40, and 56 dph ( $p<0.01$ , 0.002, 0.000, and 0.009, respectively) than larvae from protocol 1. In general, the cortisol level in larvae from all treatment groups increased until 40 dph and then decreased until 50 dph. After this, the cortisol levels increased in larvae from protocols 2 and 3, while cortisol level decreased further in larvae from protocol 1 (Fig. 6).

## Discussion

Among the three cod larval rearing protocols used in this study, protocol 2 resulted in best growth and survival. Protocol 1 used lesser resources in terms of live feed and water compared to other two protocols, but larvae reared using protocol 1 were significantly smaller than larvae from other two protocols at the end of the experiment (Fig. 2). The PC used in protocol 1 (Table 1) was within the range of nominal PCs that gave better results in other studies (Puvanendran and Brown 1999) indicating other factors such as water flow rate and feeding frequency could also play a role on the growth and survival of larval cod. Unfortunately, the experimental design of our study did not allow us to unravel the individual effects of the three factors we used. Sustainable aquaculture practices require measures of reducing water use and waste products to minimize the potential environmental impacts. While reducing the wastage, optimal growth should also be maintained during the early life stage of marine finfish and any reduction in growth during these stages could have long-term negative effects on production performances (Imsland et al. 2006; Bizuayehu et al. 2015). In our experiment, protocols 2 and 3 used more livefeed and water but the growth was significantly higher than the larvae from protocol 1. Given the long-term effects of early life stage growth on later life stages, lower growth in larvae from protocol 1 could have negative effects later in the life stage. Similar negative effects of lost growth opportunities due to inferior nutrition during larval stages of Atlantic cod has been reported in several other studies (Galloway et al. 1999; Imsland et al. 2006; Koedijk et al. 2010; Øie et al. 2017) and in gilthead seabream (Turkmen et al 2019). In cod, even a short-term feeding of

**Fig. 6** Whole body cortisol levels of larval cod raised in the three rearing protocols. Values are mean  $\pm$  SD. Different letters within each age indicates significant differences at  $p > 0.05$



copepods during the first week of start feeding resulted in significant difference in growth at juvenile stage (Koedijk et al. 2010; Øie et al. 2017) and at adult stage (Imstrand et al. 2006). Cod larvae require prey concentration in the range of 4–16 prey L<sup>-1</sup> for better foraging, growth, and survival in intensive rearing systems (Puvanendran and Brown 1999). The prey concentration and feeding frequencies in our study was well within the range in all three protocols, thus prey concentration may not have any major effects on the observed differences in growth and survival.

An optimal flow rate for larvae and early juveniles is likely to be a compromise between tank hygiene related to waste removal, feeding, and fish energy expenditure by the fish to swim against the water current (Forster and Ogata 1996). We did not measure the ammonia in the tanks. We used a flow through system, and the exchange rate was relatively high ranging from 6.4 to 19.2 from 2 to 56 dph in protocol 1. Our observations during the experiment and in later production attempts showed that not all the waste stays and accumulates in the tank bottom. Smaller wastes are usually escaped through the screens in the centre pipe. Other studies on cod using a water exchange rate of 4.8 to 12 during 2–56 dph could not detect any ammonia in their tanks (Puvanendran et al. 2009). Therefore, we believe there would have been any effect of ammonia in any of our tanks. Significantly higher survival of larvae in protocol 2 compared to protocols 1 and 3 indicates a better feeding (PC) and rearing environment (WC and WER). Increase in prey concentration may also increase the microbial load in the rearing tanks, and this could decrease the larval survival (Vadstein et al. 2018). A 50% more exchange rate in protocol 3 compared to protocol 2 could have reduced the microbial load. Still, increase in microbial load could not be ruled out in protocol 3 which could be a reason for increased mortality. The flow rate in larval rearing systems needs to be optimized to provide sufficient oxygen, meanwhile generating a proper current velocity that would not cause the larvae to swim against it. Higher than optimal current may disturb their distribution in the tank and that could affect their feeding and consequently the growth and survival (Merino et al. 2007).

Effective feeding practices are essential in reducing the waste emission in intensive aquaculture systems. In our experiment, larvae were precisely fed with feeding robots which reduced the feed wastage. The FF and PC varied among the three protocols but also had varied WER where the exchange rate was low in protocol 1 and high in protocol 3. With the varied WER, FF, and PC in all three treatments, each of the three variables compensated each other and resulted in similar prey levels in all three protocols after 1–2 h post-feeding. The amount of sediment in the tanks in all three protocols were similar throughout the experiment (Fig. 4). Typically, wastes in fish tanks first originate from the partial intake of feed by fish or the partial digestibility of feed. Soft bodied rotifers move slowly through the water column (Sarma et al. 2020), and this makes it easier for the larvae to catch and digest which could have led to a lower solid wastage during rotifer feeding phase (Fig. 4). However, *Artemia* has a thicker exoskeleton and has a faster movement through water column compared to rotifers (Garaventa et al. 2010) which make it difficult for the larvae to catch and digest. However, the efficiency of catching the *Artemia* increases with familiarity (Puvanendran et al. 2004) and the digestibility also increases with age (Perez-Casanova et al. 2006); thus, the amount of sediment decreased after a brief sharp increase at 31 dph (Fig. 4).

Larval distribution can be affected by type of livefeed used, water current, and larval developmental stage (Sakakura et al. 2006). In our experiment, during rotifer feeding and post-weaning periods, cod larvae were homogeneously distributed throughout the water column irrespective of the protocol used (Fig. 5). However, this pattern has changed in the *Artemia* and weaning stage and larvae in protocol 1 exhibited more patchy distribution,

while larvae in protocols 2 and 3 still displayed homogenous distribution. Aeration in the tanks can also affect the larval distribution (Sakakura et al. 2006) and survival (Shiotani et al. 2005); however, in our experiment, the aeration was kept constant.

Shiotani et al. (2005) reported that a moderate aeration, thus creating a moderate flow, has resulted in higher survival in seven band grouper larvae, *Epinephelus septemfasciatus*, while a higher and lower aeration resulted in increased mortality. Shiotani et al. did not measure cortisol in the fish but suggested that stress at higher aeration and less water movement in lower aeration resulting in unfavourable environmental conditions were the reasons for the higher mortality. Similar mortality patterns were observed in our experiments with the survival at moderate water flow resulting in a higher survival, i.e., protocol 2. Zhenjia et al. (2023) have reported a similar increase in cortisol levels with increasing water velocities in leopard coral grouper, *Plectropomus leopardus*, juveniles. In juvenile Japanese flounder, *Paralichthys olivaceus*, Ogata and Oku (2000) reported slow growth at higher water velocities compared to fish reared in moderate and low water velocities; however, they did not measure the serum cortisol level. However, other studies indicated that higher serum cortisol levels can impede the growth performance in fish (Gregory and Wood 1999). Cortisol levels in marine fish species including cod (Westelmajer 2008) reported to be low early larval stages and steadily increases in larvae as they grow and reach a peak level during the onset of metamorphosis (de Jesus et al. 1991; Tanaka et al. 1995; Szisch et al. 2005). In our experiment, the cortisol level followed the similar pattern and was highest between 40 and 44 dph and larvae were about 11 mm in average length and a range of 9.4–12.6 mm (Fig. 6) which is perceived as the start of the metamorphosis in Atlantic cod (Pedersen and Falk-Petersen 1992). Within 8 days post-hatch, cod larvae are physiologically able to mount a corticosteroid response after exposure to acute stress, indicating a functional HPI-axis beyond this stage (King and Berlinsky 2006). Their work, which covered developmental stages up to day 23 (also at 10 °C) post-hatch, showed a somewhat higher corticoidsteroid concentration under resting conditions at day 14, but were similar to cortisol concentrations (c. 5 ng g<sup>-1</sup> wet weight) recorded in the current study when compared at day 23.

Change in feeding regimes and dietary factors, especially the weaning period, may also cause stress and increase serum cortisol levels (Koven et al. 2003). However, in our study, the weaning period was from 45 to 56 dph which was after the peak cortisol levels that was seen between 28 and 40 dph. During this high cortisol peak, the feeding regime was co-feeding stage (38–44 dph), and during this time, the larvae were still fed with *Artemia*. Studies have also shown that co-feeding improves the performance of larval fish (Djel-lata et al. 2021). Given this, it is unlikely that the co-feeding caused the increase in cortisol level during 28–44 dph and is more likely related to other rearing conditions (see below). In eel larvae, Yamano et al. (1991) showed that cortisol levels were very high at 200 ng g<sup>-1</sup> in smaller leptocephali and cortisol levels were maintained low at less than 60 ng g<sup>-1</sup> throughout the metamorphosis in larger leptocephali. Cortisol concentrations in larvae from protocols 2 and 3 decreased from day 40 to 50, whereas only small changes were recorded for larvae in protocol 1 during the same period and they were also undergoing metamorphosis. So, our result is not conclusive if this increase in cortisol levels in larvae from protocols 2 and 3 was related to metamorphosis.

Davison (1997) in his review on the effects of exercise training in teleost fishes concluded that training speed at 1.5 body length s<sup>-1</sup> (BL s<sup>-1</sup>) or less improves the growth performance in many salmonids and striped bass. In our study, water flow/speed in protocols 1 and 2 was about 1.5 BL s<sup>-1</sup> or less and the water flow/speed in protocol 3 was 2.5 BL s<sup>-1</sup> (Fig. 5). Guan et al. (2008) reported that the critical swimming (Ucrit) speed of larval cod at 52 dph (10 °C) to be 5 BL s<sup>-1</sup> or 9.7 cm s<sup>-1</sup>. In relation to the latter, maintaining a stationary position in

the water currents used in this study, larval swimming speed may be categorized as being in the “low to moderate” range, but without being exhaustive. Increased energy expenditure and oxygen demand related to swimming may, however, require ventilatory and circulatory adaptations that will result in hydromineral imbalance, known as the “osmorepiratory compromise” (Wood and Eom 2021). Swimming induces increased ventilation rates, branchial blood perfusion, and lamellar recruitment providing an enhanced functional gill surface area promoting permeability for gas, but also unwanted changes in ion and water fluxes (Wood and Eom 2021). According to Hunt von Herbing et al (1996), cod larvae have well developed gills with numerous filaments and lamellae at the developmental stages covered in this study, providing the basis for an osmoregulatory challenge (influx of ions and loss of water) related to ventilatory- and circulatory adaptations related to increased swimming activity. Also in postembryonic fish larvae, development of osmoregulatory capacity and the ability to face osmotic challenges is under neuroendocrine control (reviewed in Varsamos et al 2005). Cortisol, which has both glucocorticoid and mineralcorticoid functions in teleosts, is an important hormone in osmoregulation in adult as well as in postembryonic/larval fish where it stimulates to increased ionocyte Na/K-ATPase activity and improved osmoregulatory capacity (Varsamos et al 2005).

A closer examination of our data revealed that water velocity, rather expressed as  $\text{BL s}^{-1}$ , changed over time with a similar pattern, but at different levels, within the different protocols (Supplementary Fig. 1). A peak in water speed ( $\text{BL s}^{-1}$ ) was seen around day 33, when protocol 3 reached almost  $3.0 \text{ BL s}^{-1}$ , and then a decline towards the end of the experiment. A similar temporal pattern was recorded for cortisol, peaking during the same period (days 28–40) as water velocity (Fig. 6). Water velocity explained 60% of the variation in larvae cortisol concentration (Supplementary Fig. 2), which may suggest a relationship between swimming effort and cortisol mobilization. Also, in support of this view, the lack of relationship between absolute water velocity and cortisol may indicate that high water velocity per se may not be perceived as stressful. From supplementary Fig. 2, it may be tempting, although speculative, to infer that a cortisol response is elicited at a water speed around  $1.75\text{--}2.0 \text{ BL s}^{-1}$  and beyond. This would also explain the temporal differences in cortisol concentrations between protocols, where protocol 1 resulted in only minor changes (Fig. 6). During the developmental stages investigated here, cod larvae do not just grow in length and weight but also display distinct morphological (and likely physiological) changes (e.g., fin development) which increase their swimming ability (Hunt von Herbing et al. 1996). Thus, as swimming ability increases, less metabolic and respiratory/circulatory adaptations may be necessary for a given swimming speed.

It should be noted that the  $U_{\text{crit}}$  is the potential swimming ability of the fish determined by forced swimming increasing velocity tests and not the optimal/preferred swimming speed ( $U_{\text{opt}}/U_{\text{pref}}$ ). At the  $U_{\text{pref}}$  speeds, the cost of transport is the minimal and the energy can be channelled to growth (Tudorache et al. 2011). Moderate water flow in fish tanks provides optimal cost of transport and provides an optimal exercise-induced growth (Davison and Herbert 2013) and the fish had lower cortisol levels (Huntingford and Kadri 2013). At a high-water flow, the fish spend more energy to maintain their position in the water column which increases their stress levels, while at a low water speed, a substantial amount of energy is lost due to higher spontaneous swimming activity (Davison 1997; Palstra et al. 2010). Therefore, water velocity used in protocol 3 would have had a negative impact on cod larval growth, while water velocity in protocols 1 and 2 would have enhanced the growth performance. However, significant differences in growth between larvae from protocol 1 and larvae from protocol 1 and 2 and nonsignificant but larger larvae in protocol 2 compared to protocol 3 suggest a moderate water flow in cod larval rearing tank is more beneficial.

The knowledge of the optimal culturing conditions is important for sustainable intensive aquaculture of marine fish species. While optimal feeding is an important factor improving the growth and survival of Atlantic cod larvae, our experiment also showed that an optimal water exchange rate and flow are also important elements to consider. Results from our experiment showed that the efficiency of the production of cod juveniles can be improved by adjusting feeding protocol (prey concentration, feeding frequencies and water flow rates) during larval rearing through improvement in the growth and survival. Our experiment showed that a moderate feeding, water flow, and exchange rate used in protocol 2 provided better growth and survival in Atlantic cod larvae. We currently use this larval rearing protocol in our facility with greater success. The standard cod larval protocols are now established but as always fine tuning of the protocols is still going on. Currently, the major contributor for the variable survival between experiments is gamete quality. The gamete quality varies significantly between different batches (cod is a batch spawner) from the same female/male and between different females/males. More research on cod broodstock husbandry and nutrition to improve the gamete quality is warranted.

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**Data availability** The authors declare that data supporting the findings of this study are available within the article in the form of tables and figures.

## Declarations

**Ethics approval and consent to participate** The manuscript is not submitted to any other journals for simultaneous consideration. The submitted work is original and has not been published elsewhere in any form or language.

**Human and animal ethics** This study was carried out in accordance with the Norwegian regulations for use of animals in experiments and was approved by the internal animal welfare committee. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

**Consent for publication** All authors agreed with the content of the manuscript and that all gave explicit consent to submit the manuscript to Aquaculture International.

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