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Effects of egg incubation temperature on survival, prevalence and types of malformations in vertebral column of Atlantic Cod (*Gadus morhua*) larvae

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Abstract
Survival, prevalence and types of malformations in vertebral column of Atlantic cod (*Gadus morhua*) larvae were assessed at hatch for six different egg incubation temperatures from 6 to 11°C, which reflected the natural temperatures experienced during the spawning season in the Shetland Isles. There were no significant differences in mortality between eggs incubated at different temperatures. A total of 152 out of 249 hatched larvae (61%) exhibited one or more out of four main types of vertebral malformations - kyphosis, lordosis, scoliosis, and severe vertebral curvature. Mean prevalence of malformations ranged from 58% to 66% for different incubation temperatures, and from 51% to 78% for each group of eggs. There were no significant differences in sum of prevalence of malformations between different groups and temperatures, but significant difference between types of malformations at different temperatures. The prevalence of malformed larvae with severe vertebral curvature increased significantly as egg incubation temperature increased.

Introduction

Malformations of fish are prevalent in many species and are particularly important in species cultivated for commercial aquaculture (Fraser et al., 2004). Malformations can have serious economic implications for both hatchery operators and on-growers, and can lead to problems ranging from lowered performance to downgrading at harvest (Barahona-Fernades, 1982; Andrades et al., 1996). The prevalence and types of malformations have been shown to be specific to the hatchery rearing conditions, but there are also general malformations associated with rearing protocols in many hatcheries (Boglione et al., 2001). Use of rearing and feeding protocols of warm-water species has been adopted, however this may not be appropriate for gadoids and lead to future problems (low survival, poor growth, malformations).

Few studies have investigated causes and types of malformations in cold water marine species such as Atlantic cod (*Gadus morhua*) mainly because culturing these species on a large commercial scale has not been successfully achieved until recently (Brown et al., 2003). Olsen et al. (2004) suggested that up to 60% of hatchery reared cod in Norway had problems associated with malformations, and thus it is recognised as a major problem.

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Malformations in other cultured species have been well documented in freshwater species such as salmon and trout (Kvellestad et al., 2000; Sadler et al., 2001), and warm-water marine species such as sea bass and sea-bream (Barahona-Fernades, 1982; Andrades et al., 1996; Afonso et al., 2000; Koumoundouros et al., 2002; Boglione et al., 2003), and barramundi (Fraser et al., 2004).

Different causes of malformations that can affect all stages from egg to adult for many different species have been suggested, including nutrition (Bell et al., 1996; Roy et al., 2002; Brown et al., 2003; Olsen et al., 2004), pollution (Muramoto, 1981; Kennedy et al., 2000), adverse temperatures (Ali and Lindsay, 1974; Weigand et al., 1989; Polo et al., 1991; Buckley et al., 2000), gas super-saturation (Chapman et al., 1988), and other diseases (Treasurer, 1992; Kent et al., 2004).

There is very limited research in this area for cultured Atlantic cod, therefore understanding the effects of initial rearing temperatures on cod larval quality may have important consequences in rearing procedures for commercial gadoid aquaculture. This study describes the effects of incubating eggs at temperatures normally occurring in winter and spring on initial survival, prevalence and types of malformations in vertebral column of larvae at hatch.

Materials and methods

Eggs were collected from local Shetland wild broodstock (8 females and 4 males) held in a 24m^3 (5kg/m^3) circular tank of 1.8m depths. Water at 6.0^{0}C ± 0.5, filtered through a 60μm was delivered to the tank at a constant flow of 60l/min giving a theoretical retention time of 6.6 hours. The broodstock were fed to satiation on sausages prepared from EWOS marine broodstock booster diet three times per week.

The cod spawned naturally between 2nd February and 28th April 2005. Viable fertilised eggs floated to the surface and were collected each morning using a custom-made 50μm mesh filter from the outflow of the surface drain of the tank. Six groups of eggs were collected on 27th, 28th February, 1st, 2nd, 5th and 7th March 2005.

After collection, the eggs were disinfected in peracetic acid (Vetroxide - Pharmaq) at a concentration of 4000ppm in seawater for 45 seconds before being transferred to sterile seawater. Each egg was pipetted onto a microscope slide for examination using a sawn off pipette together with 0.75ml of seawater before being transferred to well plates. Seawater was filtered through a 1μm filter and UV sterilised. Handling of eggs was minimised to avoid mechanical damage.

144 eggs were selected from each group that were divided equally between the six temperature treatments. Overall a total of 36 groups and a total of 864 eggs were incubated. The eggs were placed in 24 chamber well plates (volume = 3ml) with 1 egg per well. The well plates were put inside plastic boxes and submerged in controlled water baths (90l paxton chilled with ICES TAE-08 chiller for 6°C, Techne Junior TE-8J for 6.5°C, 8°C, 9°C, Griffin & George BJL-400-110F for 10°C, LMS Ltd 305 refrigerator for 11°C) inside the well plates. A temperature-logging device recorded temperature every 5 minutes (Lotek LTD1110) and every hour (Vemco mini-

logger). The six different temperatures obtained were 5.9°C ± 0.1, 6.6°C ± 0.1, 8.1°C ± 0.3, 9.3°C ± 0.1, 10.6°C ± 1.1, 11.0°C ± 0.3. Variation in temperature for 10°C was caused by initial fluctuations that affected only two groups, and this was corrected immediately.

Eggs were examined daily until one day post-hatch using a light, backlit microscope in a room kept at 6°C to assess developmental stage using Thompson and Riley's 1981 index. Malformations were assessed immediately at hatch only for larvae that were living (heartbeat observed). Malformations were classified as described by Boglione et al. (2003). One day post-hatch, the larvae were measured and photographed at a magnification of 3.2 using a digital camera (Nikon Coolpix 4500) under a light microscope (Zeiss 2000-C) to assess types and quantities of skeletal malformations.

Results for survival and prevalence for different types of malformations are expressed as means. Data expressed as percentages were arcine transformed before analysis. One-way ANOVA with Tukey's post-test was used to determine significant differences ($P<0.05$) between groups and temperatures. All statistical analysis was performed using PRISM (Graphpad, 1999).

Results

Survival

A total of 249 out of 864 larvae hatched (29%) for all groups and incubation temperatures. Mean survival varied from 21% to 35% for different temperatures, and 15% to 65% for different groups (Table 1). There were significant differences in survival between groups of larvae (ANOVA: df=5, F=3.37, $P<0.05$), but no significant differences between different incubation temperatures.

Malformations in vertebral column

The prevalence of malformations observed for each incubation temperature for all groups of larvae is shown in Table 1. A total of 152 larvae (61%) exhibited some type of malformation

Temperature (°C)	**6**	**6.5**	**8**	**9**	**10**	**11**
Eggs incubated (N)	144	144	144	144	144	144
Survival (at hatch) %	34	24	35	26	21	34
Larvae examined for malformations (N)	49	34	50	37	30	49
Normal %	41	38	36	43	34	42
Kyphosis %	27	15	28	19	23	22
Lordosis %	24	35	28	22	23	18
Scoliosis %	4	3	6	8	10	2
Kyphosis / lordosis complex %	4	6	0	0	0	0
Severe vertebral curvature %	0	3	2	8	10	16
Sum of vertebral malformations (%)	59	62	64	57	66	58

Table 1. Survival (%) and prevalence of types of vertebral malformations (%) of Atlantic cod larvae at hatch for different incubation temperatures.

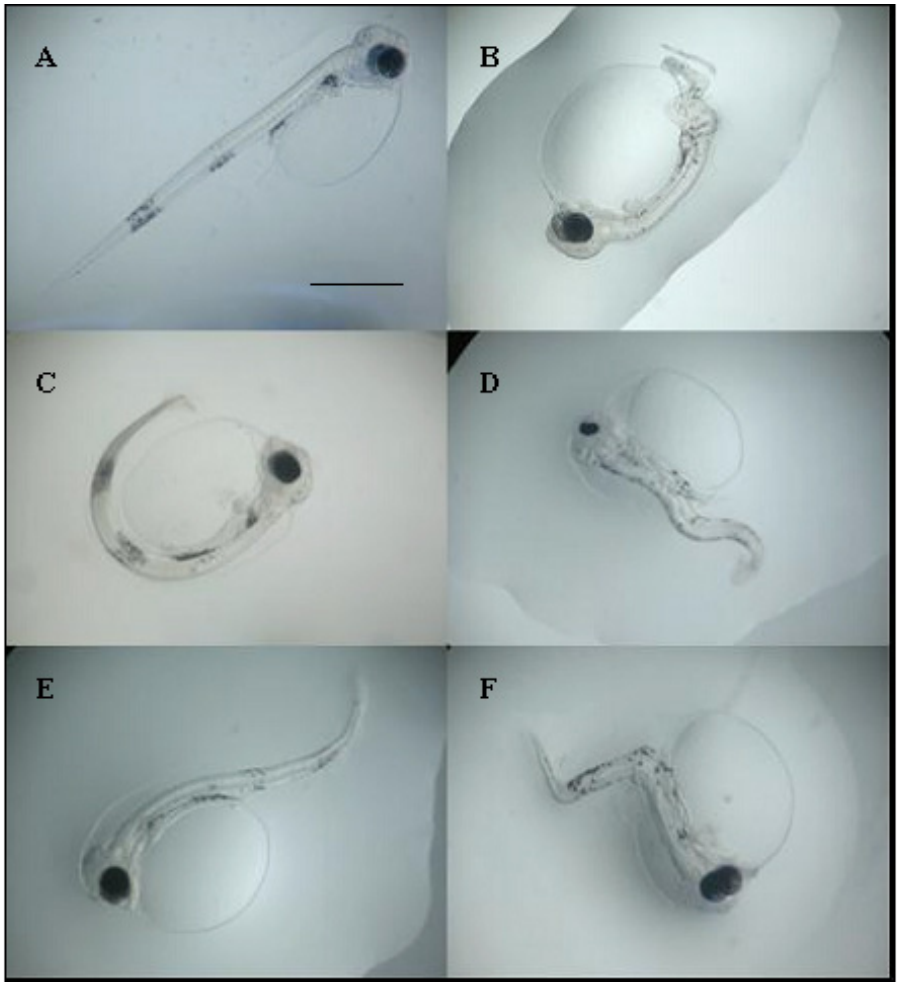

Figure 1. A – normal larvae without signs of vertebral column malformation; B – severe vertebral curvature; C – kyphosis; D – scoliosis; E – lordosis; F – lordosis / kyphosis complex. Scale bar 1mm shown in A.

at hatch. Prevalence of malformed larvae ranged from 57% to 66% for different incubation temperatures, and 51% to 77% for different groups of eggs. One-way ANOVA indicated that there were no significant differences between prevalence of malformations between groups of eggs and incubation temperature.

Types of malformations observed for all temperature treatments included kyphosis (V shape), lordosis (Λ shape), scoliosis (lateral malformation), kyphosis / lordosis complex, and larvae with severe vertebral curvature. Larvae with severe vertebral curvature resulting in shorter tails were considered as individuals exhibiting extreme curvature not shortened vertebral columns. The number of

individuals having kyphosis, lordosis, scoliosis and kyphosis / lordosis complex did not differ significantly between incubation temperatures. However the prevalence of larvae with severe vertebral curvature with shortened tails increased significantly at higher temperatures (ANOVA: df=5, F=14, P<0.001). The most prevalent anomaly for all temperatures was larvae with malformations of the vertebral column in the vertical plane (kyphosis and lordosis).

Representative examples of types of malformed larvae observed at hatch are shown in Figure 1. The severity of malformation ranged from slight to complete curvature of the vertebral column. Several larvae showed a kyphosis / lordosis complex, where the vertebral column was malformed in several directions. All larvae with types of malformations observed were considered to be non-viable in terms of rearing for aquaculture purposes. Larvae with severe vertebral curvature were alive at hatch (heart beat observed) but showed severe impairment of movements compared to normal or slightly malformed larvae.

Discussion

Malformations in cultured fish, such as Atlantic cod, have a significant impact upon commercial aquaculture (Olsen et al., 2004). Further understanding of rearing protocols, such as incubation temperature, is necessary to reduce the effect of malformations on larval quality and maximise survival rate. Brown et al. (2003) and Thomson and Riley (1981) suggested that survival rates of eggs to hatch was relatively un-affected by incubation temperatures between 6 - 12°C, and cod eggs developed abnormally and died at temperatures above 12°C. Results from these trials confirmed that although there were significant differences in survival rates between different groups of eggs, there were no significant differences in survival rate for the range of temperatures tested. The differing survival rates may be indicative of varying egg quality between different spawning female broodstock.

The prevalence of malformations in this study (over 50%) was comparable to results stated by Olsen et al. (2004) for reared cod. Four types of malformations were observed in larvae, among which kyphosis and lordosis were most prevalent. The majority of the malformed larvae were observed with only one type, but several were found with combinations of kyphosis and lordosis. Although some larvae displayed very slight lordosis or kyphosis, they potentially could cause problems during on growing. Interestingly though, larvae with severe vertebral curvature increased with increasing temperature, with eggs incubated at 11°C (close to upper thermal limit for survival) having the highest incidence of severely malformed larvae. This type of vertebral malformation was the only type significantly influenced by incubation temperature, and may suggest that although overall results showed no obvious effects of temperature on prevalence of vertebral malformations during egg development, there were subtle thermal influences on types of vertebral malformations at higher temperatures.

The high prevalence of malformations at hatch, regardless of incubation temperature has negative implications on commercial

production with regards to larval quality, and subsequent growth rates and production losses. Since the survival and prevalence of malformations was independent of incubation temperatures up to 11°C, other unknown factors in these trials were contributing to malformations observed at hatch (genetic, broodstock nutrition). This suggests that the range of different incubation temperatures tested could be used to incubate cod eggs for commercial purposes without significantly affecting survival and prevalence of malformations. However, these results also suggest that egg incubation temperature should be kept between 6 – 8°C to minimise the severity of malformations of cod larvae at hatch.

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