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Effects of temperature and lipid droplet adherence on mortality of hatchery-reared southern hake *Merluccius australis* larvae

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Abstract

Effects of exogenous (water temperature) and endogenous (lipid droplet adherence) factors were experimentally tested on early survival of southern hake *Merluccius australis* reared under controlled conditions. Experiments to determine the effect of temperature (10, 12 and 14 °C) on larval growth rates and yolk-sac absorption rates of unfed southern hake were carried out under laboratory conditions. There was no significant differences in growth rates at the temperature range tested (ANCOVA, $F=0.164$, $p>0.25$), but yolk-sac absorption rates and mortality increased with temperature (ANCOVA, $F=53.84$, $p<0.001$). A high percentage (between 31 and 81%) of hake eggs showed a lipid droplet not adhered (i.e., freely moving in the yolk, and not located in the posteriormost portion of the yolk-sac). In a second experiment, fed southern hake larvae with the lipid droplet not adhered during embryonic development did not survive after yolk-sac absorption. This study provides the first data on the influence of the lipid droplet absorption on larval survival of cultured hake, and can be used as an early indication of the quality of the batch.

Keywords: Southern hake; Survival; Temperature; Lipid droplet

1. Introduction

Southern hake, *Merluccius australis* is a gadoid fish that sustain an important industrial and artisan demersal fishery in southern Chile and Argentina (Payá and Ehrhardt, 2005). In recent years efforts have been carried out in Chile to develop a commercial culture of this species. A pilot scale hatchery produced 15,000 juveniles in 2004, and early development of eggs and yolk-sac larvae of southern hake under laboratory conditions have been described (Bustos and Landaeta, 2005); however, critical processes in early life history in captive rearing conditions are unknown.

Temperature is one of the most important regulating factors in fish growth and survival in culture conditions (Blaxter, 1992; Hart et al., 1996; Fielder et al., 2005;

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Olivotto et al., 2006). Broodstock age and nutrition may affect egg and larval quality, through variation in the amount of essential fatty acids in the yolk (Furuita et al., 2000, 2002) and/or the lipid droplet. The lipid droplet is likely the major source of energy sustaining larvae during the transition to exogenous feeding, is an important source of triacylglycerol (TAG) and affects larval survival (Norton et al., 2001; Berkeley et al., 2004). However, stress or nutritional status of broodstock may affect the mechanical absorption of the lipid droplet and the fatty acids available in it, through abnormalities in the intestinal mucosa of larvae or the formation of the yolk syncytial layer (Deplano et al., 1991; Poupard et al., 2000). The present study was conducted to investigate the effects of temperature and lipid droplet adherence (i.e., localization of the lipid droplet in the posteriormost portion of the yolk-sac) on the survival of larval southern hake after the total exhaustion of the endogenous reserves, and represents the first efforts to study this gadoid species in incipient culture.

2. Methods

Captured wild southern hakes were kept in captivity in large tanks (30 m^3) in the Quillaipe Experimental Center of Fundación Chile, Puerto Montt, Chile from May 2002, under natural photoperiod and temperature. One male (M1: 3.3 kg body weight (BW), 71.5 cm total length (TL)) and two females (female 1, F1: 3.0 kg BW, 79 cm TL; female 2, F2: 4.5 kg BW, 77 cm TL) were utilized for the experiments carried out in September 2004. Fishes were induced to spawn with gonadotropin releasing hormone (GnRH, Ovaplant®, Syndel Laboratories Inc., Canada), by intramuscular injection in the dorsal region. Fishes were kept in well-aerated tanks, with open water circulation.

When females were ripe, a gentle pressure on the abdomen from head to tail was applied to collect the spawned eggs. Eggs were fertilized by the dry method using milt obtained from the male. All batches were artificially fertilized. The eggs were transferred to a beaker to measure the percentage of buoyant eggs, as normal eggs float while poor quality or abnormal eggs sink. Buoyant eggs were stocked in 3001 cylindroconical incubation tanks. Hatching success ranged between 82 and 86% for the batches utilized in the experiments.

2.1. Experiment I

The buoyant eggs were stocked at a density of ~150 eggs l^{-1} and incubated at 11 °C in three tanks just after fertilization and the temperature of the tanks was thereafter gradually changed to 10, 12 and 14 °C, respectively. Since the spawning of hake occurs below surface waters, the incubators were kept in darkness at these temperatures for the remaining part of the experiment to reduce environmental stress. In each tank, hatched larvae were kept in three glass jars at a density of 20 larvae l^{-1}, and maintained in UV-sterilized and filtered seawater (0.5 μm). One third of the water contained in the jars was replaced daily throughout the duration of the experiment. For each temperature treatment (10, 12 and 14 °C), larvae were randomly collected each day from day 1 post-hatch (dph) to yolk-sac exhaustion, and were preserved in buffered 10% formalin. Larvae were maintained without food until death. Dead larvae were removed and counted daily from the bottom of the tank by siphoning. From this data, instantaneous mortality rates were calculated using the following formula: $Z = (\ln(N_t) - \ln(N_0)/t)\ 100$, where Z = instantaneous mortality rate (d^{-1}), N_t is the number of larvae alive at time t, N_0 is the number of larvae alive at time 0, and t is the duration in days. Notochord length (NL) of preserved larvae was measured from the tip of the mouth to the tip of the notochord. Yolk-sac volume was estimated considering the yolk-sac as an ellipsoid ($V = 4/3\pi a * b^2$, where a is half of the yolk-sac length, and b is half of the yolk-sac height). All measurements were carried out using a Sony CCD-IRIS video camera attached to a stereomicroscope connected to a PC with Optimas 6.1® software. No corrections for shrinkage in larval length were carried out.

2.2. Experiment II

Eggs with the lipid droplet not adhered (i.e., freely moving in the yolk, and not located in the posteriormost portion of the yolk-sac) and adhered were visually selected from F1 and F2 fertilizations and were placed in separate treatments (three treatments: F1 eggs with lipid droplet not adhered, F2 eggs with lipid droplet not adhered, and F1–F2 eggs with lipid droplet adhered). Early stages of *M. australis* were kept in three glass jars at a density of 20 larvae l^{-1} by treatment and maintained in UV-sterilized and filtered seawater (0.5 μm) at 10 °C, in darkness throughout the experiment (up to 17 dph). Prior to yolk-sac exhaustion, larvae were fed twice a day with *Isochrysis galbana* (~6×10^{10} cells l^{-1}) and rotifers (5000 prey l^{-1}). After 10 dph, *Artemia* were added twice per day at a density of 3000 prey l^{-1}. Dead larvae were removed and counted daily from the bottom of the tank by siphoning, and instantaneous mortality rates were calculated.

2.3. Data analysis

Linear regression models by least squares were fit between notochord length and age in order to determine growth rates at each temperature, and negative exponential models were adjusted to estimate the rate of yolk-sac absorption in *M. australis* larvae. The resulting linear regressions between larval length and age were then compared among temperature treatments by analysis of covariance (ANCOVA) (slope test, Zar, 1999). To compare yolk-sac absorption rates, negative exponential regressions were log-transformed and then compared by ANCOVA test and *q* test (Zar, 1999). Effect of temperature on mortality data was analyzed with one-way ANOVA. The residuals were analyzed for normality. Effect of lipid droplet adherence and female was separately analyzed with non parametric tests (Mann–Whitney *U* test, Zar, 1999).

3. Results

Fig. 1 shows the larval growth of southern hake cultured at three temperatures, 10, 12 and 14 °C. During the first two weeks of life, hake larvae grew in length at slow rates without significant effects of temperature on the growth rate (ANCOVA, $F=0.164$, $p>0.25$). Larvae cultured at 10 °C grew at a linear rate of 0.04 mm d^{-1} (Fig. 1a). At 12 °C, the estimated larval growth was 0.03 mm d^{-1} (Fig. 1b), and 0.04 mm d^{-1} at 14 °C (Fig. 1c). However, a rise of water temperature increased the rate of yolk-sac absorption and reduced the time of endogenous feeding (Fig. 1d–f): yolk-sac absorption

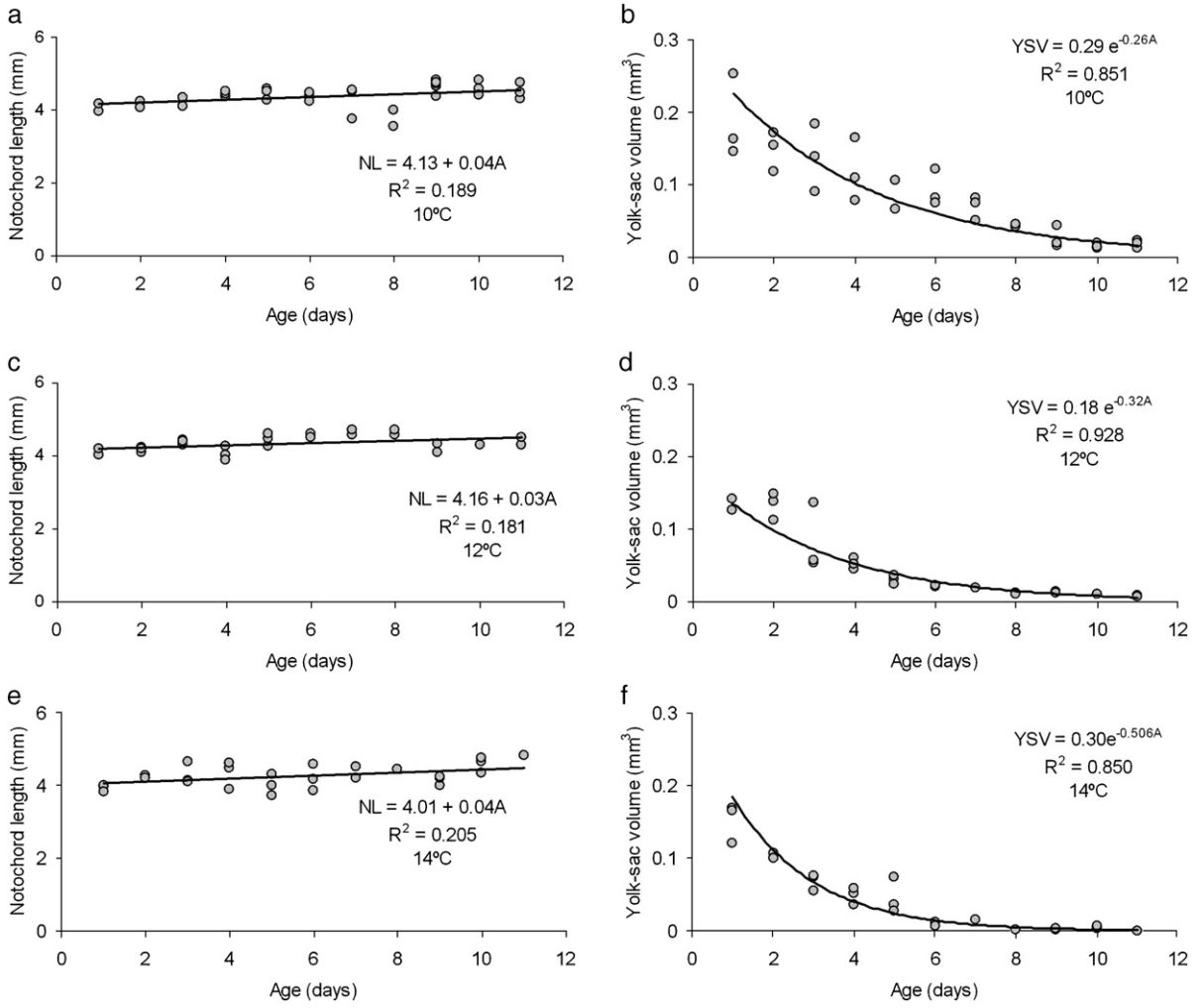

Fig. 1. Effect of temperature on larval growth in length and yolk-sac absorption of southern hake at a, b) 10 °C, c, d) 12 °C, and e, f) 14 °C. NL = notochord length, A = age, YSV = yolk-sac volume.

was significantly higher at 14 °C than at 10 and 12 °C (ANCOVA, $F=53.84$, $p<0.001$; $q_{2,\ 60}=4.894$; q 10 °C–12 °C=5.99; q 10 °C–14 °C=18.45; q 12 °C–14 °C=16.42; $p<0.001$). Endogenous feeding lasted 9, 6 and 5 days at 10, 12 and 14 °C, respectively (Fig. 1d–f).

Temperature also affected the behaviour and survival of early larvae of *M. australis*. At higher temperature, larvae showed higher locomotive activity. Eye pigmentation and mouth opening of southern hake also occurred earlier at higher temperatures that at lower, before the yolk exhaustion. Cumulative mortality showed that hake larvae survived better at lower temperatures (Fig. 2a). One-way ANOVA test showed that instantaneous mortality was significantly affected by temperature ($F=3.96$, $p=0.024$). Mortality rates at 14 °C were significantly higher than at 10 °C (Tukey test).

During southern hake production in cycle 2004, the percent of mean attached lipid droplet was 44% (±17% standard deviation), and a coefficient of variation of 38% (unpublished data), with a minimum percent registered of 19%, and a maximum of 69% (sample from 12 batches of larvae harvested).

In the second experiment, larvae with the adhered lipid droplet survived longer (5–6 dph) than larvae with a freely lipid droplet in the yolk-sac (Fig. 2b), and showed lower instantaneous mortality rates ($U=15$, $p=0.002$, Table 1). No significant differences were detected in Z of

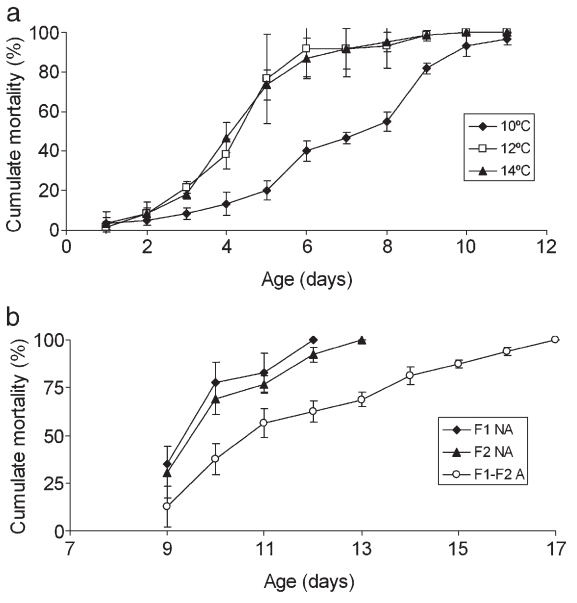

Fig. 2. a) Cumulative mortality experimented by southern hake larvae reared at 10, 12 and 14 °C. b) Cumulative mortality observed in southern hake larvae with a lipid droplet non adhered (female 1 F1 NA and female 2 F2 NA) and with the lipid droplet adhered to the backward portion of the yolk-sac (F1–F2 A).

Table 1
Range of instantaneous mortality rates (Z) estimated for *Merluccius australis* larvae reared at different temperatures and with the lipid droplet adhered/not adhered

Instantaneous mortality rates (d^{-1})		
	Min	Max
Temperature		
10 °C	0.017	0.211
12 °C	0.017	0.209
14 °C	0.016	0.329
Oil globule		
F1 non adhered	0.061	0.733
F2 non adhered	0.074	0.511
F1–F2 adhered	0.036	0.168

larvae with non adhered lipid droplet between females ($U=30$, $p=0.564$). Just before the onset of the oil absorption, lipid droplet in normal larvae changed in color from shiny orange to opaque brown. For early stages originated from both females utilized in this study, when the lipid droplet was not located in the posterior margin of the yolk-sac, the larvae failed to accomplish mouth opening and eye pigmentation, and died shortly after yolk-sac absorption. Also, the lipid droplet did not change in color.

4. Discussion

The transitional feeding period or endotrophic period in larvae is an interval in which feeding ability being developed and the fish starts to eat with some reserves still present to meet the energetic demands of prey capture (Poupard et al., 2000). At higher temperatures there was an increase in the rate of yolk-sac absorption, a reduction in the duration of the endogenous feeding and an acceleration of the sequence of development of morphological characters (mouth opening and eye pigmentation) and larval activity, probably due to an increase in the metabolic demand, as has been observed in several marine fish species (Fukuhara, 1990). Although temporal changes of larval length were not significantly different among water temperature treatments, at least during the yolk-sac stage, larval mortality of southern hake showed a significant increase at 14 °C. It is well known that temperature has important effects on yolk utilization, starvation, feeding success, and early survival (McGurk, 1984; Fukuhara, 1990); in this sense, these results may be of value to improve the biologic knowledge of this new culturing species.

Another factor that influenced larval mortality of southern hake was the failure of the lipid droplet

absorption. The lipid droplet contains non-polar lipids, i.e., triacylglycerols, wax esters or cholesteryl esters, rich in polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid (EPA, DHA and AA, respectively), which have a critical role in normal development and functioning of the visual and neural systems, particularly during and immediately following first feeding (Mourente et al., 1991; Wiegard, 1996). During embryogenesis, a yolk syncytial layer (YSL), a structure unique in teleosts, forms by the collapse of open marginal blastomeres into the immediately adjoining cytoplasm of the yolk cell, and encapsulates the lipid droplet. This process leads to the differential absorption of the yolk mass and the oil globule (Poupard et al., 2000; Diaz et al., 2002a), increasing the neutral lipid metabolism (Williams et al., 2004). Due to a lack of vascular network in the yolk sac of fish larvae, and since red blood cells do not appear until late development, lipids of the oil globule are mobilized to the embryo through lipoprotein particles synthesized by the YSL and triggered by the apolipoprotein (*apoE*) gene expression (Babin et al., 1997; Poupard et al., 2000). Therefore, non-adherence of the lipid droplet and subsequent death of larval southern hake may be caused by the failure of the formation of the YSL or the failure of synthesis of lipoproteins. In the gilthead sea bream, *Sparus aurata*, the failure of lipid droplet absorption is accompanied by the accumulation of lipoproteins in cavities of the endoplasmic reticulum (Diaz et al., 2002b). In this study, a low proportion of adhered lipid droplet in embryonic southern hake was noticed (from 19%), and all larvae without such feature died shortly after yolk-sac exhaustion. However, the relationship between the failure of lipid droplet absorption and eye pigmentation in southern hake larvae needs to be addressed since in massive culture conditions larvae with globule not adhered and pigmented eyes are frequently detected. Although the mechanisms by which the failure in the adherence of the lipid droplet of larval southern hake remains unknown, this characteristic may be used as an early indication to determine the quality of the cohort recently produced in massive aquaculture systems.

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