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Osmoregulation, nutritional effects and buoyancy of marine larval fish: a bioassay for assessing density changes during the earliest life-history stages

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Abstract It has been hypothesized that marine fish larvae in the advanced stages of starvation would show increased density (ρ = mass volume^{-1}) from water loss due to osmoregulation failure. Changes in larval buoyancy are currently attributed to swim bladder regulation and protein synthesis or catabolism. Osmoregulation-related changes in density is an alternative mechanism, the importance of which remains untested in the laboratory and the influence of which on vertical distributions is unknown. We provide evidence that loss of osmotic control is a plausible mechanism for increased density of larval cod (*Gadus morhua* L.). Furthermore, our results show that this mechanism is not restricted to larvae in the advanced stages of starvation. "Relative" larval densities are estimated using a modified density gradient. We use a gravimetric method to separate the effects of nutrition from osmoregulation failure. We assessed the importance of sampling strata on estimates of larval density. Proportional sampling within three depth strata (stratified sample) produced the least biased method for determining the "average" density of a population of larvae in laboratory culture. Larvae sampled from the bottom third of the culture tank were significantly more dense then those sampled from the surface. This was true for larvae of all ages. The average change in density from hatching till death from starvation for larvae sampled in the surface stratum was nominal ($\Delta\rho = 5.0 \times 10^{-4}$ g cm^{-3}), while the change for those sampled from the bottom stratum was large ($\Delta\rho = 3.8 \times 10^{-3}$ g cm^{-3}). These large density differences suggest that larvae sampled from the bottom stratum were either osmotically stressed or were facultatively changing their density via regulatory pathways. Preliminary observations suggest that vitality is lower amongst those larvae which are sampled near the bottom. The small change in average density of larvae sampled from the surface stratum was due to starvation. The density differences we observed between "osmotically stressed" and "starving" larvae could readily have been misconstrued as differences in feeding and growth experienced by individual larvae. The potential bias of increased density from osmoregulation failure must be considered as a factor in experimental designs developed to assess the effect of fed and starved treatments on buoyancy for larvae of all ages. The simple bioassay we describe may prove useful both as a means of assessing larval condition and as a mechanism for evaluating factors affecting larval vertical distributions in the field.

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Introduction

Factors that regulate the buoyancy of many temperate marine larval fish species also determine their vertical position in the water column and, in turn, their patterns of drift, their association with prey, and interactions with predators (Blaxter and Ehrlich 1974; Fortier and Leggett 1982, 1983; Henri et al. 1985; Munk et al. 1989; Sclafani et al. 1993). Field and laboratory studies indicate that feeding success may strongly influence larval buoyancy (Blaxter and Ehrlich 1974; Neilson et al. 1986; Yin and Blaxter 1987; Frank and McRuer 1989; Sclafani et al. 1993). For example, laboratory studies have shown changes in the sinking rates of larval fish during yolk-sac resorption (Blaxter and Ehrlich 1974; Ellertsen et al.1980; Yin and Blaxter 1987). Food deprivation following yolk-sac absorption can result in a density (mass volume^{-1}) decrease and a reduction in their sinking rates (Blaxter and Ehrlich 1974; Ellertsen et al. 1980; Neilson et al. 1986; Yin and Blaxter 1987). These changes are

reported to be related to simultaneous increases in water content and decreases in protein concentration of the larvae (Blaxter and Ehrlich 1974). Alternatively, feeding appears to result in an increase in larval sinking rates (Blaxter and Ehrlich 1974). Since feeding and starvation have opposite effects on larval density, Neilson et al. (1986) suggested that buoyancy of larval cod (*Gadus morhua* L.) may be a better index of larval feeding condition than morphometric indices.

To date, the literature suggests that feeding and starvation are the main factors determining the changes in larval buoyancy; nonetheless, loss of osmotic control may also influence larval buoyancy (Shelbourne 1957; Blaxter and Ehrlich 1974). Shelbourne (1957) proposed that larvae will experience a breakdown in their ability to osmoregulate when feeding conditions preclude larvae from meeting their metabolic demands (''osmotic breach''). Because marine larvae occupy a hypertonic salt water environment (Holliday and Blaxter 1960; Mangor-Jensen 1986) an ''osmotic breach'' would lead to a net loss of hydrostatic lift, causing an increase in sinking rate (Shelbourne 1957; Blaxter and Ehrlich 1974). However, information on the ''potential'' effects of osmotic-related changes on larval fish buoyancy is largely anecdotal and generally related to increased sinking rates of larvae in the advanced stages of starvation (e.g. Blaxter and Ehrlich 1974; Ellertsen et al. 1980; Yin and Blaxter 1987). The osmoregulation failure mechanism for increased larval density has not been tested experimentally and consequently its contribution to estimates of larval density is not known.

The experiments detailed below suggest the increased density of some larvae may be due to loss of osmotic control, supporting Shelbourne's (1957) ''osmotic breach'' hypothesis. The effects of feeding and starvation on larval buoyancy can be separated in experimental design, but osmotic effects may confound the interpretation of either treatment. We present evidence, in a single treatment, that the effects of nutrition and osmoregulation failure occur simultaneously at all stages of larval development. To date, these effects have not been separated. We describe a bioassay designed to separate these effects on larval density that can be used in the laboratory and field, thus providing reliable estimates of average larval density.

Materials and methods

Egg and larval rearing

Throughout this paper we use the word ''density'' (mass volume^{-1}, e.g. ρ = g cm^{-3}) rather than ''buoyancy'' because: (1) it is the density difference between a larva and its surrounding fluid medium that determines the vertical velocity of the larva and (2) density is the variable we measure.

Cod eggs were collected in egg baskets located in the sea water outflow pipes of spawning brood stocks maintained at Memorial University's Marine Science Centre in Newfoundland, Canada. The eggs were rinsed with sea water containing streptomycin (50 mg l^{-1}) and penicillin-G (30 mg l^{-1}), placed in plastic bags (6 litre) containing filtered (20 μm) sea water and shipped by air-freight in an ice cooler (~1 to 2 °C) to our laboratory at McGill University in Montreal, Quebec. There, they were gradually acclimated to Instant Ocean (Mentor, Ohio, USA) that matched the salinity, temperature and pH of the transfer water (salinity = 32‰, temperature = 4 to 4.5 °C and pH = 7.9). The eggs were incubated in eight, 20-litre static tanks which were siphoned daily and aerated. An 18 h light:6 h dark photoperiod was maintained. We employed several shipments of eggs in our experiments, but statistical comparisons were restricted to larvae from a single shipment.

At hatch, larvae of equal age were collected with a wide-tipped plastic pipette and transferred to four, 20-litre static holding tanks (n = 500 tank^{-1}). Twenty percent water changes were performed daily by siphoning from the bottom of the tanks. To control against bacterial infections, a stock solution of Instant Ocean mixed with penicillin-G (30 mg l^{-1}) and streptomycin (50 mg l^{-1}) was added from a misting bottle on alternate days. All larvae were starved. One hundred percent mortality occurred by Day 12 post-hatch.

Modified density gradient column apparatus

Larval density was measured using a linear density gradient column originally described by Coombs (1981); Coombs created a linear salt water density gradient by mixing high- and low-density water, using the apparatus shown in Fig. 1. Live larvae, when added to the column, typically sink through the density gradient to the depth at which they are in hydrostatic equilibrium with the surrounding fluid (i.e. neutrally buoyant). Their position relative to that of calibration beads of known density facilitates determination of their ''relative'' density. We modified Coombs' (1981) method as follows: (1) we reduced the costs of the apparatus by using less expensive materials and eliminating some of the hardware, (2) we devised a new retrieval system for the rapid removal of individual eggs and larvae from the column and (3) for some trials we replaced the hypertonic salt water medium with an isotonic medium. These modifications are briefly described in the following sections and are illustrated in Fig. 1.

Modified column apparatus

One litre glass graduated cylinders (i.d. = 6 cm) with 10 ml graduations replaced Coombs' (1981) elongated, smaller-diameter tubes. These wider-diameter columns reduced drag from side-wall friction (Vogel 1981). To increase the precision of each observation, a copy of the cylinder's graduations was taped to the rear of the cylinder at a point 180° from the front etchings. We replaced Coombs' (1981) retrieval basket with a motor-driven pipetting system equipped with a rheostat for speed control of coarse vertical positioning of the pipette, and a hand-manipulated micromanipulator for fine-scale, three-dimensional movements (Fig. 1). Once the pipette (hollow glass rod, i.d. = 0.25 cm) had been positioned directly above the larva to be sampled, a 20 cm^3 water-filled syringe attached to the exposed end of the pipette was gently activated to draw the larva into the pipette.

Modified gradient solutions

We created salt water density gradients following Coombs' (1981) methods. We also modified some density gradients to be isotonic to that of a larval cod (~330 mOsm: Mangor-Jensen 1986). To create these isotonic gradients we replaced the salt water solutions with a mixture of the low osmolality solute, Percoll (Pharmacia Fine Chemicals, Piscataway, N.J., USA: ρ = 1.130 g cm^{-3}, < 25 mOsm kg^{-1} H_2O), and a Ringer saline solution (~330 mOsm: Mangor-Jensen 1986). The high-density reservoir beaker contained both Percoll and Ringer at a ratio of 2:1, while the low-density reservoir beaker contained only Ringer solution (Fig. 1). By mixing these two solutions we created a stable linear density gradient of

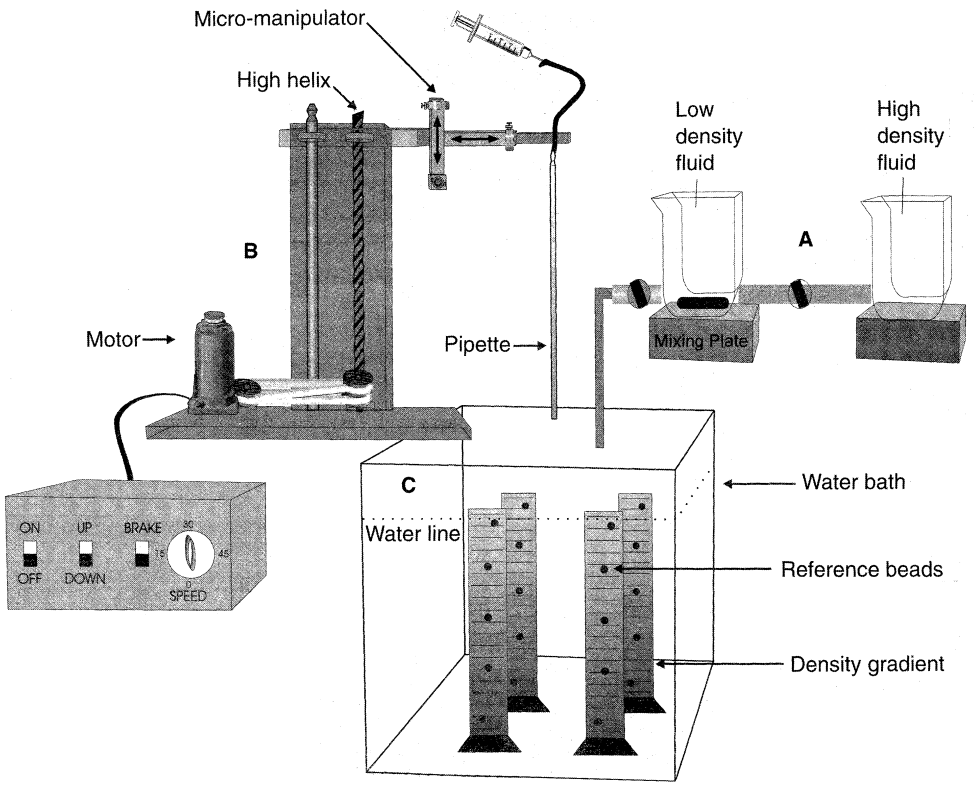

Fig. 1 Modified density gradient apparatus (after Coombs 1981). Mixing containers (*A*), motorized larval retrieval apparatus (*B*) and water bath containing density gradient columns (*C*)

approximately 1.010 to 1.051 g cm^{-3} in the graduated cylinder. All gradient columns used contained 25 mg l^{-1} of MS-222 anesthesia, which was added to both reservoir beakers prior to mixing. This ensured passive settling of the larvae to their point of neutral buoyancy. We also note that larvae removed from the gradients after 1.5 h fully recovered when placed into fresh Instant Ocean.

Five calibration beads of "known" density rinsed in distilled water were added to the column. Density gradients were used only if the coefficient of determination (r^2) values for the calibration beads in the linear density gradient was >0.98. The graduated cylinders were housed in a Plexiglas (0.5 cm thick), circulating-water bath maintained at a constant temperature of 4.5 °C (± 0.5). The accuracy of density measures within the Percoll and salt water gradients was tested by repeatedly determining the density of five individual yolk-sac larvae that were added and removed from the column five times (accuracy $= 1.6 \times 10^{-4}$ g cm^{-1}). The same test, when applied to the calibration density beads, yielded similar accuracy.

Experiment 1: test of density changes associated with loss of osmoregulation

The exact mechanisms involved in larval fish osmoregulation are not fully understood, however, mitochondria-rich chloride cells in the integument are believed to play an important role in ion-regulation (Alderdice 1988), and other mechanisms have been proposed (Tytler and Blaxter 1988; Tytler and Bell 1989). Even though the mechanism of osmoregulation is not completely known, we attempted to use a pharmacological agent to inhibit ion transport and decouple the active osmoregulation process. We treated 8-d-old larval cod sampled from the tank surface for 1 h with 0.01% sodium azide, a commonly employed metabolic inhibitor. Larvae from both the untreated control and sodium azide treatment were then measured in Percoll gradients to test for density differences. We found a small, but significant, average increase in the density of larval cod that were treated with the sodium azide (mean difference $= -0.00121$ g cm^3, $t = -4.72$, $df = 2$, $p = 0.04$). We attempted to use this method with older starved larvae, but were unsuccessful in attaining their density measures because of their "weakened" state prior the addition of the sodium azide. Most older larvae did not survive long enough to be measured in the density gradient. The few that appeared robust generally died within the gradient. Attempts to adjust the concentrations of sodium azide (i.e. lower concentration and longer incubation time) failed to ameliorate this problem. We therefore decided to use the data collected on the 8-d-old larvae as preliminary evidence for increased density associated with loss of osmotic control and developed the following test for buoyancy-related changes associated with a putative osmotic breach.

In the following experiment we tested the ability of starved larvae (11-d post-hatch) to maintain osmotic balance in a hypertonic salt water gradient (S1). As a control we exposed starved larvae (11-d post-hatch) to an isotonic density gradient (P1, Percoll and Ringer solution). Our a priori expectation was that: (1) the larvae exposed to the hypertonic gradients would continually increase in density if unable to maintain osmotic balance and (2) larvae placed in an isotonic control gradient would maintain their density even if unable to sustain osmotic control. In contrast to the starved larvae, 2-d post-hatch larvae, when placed into a separate hypertonic salt water gradient (S2), were expected to maintain their osmotic control and, hence, their density. These larvae possessed yolk sacs and were assumed to be well nourished.

Both yolk-sac and starved larvae were randomly sampled from the holding tanks. These larvae were quickly placed under a dissecting scope to verify a heart beat, gently rinsed with sea water to remove any air bubbles that might be attached to the surface of their body, and then gently pipetted into the density gradient columns. Twenty-five larvae were placed into both isotonic (P1) and hypertonic (S1) columns. The yolk-sac group was placed into another hypertonic gradient (S2). A separate timer was activated for each column at the time the larvae were introduced. The vertical position of the larvae in each column, relative to the calibration beads, was recorded at approximately 1.5-h intervals for 10 h and again at 24 h. Hence, a change in larval density was calculated as a

movement in vertical position of the larvae after reaching their initial depth of neutral density. After 29 h all larvae were removed from the columns, placed in fresh sea water, and checked for a heart beat. The experiment was repeated twice. Student's *t*-tests ($df = 2$) were used to compare the distributions at each time interval. Data from the hypertonic gradient, for times >7 h, were corrected to account for larvae that had settled to the bottom of the column. No such settling occurred in the isotonic column.

Experiment 2: bioassay for separating the effects of starvation and osmoregulation failure to achieve unbiased estimates of larval density in an experimental unit (i.e. aquaria)

In these trials, we tested for non-uniform vertical distributions in the tanks, and assessed whether systematic differences in larval density within particular depth strata occur as a result of physiological factors. If systematic differences occur, then it is necessary to determine how sampling the vertical depth strata would affect average estimates of density within a sampling unit such as an aquarium.

Twenty larvae were sampled at random depths from each of the four culture tanks on Days 2, 5, 9 post-hatch, and 15 larvae were sampled on Day 11 post-hatch. On each sampling day these 80 larvae were placed in a 2 litre glass beaker (height = 21.5 cm, i.d. = 15.5 cm) containing water drawn from the holding tanks and maintained at identical temperature (note: the 2 litre beaker did not contain any MS-222). The beaker was marked to indicate three, 7-cm zones: surface, intermediate and bottom. Light levels were identical to those in the holding tank ($\sim$20 W cm^{-2}). The larvae were allowed 1.5 h to stabilize their depth in the beaker. Visual observation revealed that few larvae changed their vertical position once a given depth had been assumed.

After 2 h the number of larvae occurring within each of the three, 7-cm depth strata was recorded. Larvae were then sampled from each of the three strata as follows ($n = 20$ sample^{-1}, except for Day 11: $n = 15$): (1) as a stratified sample from all three strata in proportion to the numbers in each stratum, (2) exclusively from the surface stratum and (3) exclusively from the bottom stratum. Due to the low occurrence in the intermediate stratum (<5%, see Fig. 3), larvae were sampled from this stratum only in the stratified sample. The bimodal, vertical-distribution data were consistant with our observations of limited swimming between strata. The larvae were then placed into an isotonic gradient column to determine their density. In several trials, visual observations for position maintenance and response to the suction of a pipette tip were recorded for the remaining larvae in the 2 litre experimental tank.

Bootstrapping tests

In the above experiment, we sampled from two depth strata (surface and bottom), and had three estimates for average larval density: surface, bottom and stratified. Our goal was to determine which of the average estimates of larval density within the experimental unit was least biased. We used resampling techniques (Simon 1992; Dixon 1993) to address the following questions: (1) how reliably did stratified sampling estimate the average density of the larvae in the larval population from which the sample was drawn, (2) did the surface and bottom samples provide reliable estimates of average larval density within the sampled population and (3) did the observed differences in average densities of larvae sampled from the surface and bottom strata represent extreme values from the ranges of a single population?

To answer the first question we tested the null hypothesis: $H_0 : \bar{x}_{\text{Obs Strat}} = \bar{x}_{\text{Boot Strat}}$, by comparing the mean of the observed values from the stratified samples ($\bar{x}_{\text{Obs Strat}}$) with the mean of the bootstrapped distribution of stratified samples ($\bar{x}_{\text{Boot Strat}}$) for each age. We bootstrapped the data from our stratified sample by randomly resampling 20 values from the data 1000 times (with replacement) for each age. The mean of the bootstrapped distribution was calculated and compared to the mean of the observed stratified distribution. If the means were equal, or their difference small, we concluded that the stratified sample was an unbiased estimate of average larval density in the total population.

We next calculated the 95% confidence interval (percentile methods, see Dixon 1993) of the bootstrapped distribution for the stratified samples. If the stratified sample was an unbiased estimate of population mean density then we addressed the second question by summing the number of times the bootstrapped average values of the surface and bottom strata (using the same resampling method as above) fell within the confidence intervals of the unbiased bootstrapped distribution for the stratified samples. If the overlap of the two distributions being compared was >5%, we concluded that the surface or bottom samples were also unbiased point estimators.

To answer the third question, we combined the data from the two distributions to be compared (e.g. surface and bottom or stratified and surface) and drew a random sample ($n = 20$), which was then averaged. Two averaged values were then subtracted one from another to determine the mean difference. This process was repeated 1000 times to obtain a random distribution for the mean differences between the two groups being evaluated. This distribution was then compared with the observed differences between the two groups. The 95% confidence intervals for the bootstrapped mean differences were also calculated using the "percentile method" (Dixon 1993). We expected that if 95% of the bootstrapped data exceeded the observed mean differences of the two groups being compared, then the observed differences may have occurred by chance (i.e. the differences were random). This process was repeated for each age group sampled.

Results

Experiment 1: osmoregulation and larval density

Larvae placed into both the salt (hypertonic) or Percoll (isotonic) linear density gradients settled to their neutral density position in approximately 1 to 1.5 h. Approximately 0.5 h after reaching this depth of neutral buoyancy, the starved larvae in the hypertonic environment (S1) began to sink deeper in the increasing salt water gradient as their density changes (Fig. 2). After 7.5 h they were significantly more dense than the starved larvae in the isotonic gradient (P1) and the yolk-sac larvae in the hypertonic salt water gradient (S2) (*t*-tests: $p < 0.05$, $df = 2$, Fig. 2). The starved larvae in the isotonic gradient (P1) showed a significant decrease in density over time (linear regression: $p < 0.01$, $df = 16$). The yolk-sac larvae in the hypertonic gradient (S2) did not exhibit significant trends (linear regression: $p > 0.06$, $df = 16$). After 29 h, all but three of the starved larvae in the hypertonic gradient (S1) had settled to the bottom of the column. Their densities were significantly different from the larvae in columns P1 and S2 at all time intervals >7.5 h (Fig. 2). No significant density differences were observed between the starved larvae in the isotonic gradient (P1) and the yolk-sac larvae in the hypertonic column (S2) up to 11 h. A small, but significant interaction between P1 and S2 occurred at 29 h (Fig. 2). It is possible that our Percoll and Ringer saline solution had a lower osmolality than that of the average larval cell, reversing the osmotic exchange and decreasing their density. The small increase in density of the yolk-sac larvae at 29 h may have occurred as a

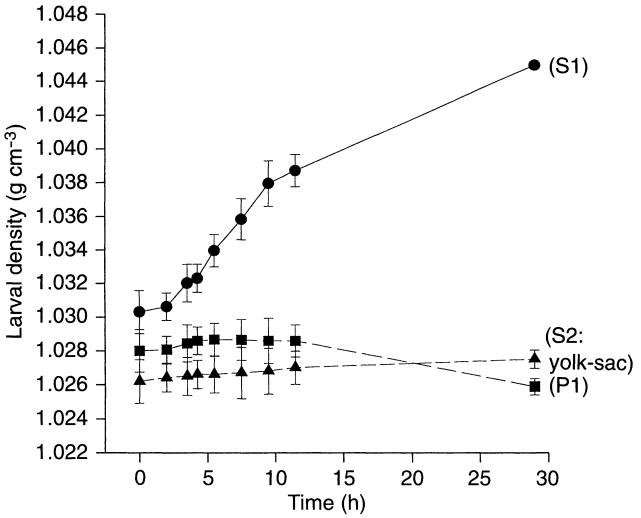

Fig. 2 *Gadus morhua*. Changes in density (g cm^{-3}) of starved cod larvae (11-d post-hatch) through time in an isotonic Percoll density gradient (*P1*) and a hypertonic salt water gradient (*S1*). A third group, yolk-sac larvae (2-d post-hatch), occupied a hypertonic salt water gradient (*S2*). All columns contained 25 mg l^{-1} of MS-22 to ensure passive settling of the larvae. *Data points*: means ± 1 SEM

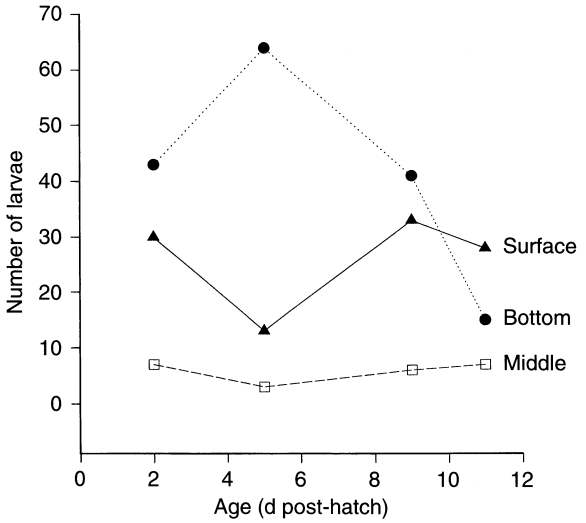

Fig. 3 *Gadus morhua*. Number of cod larvae occurring within each of three equal depth strata (*surface*, *middle* and *bottom*) in a 2 litre beaker ($n = 80$ on all days, except for Day 11: $n = 50$). The stratified sample removed larvae in proportion to the numbers in each depth stratum

consequence of utilization of yolk reserves during the experiment. After 7 h, the starved larvae in both the hypertonic (S1) and isotonic columns (P1) began to exhibit visible signs of stress. By 11 h several larvae in both columns had become opaque, a condition associated with death. At 29 h, only the yolk-sac larvae in the hypertonic column (S2) and a small number of the starved larvae in the isotonic column (P1) remained alive. Hence, we do not emphasize the significance of the 29-h data points and the switch in the density of S2 and P1 after 11 h.

Depth sampling

Larvae sampled from the surface stratum were less dense than those sampled from the bottom stratum, at all stages of development (Fig. 4). The magnitude of the differences between strata increased with age. Larvae sampled from the bottom stratum exhibited a greater change in density through time relative to those sampled from the surface. The mean density of the stratified sample was intermediate to those of the surface and bottom samples at all stages of development and closely followed the pattern of larval numbers found at depth (Figs. 3, 4).

We did not specifically test for differences in survival between larvae sampled within a particular stratum. However, we did examine the escape response of larvae to the suction of a Pasteur pipette tip and we checked for weak, or irregular heart beats. Up to 50% of the larvae sampled from the bottom stratum had highly irregular heart-beat patterns, were "head heavy" (Blaxter and Ehrlich 1974), showed limited escape response and rested on their sides on the tank bottom. These observations suggest that many larvae in the bottom stratum were near death. Larvae sampled from the surface stratum, although weak by the end of the experiment, were noticeably more reactive to suction from pipette tips and exhibited less irregular heart beats than those sampled from the bottom of the tanks. In summary, the increase in density for many larvae sampled from the bottom third of the tank was consistent with a breakdown in their osmoregulation as shown in Experiment 1 (Fig. 2), which is associated with decreased vitality.

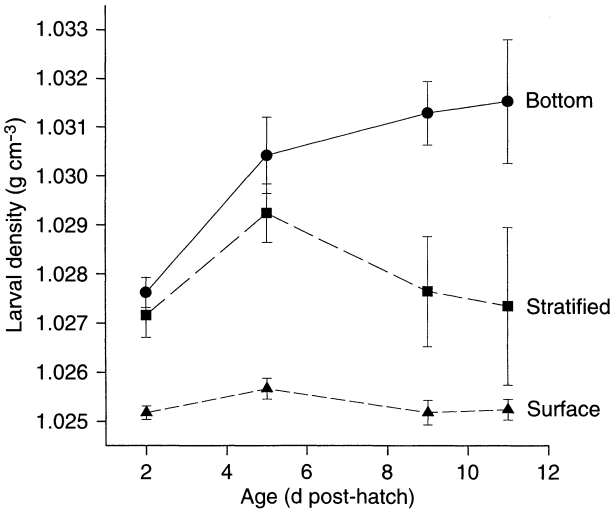

Fig. 4 *Gadus morhua*. Mean densities of cod larvae sampled from a 2 litre glass beaker divided into equal depth strata. Groups of 20 larvae were randomly sampled from the surface, the bottom and with respect to their proportion at depth (*Stratified*) throughout the starvation period. *Error bars:* ± 1 SD

Table 1 *Gadus morhua*. Observed and bootstrapped (1000 trials) data for cod larvae sampled from the surface and bottom of experimental chambers, in proportion to their numbers within each stratum (*stratified sample*). Observed and bootstrapped values for the stratified sample are unbiased. An *asterisk* denotes that the observed values for the surface or bottom samples differed significantly ($\alpha = 0.05$) from the bootstrapped stratified data (which is the best estimate for the average larval density in the tank)

Age, distribution	Sample size	Mean density:		
		Stratified sample	Surface sample	Bottom sample
2 d				
Observed	20	1.0272	1.0252	1.0276*
Bootstrapped		1.0272	1.0252	1.0276
5 d				
Observed	20	1.0292	1.0257*	1.0304*
Bootstrapped		1.0287	1.0266	1.0295
9 d				
Observed	20	1.0276	1.0252	1.0313
Bootstrapped		1.0277	1.0252	1.0313
11 d				
Observed	15	1.0273	1.0252*	1.0315
Bootstrapped		1.0273	1.0262	1.0296

Bootstrapped comparisons

In all but one case, the mean density of the observed stratified distribution was identical to that of the bootstrapped stratified distribution (Table 1). The samples taken from the bottom and surface strata were reliable for estimating the mean density of larvae 50% of the time (Table 1). Bottom samples tended to overestimate average larval density while surface samples had the opposite effect (Table 1). The differences between the means of the surface and bottom samples were significant at all ages, indicating that there were distinct densities (Table 2). Generally the mean differences between the stratified sample and either the surface or bottom sample did not differ significantly (Table 2). The stratified samples typically had greater variability because they included individuals from both the surface and bottom strata. Furthermore, the weighting of the stratified samples influenced the mean values of larval density. For example, the average density of the stratified sample was like that of a surface sample when more larvae were present in the surface stratum relative to the bottom stratum (Table 2; Figs. 3, 4; age 11 d). This further indicates the suitability of employing stratified sampling for determining average larval density in this kind of experiment.

Table 2 *Gadus morhua*. The left hand column indicates the data sets combined and bootstrapped (1000 trials) to generate a random distribution of mean differences. The right hand column indicates the number of times the bootstrapped differences exceed the observed differences

Age, bootstrapped mean difference comparisons	Number of times the bootstrapped differences > observed differences
2 d	
Stratified vs Surface	118
Stratified vs Bottom	1000
Surface vs Bottom	18
5 d	
Stratified vs Surface	1
Stratified vs Bottom	1000
Surface vs Bottom	0
9 d	
Stratified vs Surface	335
Stratified vs Bottom	86
Surface vs Bottom	2
11 d	
Stratified vs Surface	678
Stratified vs Bottom	212
Surface vs Bottom	23

Discussion

The literature on larval fish buoyancy has emphasized the effects of changes in the proximate condition of larvae during feeding and/or starvation on their vertical velocities (e.g. Blaxter and Ehrlich 1974; Ellertsen et al. 1980; Yin and Blaxter 1987; Sclafani et al. 1993). Alternatively, differences in vertical distributions between feeding and starving larvae have been detected for field-sampled specimens and these have been attributed to passive buoyancy effects (e.g. Frank and McRuer 1989). These observations have led to the suggestion that buoyancy may be a reliable index of larval condition as well as an important determinant of vertical distribution for the larvae of some species in the laboratory and in the field (Neilson et al. 1986; Frank and McRuer 1989; Sclafani et al. 1993).

Our experiments show that density changes associated with osmoregulation can have a profound effect on the density of individual larvae and may indeed have the most significant influence on their buoyancy. Our results also indicate that loss of osmotic control by larvae in a weakened state produces a predictable change in their density. These findings support Shelbourne's (1957) "osmotic breach" hypothesis and the other anecdotal studies that followed (Blaxter and Ehrlich 1974; Ellertsen et al. 1980; Yin and Blaxter 1987). While our experiments were not designed to determine whether solvent loss or solute gain is the main cause of this effect, comparisons of density changes of weakened larvae in

hypertonic and isotonic gradients clearly demonstrated that maintaining osmolality is critical to buoyancy regulation. We expect that with an increased understanding of the osmoregulation processes in larval fish more controlled physiological studies can be performed to determine the rates and mechanisms involved with the associated density changes. Nevertheless, our results indicate that osmoregulation failure of weakened larvae is associated with an increase in their density and that this density increase will strongly influence their vertical position in a hypertonic environment such as sea water.

The larvae used in our experiments were progressively starved throughout the trials. We therefore expected them to become less dense through time as shown in other studies (e.g. Blaxter and Ehrlich 1974; Neilson et al. 1986; Yin and Blaxter 1987). However, only larvae in the surface stratum, in Experiment 2, displayed such changes (note: compressed scale in Fig. 4). As early as 2-d post-hatch, however, all larvae sampled from the surface stratum were less dense than those sampled from the bottom of the tanks. Furthermore, density was smaller in the surface stratum then in the bottom stratum. These results imply that larvae sampled near the surface, while starving, were able to maintain osmotic balance, whereas those sampled near the bottom had become osmotically stressed. Yolk-sac depletion produced a sharp increase in the density of larvae from the bottom stratum. This suggests that in the first 4 d the yolk-sac material provides a positive buoyancy force which counteracts density increases associated with osmotic complications. An alternative explanation might be that behavioural differences due to swimming within their culture and 2-litre observation tanks created these patterns. This explanation is unlikely given the low numbers of larvae observed in the middle stratum (Fig. 3). Moreover, visual observations revealed low activity levels of the larvae during the trials. Even though larval cod are believed to be unable to regulate their swim bladder for the first several weeks of life (Morrison 1993; Hunt von Herbing 1996), we can not fully dismiss facultative regulation through other physiological processes (perhaps including the swim bladder) as an alternate explanation for the observed density differences. This remains an interesting possibility.

In the absence of the results of Experiment 1, the density differences observed between the starved larvae sampled from the surface stratum and from the bottom stratum could have been attributed to density differences due exclusively to differential feeding success or use of energy reserves by larvae. Such conclusions have repeatedly been drawn (see Neilson et al. 1986; Frank and McRuer 1989; Sclafani et al. 1993). Between the beginning and end of their experiments, Neilson et al. (1986) reported an average density difference of 1.5 σ_t [where $\sigma_t = (\rho - 1) \times 10^3$] units between starved and well-fed larvae. This difference was attributed to feeding effects. In this experiment we observed an average difference of 0.5 σ_t units between osmotically stressed and starved larvae. These data indicate that density differences between osmotically stressed and starving larvae can be equal to or greater than density differences between feeding and starving larvae. Furthermore, these experimental results provide an alternative to nutritional explanations of buoyancy changes. Osmoregulation changes could easily confound inferences about nutritional factors regulating individual buoyancy. For example, all of Neilson et al.'s (1986) fed larvae in their high-food treatment suffered unusually high mortality around the same time that their starved larvae died, yet they conclude that the observed density differences were the result of feeding effects. An alternative explanation is that the larvae in Neilson et al.'s (1986) high-food treatment may not have been feeding after yolk-sac absorption and thus experienced an increase in density due to loss of osmotic control. The bioassay we describe has the potential to separate effects due to feeding, starvation and osmotic-stress on larval buoyancy.

The results of our bootstrapping tests confirm that the sampling regime typically employed in laboratory experiments can significantly influence the outcome of buoyancy studies. Sampling which fails to account for the proportional depth distribution of larvae in the experimental chamber can clearly bias estimates of average density, and could dramatically elevate variance measures. Our results suggest that a stratified sampling protocol can eliminate this potential bias. Similarly, such estimates may have important consequences about the inferences we draw about mean larval condition in the field. If samples are drawn from only one depth stratum they may be misrepresenting the true "average" condition of the population of interest in statistical analyses.

Our findings also provide useful guidelines for the design of sampling protocols in future experiments. In comparative studies of growth and/or performance in fed and starved larvae (e.g. Neilson et al. 1986) it would be important to avoid sampling from the bottom stratum of the experimental tanks since this stratum may well contain osmotically stressed larvae. In experiments involving feeding larvae, sampling complications could occur due to the interactions of search behaviour and osmotic failure. For example, if prey items sink toward or aggregate at the bottom of the tank it will become difficult to distinguish between larvae occupying these strata in search of prey (i.e. feeding behaviour) and those that are osmotically stressed and sinking out of the water column.

One effective way to minimize the sampling bias would be to sample with respect to the proportion of larvae occurring at depth and then assess their buoyancy in an isotonic density gradient. However, most larval rearing tanks are not transparent and are optimized for the feeding success of heavily stocked tanks. This can make stratified sampling difficult. In such cases, we strongly recommend that larvae be sampled randomly across all depths to obtain an unbiased estimate of "tank" density. Conversely, if one is interested solely in determining the density differences between fed and starved larvae, confounding osmotic effects can poten-

tially be avoided by sampling exclusively within the surface depth stratum. This of course is not representative of the "tank" average density, but may also lead to an unconfounded estimate of nutritional effects when stratified sampling is not possible because osmotically stressed larvae are least likely to be observed within that stratum. Another alternative would be to isolate larval fish from the bottom stratum and discriminate their viability with methods such as: behavioural performance (e.g. Neilson et al. 1986), position maintenance (Blaxter and Ehrlich 1974; Batty 1987) or biochemical condition indices (e.g. Clemmesen 1987, 1993; Ferron and Leggett 1994). One may also detect physiological differences by subjecting the bottom sampled larvae to short-term (< 6 h) buoyancy trials in hypertonic gradients, in which osmotically stressed larvae will sink continuously. A more rigorous test correlating larval density within particular strata with larval survival is warranted.

It is important to recognize that a significant increase in larval density may occur at any stage of development, and that an "osmotic breach" (Shelbourne 1957) does not ultimately depend on starvation. It is likely that an "osmotic breach" occurring at any stage of development will lead to variable sinking rates and will impact their vertical and horizontal distributions in the field.

To illustrate this effect, we conducted a simple comparison of sinking rates of "healthy" and "osmotically stressed" larvae of the same age (3-d post-hatch). The "healthy" yolk-sac larvae employed in our experiments had an average density of 1025.5 kg m^{-3} and the density of "osmotically stressed" larvae averaged 1028.0 kg m^{-3} (Fig. 4). Assuming a water density of (1025.33 kg m^{-3}, Parsons et al. 1984), a sea water viscosity of 1.61×10^{-3} kg m^{-1} s^{-1} (Dorsey 1940), a gravitational constant of 9.8 m s^{-1}, and the radius and oblate spheroid dimensions of larval cod as shown in Sclafani et al. (1993), a Stoke's terminal velocity prediction (Batchelor 1967) for "healthy" larvae would be −0.06 cm s^{-1}, while that of "osmotically stressed" larvae would be −1.0 cm s^{-1}. These calculations clearly show that osmotically stressed larvae will quickly descend through the water column. However, this calculation may overestimate the effect of an "osmotic breach" on larval sinking rates. A gradual loss of osmoregulation and a somewhat slower increase in the density of the larvae is more likely. Nevertheless, loss of positive buoyancy forces due to an "osmotic breach" may explain the paucity of "poor" condition larvae that has been repeatedly observed in the field (O'Connell 1980). Such larvae may simply achieve high density due to loss of osmotic control, sink continuously, and become lost from the "healthy" population.

The bioassay we describe can be used to determine the density differences of larvae in various physiological states in the laboratory. It can also be used to define the proportion of individuals that experience loss of osmotic control from those which are experiencing nutritional effects in the field. This can be done simply by measuring the density of larvae in a density gradient in situ (see Coombs 1981). Those larvae which are extremely dense are osmotically stressed while the remainder are not and can be further assessed for their nutritional status. However, to the best of our knowledge, the definitive test for density differences between fed and starved larvae (nutritional effects) controlling for osmotic effects has not been performed. Osmoregulation failure remains a potentially important ecological factor: loss of osmotic balance may be a survival point-of-no-return in the laboratory and field. The buoyancy bioassay we described may therefore prove useful both as a means of assessing larval condition and as a means of evaluating factors affecting vertical distributions of larvae in the field.

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