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The relationship of embryonic development, mortality, hatching success, and larval quality to normal or abnormal early embryonic cleavage in Atlantic cod, *Gadus morhua*

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ABSTRACT

A reliable method for assessing the viability of fertilized eggs early in development would be beneficial for the aquaculture industry, allowing egg batches with a high probability of low hatching success to be discarded before costly resources are devoted to their culture, and for recruitment models where egg viability is used predictively. During the last decade, the observation of cellular morphology during embryogenesis has received increased attention as a potential early indicator of embryo quality. However, most often, abnormally cleaving eggs are assessed *en masse* even though noticeable differences in cleavage patterns are generally present between individual eggs. We separated six egg batches of Atlantic cod, *Gadus morhua* Linnaeus, 1758, into normal and abnormal cleavage patterns, reared eggs individually in a temperature-controlled room, and recorded daily egg mortality until hatch, hatching success, larval deformation, and larval mortality. Seven abnormal cleavage patterns were readily distinguishable and all showed moderate variability in egg mortality. Both normal and abnormal eggs had similar mortality-rate trends, consisting of an initial high mortality that became asymptotic at about day 8 of development at 6.5 °C. Specific cleavage patterns showed variable mortality-rate trends. No significant differences in cumulative egg mortality were found between any abnormal cleavage patterns, but overall, abnormal eggs had significantly greater egg mortality than normal eggs. Hatching success was high in all groups and not significantly different between normal and abnormal eggs. Few larvae were deformed within any egg batch or pattern and no consistent trends were noted. A severity index was calculated and a suggested severity order determined as asymmetry < adhesions < margins ≤ inclusions < blastomere size.

1. Introduction

Egg quality among batches in serial-spawning, marine fishes is highly variable (e.g. Bromage, 1995; Kjesbu et al., 1996; Larsson et al., 1997; Manning and Crim, 1998), and is thought to be influenced by many factors including diet, stress, genetics, egg over-ripening, water quality (such as pollution), and physical drivers (e.g. Westernhagen et al., 1988; Bromage et al., 1994; Cameron et al., 1996; Brooks et al., 1997; Makhotin et al., 2001; Thorsen et al., 2003). When dealing with captive broodstock and egg rearing, a reliable method for assessing the viability of fertilized eggs early in development would be beneficial for the aquaculture industry, allowing batches of eggs that indicate high probability of low hatching success to be discarded before costly resources are devoted to their culture (e.g. Avery and Brown, 2005). As well, egg quality as it affects survival is key to recruitment success for wild populations although the factors that reduce egg quality may be quite different from those that affect aquaculture.

As many marine eggs are transparent and can be assessed through visual observation, cellular, morular, or blastular morphology during embryogenesis have received attention as potential early indicators of embryo quality (Westernhagen et al., 1988; Cameron and Berg, 1992; Pickova et al., 1997; Shields et al., 1997; Vallin and Nissling, 1998; Rideout et al., 2004a; Avery and Brown, 2005; Penney et al., 2006) since it was first suggested by Kjørsvik et al. (1990). After an egg is fertilized, early embryonic development proceeds with a series of mitotic cellular cleavages (divisions) of the zygote which form a blastodisc[1] normally

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[1] Some studies mistakenly refer to the blastodisc as the blastula when the blastodisc is actually the animal pole region of a yolky egg and apparent during the morula stage, although it can be used to describe the same embryo area in late stages, such as the blastula stage (Kane and Warga, 2004). Since there is no blastocoel during the morula stage, it is incorrect to refer to the embryo during the early cleavage period where cell divisions can easily be seen as a blastula.

composed of symmetrically arranged blastomeres (cells) that become smaller and arranged differently with each cleavage, while the blastodisc size remains unchanged. After about the 16- or 32-cell stage of development (4th or 5th cleavage), symmetry is lost, and assessments of egg morphology as a quality indicator become suspect.

In contrast to the described normal cleavage pattern, several cleavage abnormalities may occur during embryogenesis, including asymmetrically arranged blastomeres, differences in blastomere shape (margins) and size, or poor adhesion between adjacent blastomeres (Shields et al., 1997). Abnormal blastomere cleavage has been correlated with egg mortality during embryogenesis, low hatching success, and larval abnormalities in serial-spawning, marine fishes such as the Atlantic cod (Kjørsvik, 1994; Pickova et al., 1997; Vallin and Nissling, 1998; Penney et al., 2006); Arcto-Norwegian cod (Solemdal et al., 1995, 1998; Makhotin et al., 2001); Atlantic halibut, *Hippoglossus hippoglossus* (Linnaeus, 1758) (Shields et al., 1997; Mazorra et al., 2003); dab, *Limanda limanda* (Linnaeus, 1758) (Cameron and Berg, 1992); haddock, *Melanogrammus aeglefinus* (Linnaeus, 1758) (Rideout et al., 2004a); turbot, *Psetta maxima* (Linnaeus, 1758) (Kjørsvik et al., 2003); and yellowtail flounder, *Limanda ferruginea* (Storer, 1839) (Manning and Crim, 1998; Avery and Brown, 2005). The postulate is that blastomere abnormalities during early embyrogenesis adversely affect subsequent development, thus leading to egg death before hatching, and (or) deformed larvae. If a link exists, the main implication would be a loss of larval recruitment, and increased biofouling potential from liberated egg nutrients during aquaculture (Thorsen et al., 2003).

The relationship between abnormal cleavage patterns and egg mortality may be more complex than has previously been suggested. For example, Avery and Brown (2005) showed in yellowtail flounder an increase in mortality rate for abnormal eggs during the first 3 days post-fertilization over normal eggs at the 4- to 8-cell stage, and similar rates thereafter with no difference in the standard length or morphological abnormalities of the larvae, but high variability among batches. Vallin and Nissling (1998) found higher overall mortality in abnormal eggs of Atlantic cod over normal eggs and substantial variation among batches, but batches with high proportions of abnormal eggs often produced considerable numbers of viable larvae. Vallin and Nissling (1998) suggested that early cleavage abnormalities may be corrected during later stages of embryogenesis, thus allowing subsequent development to proceed normally, and cells later in development should be more sensitive to deviations than early, undifferentiated cells; a hypothesis in contrast to that of Kjørsvik (1994) who suggested that abnormalities of early, undifferentiated cells will influence development more than single-cell abnormalities later in development, and, presumably, all early abnormal eggs will die later in development (similarly suggested by Westernhagen et al. (1988)). Taken together, the results of Avery and Brown (2005) and Vallin and Nissling (1998) suggest that many so-called "abnormal" embryos may actually develop into viable larvae. Unfortunately, most previous studies provide assessments based on the proportion of abnormal eggs within a batch or eggs grouped in some manner; thus, there is a paucity of literature on the relationship between cleavage abnormality patterns and mortality in individual eggs. In fact, studies on individual eggs and early life-history traits in general are rare – Chambers et al. (1989) on capelin, *Mallotus villosus* (Müller, 1776); Pickova et al. (1997) comparing Baltic cod, *Gadus morhua callarias* Linnaeus, 1758 and Skagerrak cod; Solemdal et al. (1995, 1998) and Makhotin et al. (2001) on Arcto-Norwegian cod; Vallin and Nissling (1998) on Atlantic cod;

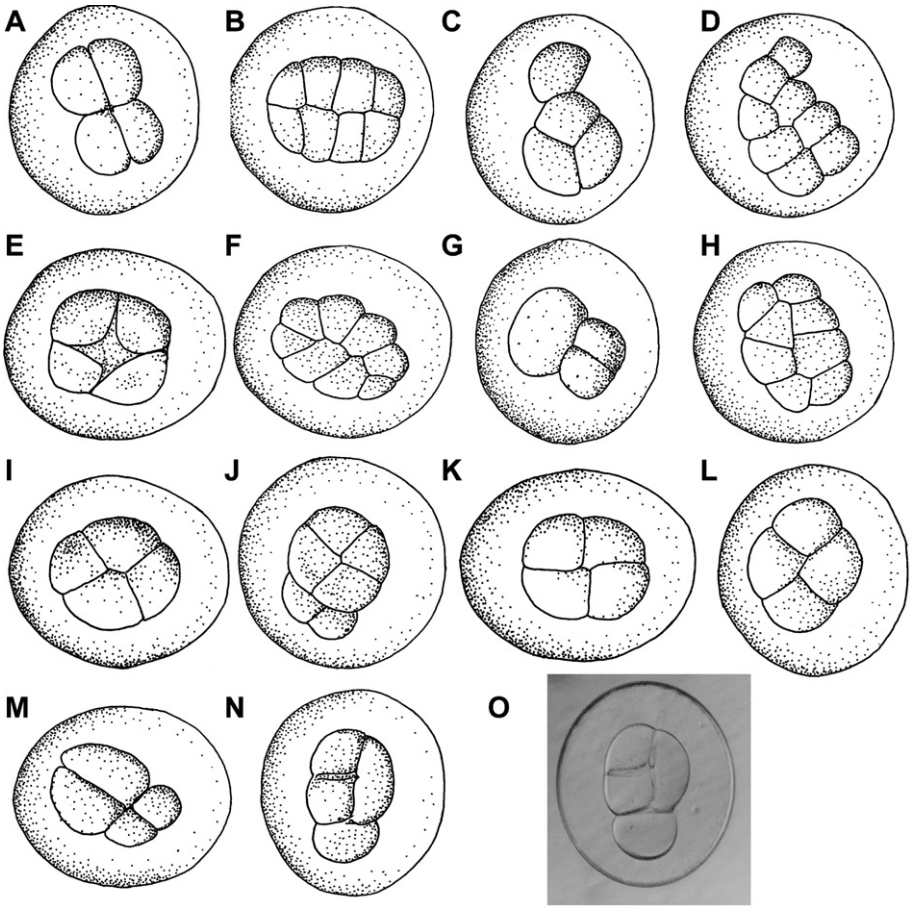

Fig. 1. Illustrations of normal and abnormal blastodiscs of eggs of Atlantic cod (*Gadus morhua*) during early cleavage stages. Development at the 4- and 8-cell stages (in pairs) for normal (A, B), Marginal (C, D), Donut (E, F), Triple (G, H), and Pie (I, J) patterns, with a possible intermediate pattern (K) between Pie (I) and Offset (L), and Unequal (M) at the 4-cell stage only. The least common Jumbled (N) pattern with the original digital image (O) for comparison.

Panini et al. (2001) on seabream, *Diplodus sargus* (Linnaeus, 1758) and European seabass, *Dicentrarchus labrax* (Linnaeus, 1758).

Researchers have characterized cleavage abnormalities into groups such as asymmetry, poor cellular adhesion, and (or) poor differentiation of margins (Shields et al., 1997; Rideout et al., 2004a; Avery and Brown, 2005; Penney et al., 2006), but none have examined specific blastomere patterns of the blastodisc or categorized patterns for severity of their effect on embryogenesis. A clear understanding and quantification of the effects of specific abnormal cleavage patterns on egg quality measures is necessary, because the assessment of all abnormal embryo patterns combined may confound estimates of embryonic mortality if more "severe" patterns of abnormality are responsible for a large proportion of the observed mortality, while less "severe" abnormalities may have little effect on embryogenesis and subsequent hatching success. We refine the general suggestions of Kjørsvik (1994) and Vallin and Nissling (1998) that some patterns of abnormalities will be more serious or occur at critical periods of development and may cause immediate egg death, while others are less critical or occur at noncritical development periods and may be "corrected", thus allowing subsequent development to proceed normally. The present study will examine the development of individual eggs of Atlantic cod classified to identifiable abnormal cleavage patterns in relation to egg mortality, hatching success, larval deformations, and larval mortality.

2. Materials and methods

Eggs were collected from a broodstock Atlantic cod maintained at the Ocean Sciences Centre, Logy Bay, Newfoundland, Canada, in 2004 and housed in the Joe Brown Aquatic Research Building. Each of two common ~38 m^3 tanks supplied with degassed, filtered seawater contained about 50–60 communally spawning males and females of 5–7 years of age. After each spawning event, buoyant eggs were retrieved from an automatic egg collecting device each morning between 0900 and 1000. Six batches were collected from 27 May to 16 June with volumes were between 2500 and 4750 mL and eggs were at various stages of development including 2-, 4-, 8-, 16-, 32-cell and morula stages owing to apparent multiple spawning events within the communal tanks. If a batch had sufficient numbers of fertilized, developing eggs, they were transported immediately to a cold room for sorting at the 2- to 4-cell stage. Ninety-six fertilized eggs (48 normal and 48 abnormal consisting of several abnormal patterns) were selected from each of six batches except for Batch 6 where normal $n=24$. Each egg was transferred with a modified plastic pipette tip into a separate well (~1 mL in volume) of a spectrophotometric plate filled with seawater containing 0.1 g/L Streptomycin sulphate and 0.06 g/L Penicillin G to reduce bacterial contamination, and were maintained in a cold room at 6.5 °C throughout the experiment. Half of the water in each well was changed daily using a glass pipette without contacting the developing egg.

Abnormal eggs were classified based on the nature of the blastodisc arrangement at the 4-cell stage of development. Where Shields et al. (1997) assigned eggs a qualitative score from 1 to 4 describing the degree to which eggs appeared normal at the 8-cell stage (used subsequently by Mazorra et al., 2003), we assigned descriptive names to all observed abnormal cleavage patterns that resulted from variation in one or more egg quality parameters at the 4-cell stage with no qualitative scoring, opting for a binomial approach where eggs either exhibited a parameter or not. Eggs were observed until the 16-cell stage every 6–8 h on an irregular schedule after which it was difficult to discern if the pattern was different from normally cleaving eggs as described by Hall et al. (2004), followed by at least daily observations at later stages of development, until mortality or hatch. Once the terminal condition of each egg was recorded, it was removed from the plate and

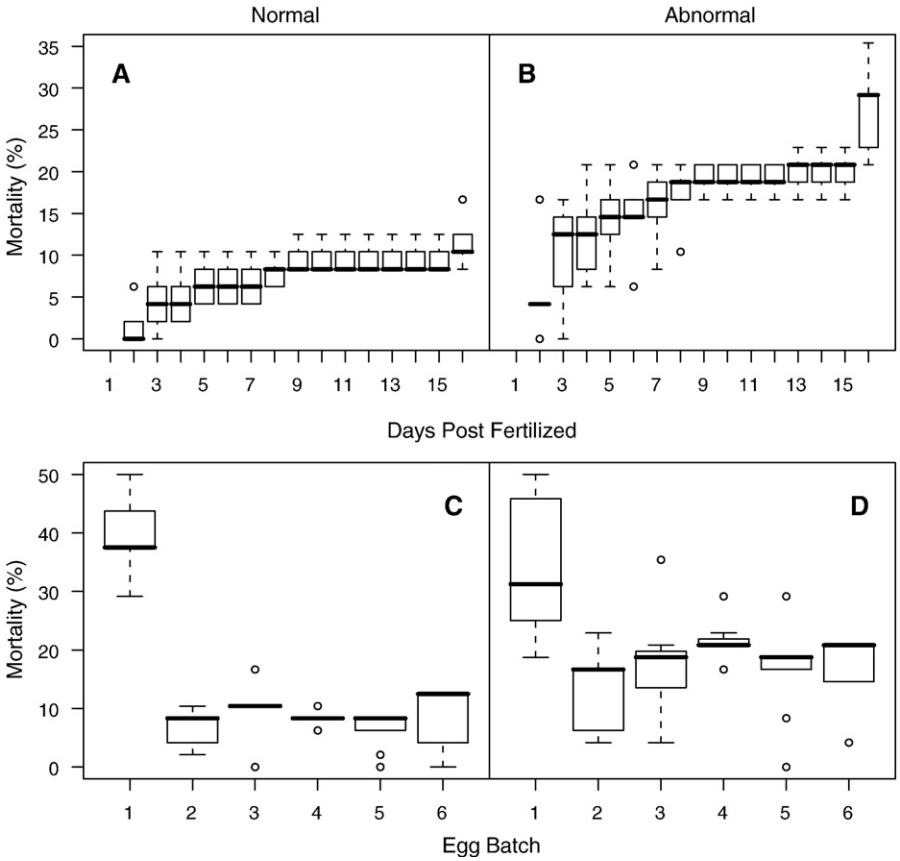

Fig. 2. Egg mortality trends for Atlantic cod (*Gadus morhua*) for normal (A) and abnormal (B) cleavage patterns at the 4-cell stage of development, and batch egg mortality for normal (C) and abnormal (D) eggs. Boxplots consist of median (dark bar), lower and upper quartiles (box), whiskers (T bars; 1.5 interquartile range), and outliers (circles).

the well water removed to prevent possible bacterial cross-contamination from dead eggs or larvae.

During each observation period, we recorded egg and larval quality measures as follows: egg mortality, hatching time, hatching success, larval deformations, and larval mortality within 24 h of hatch. Egg mortality was calculated based on the original number of eggs (48) within wells. Hatching time was calculated as the weighted mean time to hatch in days. Hatching success was calculated as the proportion of eggs that hatched of those eggs that survived to the weighted mean day of hatch. Note that our definition of "hatching success" differs from that used in some previous studies, in which hatching success was defined as the proportion of eggs that hatch of all eggs within their respective group. In such cases, hatching success would simply be synonymous with egg survival (a measure that can easily be inferred from our measure of egg mortality). Larvae with deformations or those that subsequently died after hatch were expressed as a proportion of the number of eggs which hatched. Mortality, as a proportion, is herein referred to as "egg mortality" to distinguish it from larval mortality rates. Egg mortality rates (egg mortality per unit time) were calculated by linear regressions.

Illustrations were created of each abnormal pattern compared with normally developing eggs for 4- and 8-cell stages of embryogenesis as a visual guide (Fig. 1). Illustrations are representative of the described patterns, as some variability existed that was most likely due to some continuity between patterns, e.g., Pie and Offset have similar adhesion issues, but remain distinguishable. Severity of patterns was evaluated (1) by assigning an "abnormality score" (AS) according to the number of abnormal parameters present at the 4-cell stage, where rank 0 is no abnormal parameters, rank 1 is 1 parameter, etc. and (2) by calculating a "severity index" (SI) defined as the mean of AS and the ranks of the recorded or calculated quality measures (see Table 2; where rank 1 is the best case, and tied ranks are averaged).

Egg mortality, hatching success, deformed larvae, and larval mortality at day 14 (weighted mean day of hatching, days post-fertilization) were analyzed with a paired Wilcoxon rank sum tests to preserve batch association, or a Kruskal–Wallis test when comparing more than two groups (pairing is not possible with Kruskal–Wallis). Mortality rates were compared on ranked data with ANCOVA using time as the covariate (commonly referred to as a homo- or hetero-geneity of slopes procedure) after partitioning temporal (daily) egg mortality into groups based on graphical analysis. Larval quality was assessed by visual observation of larval morphology (incomplete or pinched fins, lordosis or scoliosis, pigment abnormalities, irregular body undulations, or a combination thereof), and by recording larval death within the first 24 h after hatch. Spearman rank correlations were used for correlation analysis. All analyses were completed with R (R Development Core Team, 2008).

3. Results

Each blastodisc was classified to a pattern based on five main parameters that are easily distinguishable at the 4-cell stage of development; namely symmetry, blastomere size (equal cell sizes were considered normal), margins, adhesions, and inclusions which are all present in eggs of Atlantic cod and, for the most part, appear to be persistent during early cleavage stages (Fig. 1). Symmetry was assessed on both a bilateral basis (along at least one axis; e.g., Fig. 1M, Unequal) and a radial basis (e.g., Fig. 1E, Donut). This assessment differs from Shields et al. (1997) and Penney et al. (2006) where only bilateral symmetry was assessed about all axes at the 8- and 4- to 32-cell stages, respectively. Margin errors were seen at the blastomere and (or) blastodisc level characterized by misshapen blastomeres or one or more blastomeres on the periphery of the blastodisc with little surface contact for adhesion to adjacent blastomeres (e.g., Fig. 1C). Shields et al. (1997) defines margins at only the blastomere level, not the blastodisc as a whole. Offset and Pie patterns may be only slightly different as intermediate patterns were occasionally noted indicating that some plasticity in patterns may exists (Fig. 1I and K–L). In fact, there appears to be little difference between normal, Pie, and Offset. Abnormalities generally continued through the 16-cell stage. By the 32-cell stage, the blastodiscs contained too many cells to make an accurate comparison between normal and abnormal eggs; however, they appeared to be indistinguishable. By the 64-cell stage, all embryos looked the same.

The abnormal eggs of each batch consisted of a variable number of eggs of specific patterns with all patterns (other than Jumbled) represented in each batch except Batch 1 that had no Offset and only one Marginal egg. Batch 1 consisted primarily of Jumbled eggs (Fig. 1N and O; $n=17$ of 48 abnormal eggs), and had the highest egg mortality rates overall (Fig. 2C) and for each day; however, all 11 Jumbled eggs that survived to hatch produced a normal larva. In addition to 30% of Batch 1 normal eggs dying after the first day (compared with <7% for each of Batches 2–6), and since this batch also exhibited additional indicators of poor quality (some translucent or cloudy eggs, reduced egg buoyancy, higher proportion of unfertilized eggs, markedly higher abnormal proportion of eggs than the other 5 batches) suggesting some other destructive agent (possible bacterial infection, increased temperature shock or mechanical stress, or sitting in the egg collector too long before transport and sorting), it was discarded from group

Table 1
Blastomere cleavage patterns of the blastodisc described at the 4-cell stage of development, mean number of eggs within each group (sample size), abnormality score (number of abnormal parameters present at the 4-cell stage) and severity index (average of abnormality score and ranks of quality measures from Table 2) for Atlantic cod, *Gadus morhua*

Pattern	Description of blastomere arrangement	$\bar{X}\pm$ SD[a]	Abnormal parameters[b]	Abnormality score	Severity index
Normal	Symmetrically arranged, and are of equal size with clearly defined margins and no inclusions.	48[c]	n/a	0	3.1
Abnormal		48	n/a	2[d]	4.4
Donut	Similar size, but an inclusion towards the centre of the blastodisc. Radial symmetry.	6.8±1.1	Inclusions	1	4.6
Jumbled	Random cell arrangement with no discernible pattern with regard to symmetry or adhesion. Cells may or may not be of equal size.	n/a[e]	Symmetry, adhesions, margins, inclusions	4	n/a
Marginal	One of the four cells is attached to only one other cell and becomes peripheral on further cleavages. Asymmetric.	6.8±4.2	Symmetry, adhesions	2	2.2
Offset	Cells shifted along one axis disrupting symmetry, otherwise normal.	10±4.2	Symmetry	2	3.4
Pie	Similar sized cells and radial symmetry, but poor differentiation of blastodisc margins resulting in a circular shape with wedge-shaped cells.	11±3.8	Margins	1	4.1
Triple	Three cells are present instead of four. Bilateral symmetry bisecting larger cell.	8.0±4.1	Symmetry, blastomere size, adhesions	3	3.7
Unequal	Two cells are relatively large in size while the other two are smaller. Bilateral symmetry.	5.8±0.8	Blastomere size	1	5.7

[a] Batch 1 excluded.
[b] Abnormal parameters as outlined in Shields et al. (1997). Multiple parameters indicate eggs that display more than one abnormality.
[c] Except Batch 6 where $n=24$.
[d] Mean rank of all abnormal patterns.
[e] Jumbled pattern only occurred in Batch 1.

Table 2
Egg mortality until weighted mean day of hatch (14 days post-fertilization; dpf), hatching success, deformed larvae, larval mortality, and weighted mean time until hatch for normal and abnormal cleaving eggs (categorized at the 4-cell stage) of Atlantic cod *Gadus morhua*, reared at 6.5 °C

	Mortality[a] (%)	Hatching success[a] (%)	Deformed larvae[a,b] (%)	24-h larval mortality[a,b] (%)	Time until hatching[c] (dpf)
Normal	9.6±1.9	98±2.9	17±6.1	17±5.8	13.7±0.52
Abnormal	20±2.4*	91±6.8	16±6.3	15±5.3	13.8±0.56
Donut	16±17	88±12	15±16	18±19	13.9±0.63
Marginal	22±25	100±0	7.0±10	3.6±8.1	13.8±0.48
Offset	12±13	83±18	8.3±12	5.8±8.1	13.7±0.58
Pie	11±9.4	82±13	20±20	17±15	13.7±0.61
Triple	27±9.9	98±5.0	11±17	11±17	13.7±0.63
Unequal	27±13	93±15	29±33	29±33	13.8±0.48

Values are means across five batches of fertilized eggs (±SD). Batch 1 was excluded.
*Significantly different than normal eggs using paired Wilcoxon rank sum test at α=0.1.
[a] Quality measure used to calculate severity index.
[b] Expressed as a proportion of the total number of hatched eggs.
[c] Weighted mean day of hatch.

analysis. The Jumbled pattern generally contained several errors and had the highest abnormal parameter score (Table 1). SI could not be calculated because of incomplete or inconsistent quality measures. The highest proportion of any abnormal pattern (excluding Batch 1 Jumbled) was Batch 3 Offset (n=16) followed by several patterns scattered throughout several batches with n=13.

Egg mortality was initially high (days 1–8; up to 48 degree-days), but reached an asymptote at around day 8 for both abnormal (combined across patterns) and normal eggs (Fig. 2A–B). All eggs had either hatched or died by day 15 with a weighted mean day of hatch of ~14 or ~91 degree-days (Table 2). Mortality rates (slopes) from normal and abnormal eggs were not significantly different from days 1 to 8 (p=0.917) or from days 8 to 15 (p=0.963), using day 8 in each analysis. Day 15 showed increased egg mortality because some eggs died after day 14 (Fig. 2A–B), but with no consistency among which pattern or batch displayed the increase. Dropping day 15 from the analysis resulted in no significant difference in the slope (p=0.993) from days 8 to 14. The intercepts (mean egg mortality) for abnormal eggs were significantly greater than normal egg intercepts for days 1–8 (p<0.001) and days 8–14 (p<0.001), respecting that days are not independent and a repeated-measures ANCOVA might be a better model choice. The important points are (*i*) to determine when eggs reach an asymptote and thus provides an indication of when mortality stabilizes (which can be done visually with no statistical analysis) and (*ii*) to determine cumulative egg mortality (surviving eggs) at hatch. Egg mortality at day 14 was greater for abnormal eggs over normal eggs when taking into account that p=0.063 is the lowest possible p value obtainable from the Wilcoxon rank sum analysis and n=5 batches for each group. In consideration of the magnitude difference between 0.063 and other p values in the same family of tests, and the significant differences in intercepts from ANCOVA, the difference appears valid. Conversely, the opposite position could be argued, as the specific abnormal patterns were highly variable. However, the high variability could be due to sample size for each pattern (Table 1), albeit no consistent trend was apparent. For example, Pie had the lowest egg mortality among all abnormal patterns, lowest hatching success, second highest percentage of deformed larvae, and third highest larval mortality while having the highest proportion of abnormal eggs in each batch. For egg mortality, each abnormal pattern was not significantly different from normal eggs (p=0.122). Excluding Batch 1, egg batches showed relatively consistent egg mortality, with abnormal eggs consistently greater and with higher variability than normal eggs (Fig. 2C–D; Table 2), which supports a true difference. High variability exists at the specific pattern level, but becomes markedly less when all abnormal patterns are combined.

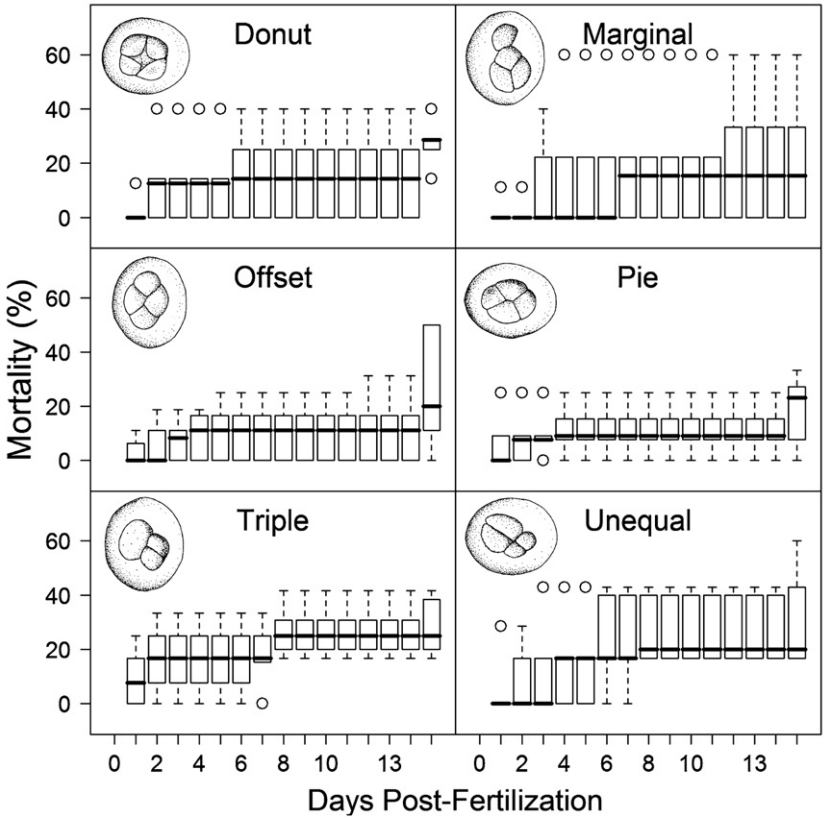

Fig. 3. Egg mortality trends for six abnormal patterns of eggs of Atlantic cod (*Gadus morhua*). Patterns were determined at the 4-cell stage of development. Boxplots consist of median (dark bar), lower and upper quartiles (box), whiskers (T bars; 1.5 interquartile range), and outliers (circles).

Egg mortality and rates of specific patterns differ from normal and abnormal trends (Fig. 3). Pie and Donut show uniform mean egg mortality with increased variability occurring at days 4 and 6, respectively. Marginal, Triple, and to a lesser extent, Unequal show saltatory changes but at different development times. The trend of offset eggs is similar to the mean trend of normal and abnormal eggs, but the asymptote is reached at day 4 rather than at day 8. All patterns except Marginal show either an increase in mean egg mortality or an increase in variability or both on day 15, and all patterns show changes in variability during development; most increasing ontogenetically.

Hatching occurred in all batches at about day 14 (Table 2), and was remarkably consistent. Hatching success was high in all groups, but not significantly different between normal and abnormal ($p=0.100$) or between normal and any abnormal pattern ($p=0.237$). Abnormal eggs were more variable in hatching success than in egg mortality with no significant correlation when all groups were analyzed together ($p=0.565$; Fig. 4). Although the overall median hatching success was 100% (mean 93%) for normal and individual abnormal patterns (the pooled abnormal group was discarded for this analysis), 40% of cases were <100% with a low of 60% for Offset and 67% for Unequal (Table 2). In addition, variability within and among patterns was inconsistent (e.g. 83±18 for Offset, and 100±0 for Marginal; Table 2).

The proportion of deformed larvae and larval mortality were both highly variable among abnormal patterns, but fairly consistent for normal eggs. Relatively few larvae were deformed or died after 24 h compared with the number of eggs that hatched (Table 2). No difference existed between normal and abnormal deformed larvae ($p=0.623$) or larval mortality after 24 h ($p=0.347$). However, a significant correlation between deformed and dead larvae ($p<0.001$) suggests that a high percentage of larvae that were deformed died. This association tended to be verified with visual observations, but no quantitative data were collected. The most common larval deformations were spine curvatures (scoliosis and lordosis) and pinched or underdeveloped fins (Fig. 5). Some pigmentation differences were noted, but clear differences were not observed.

SI included both AS (Table 1) and the ranks of quality measures (Table 2). The mean of ranks provides a qualitative, nonparametric indicator of blastodisc pattern severity of the combined effect of the abnormal parameters. The extreme values belong to Marginal (2.2) and Unequal (5.7) patterns, and only Marginal had a lower SI value than normal. The three highest abnormal SI values had only one abnormal parameter each (blastomere size, inclusions, or margins). Note that Unequal could arguably include asymmetry, as they are only symmetric in one plane, and margins, as the large blastomere may not have divided completely, which would increase AS. The three lowest abnormal SI values have asymmetry in common, and adhesion issues in two of the three cases. Oddly, when blastomere size is not the sole quality measure, as in Triple, SI decreases to 3.6 suggesting that combining parameters could have mitigating or masking effects. A suggested severity order is asymmetry<adhesions<margins≤inclusions<blastomere size.

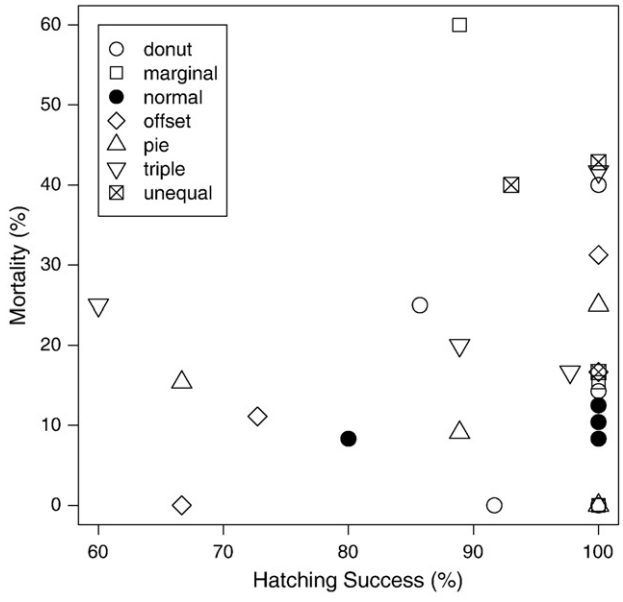

Fig. 4. The relationship of hatching success and mortality of normal and each abnormal egg patterns across batches for Atlantic cod (*Gadus morhua*).

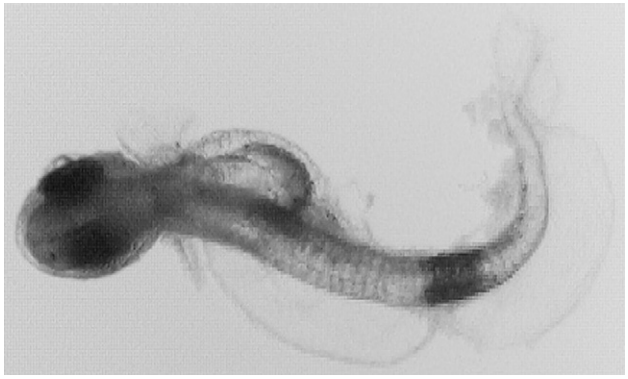

Fig. 5. Typical larval deformations found in live larvae hatched from eggs having normal and abnormal cleavage patterns in Atlantic cod (*Gadus morhua*). Note the curved spine and incomplete (pinched) dorsal fin.

4. Discussion

4.1. Atlantic cod eggs

Overall, abnormal eggs were not markedly present in any batch (T. Avery, pers. obs.); certainly no more than the 13% (14 of 16 batches below 20%) shown for Atlantic cod by Vallin and Nissling (1998); a mean of <20% for Arcto-Norwegian cod from 6 of 8 females spawned for two consecutive years (Solemdal et al., 1998); or 22.9% and 28.7% for dab and whiting, *Merlangius merlangus* (Linnaeus, 1758), respectively, shown by Cameron and Berg (1992). We estimate that the proportion of abnormal eggs was on the order of 10% as shown by Penney et al. (2006) using broodstock Atlantic cod maintained in the same facility. The number of eggs within each pattern may not be proportionately representative of actual because we did not try to select a set number of eggs of a particular pattern, but selected abnormal eggs at random as they were searched within the petri dish. Still, the numbers are suggestive of some abnormal patterns being more or less common than others. Seventeen Jumbled eggs were found only in Batch 1 and was the highest proportion of any "randomly" selected pattern and unlikely to occur by chance alone. Batch 1 was discarded from analysis for several factors relating to traditional poor egg quality, but nonetheless, had the worst combined mean egg mortality and early egg mortality rate. To suggest that Jumbled egg patterns are most severe and indicative of poor eggs, however, may be premature. Westernhagen et al. (1988), Vallin and Nissling (1998), Makhotin et al. (2001), and Thorsen et al. (2003) all show images of patterns characteristic of Jumbled described herein, so this pattern is likely not an isolated event. All of their images include asymmetry, poor margins of both cells and blastodisc, and differences in blastomere size. In addition, Thorsen et al. (2003) note in their Fig. 5 that the abnormal egg hatched normally (presumably producing a normal larva). In comparison, 11 of the 17 Jumbled eggs herein hatched (6 died before hatch) and produced normal larva.

Egg mortality was variable among and batches and patterns. Some patterns reached an asymptotic, low or stable egg mortality as early as day 2 (Donut and Triple), but the overall normal and abnormal

mortality trend was a high rate (slope) early in development before reaching an asymptote around day 8. Sample size does not appear to be a factor in the pattern trend differences, as Donut and Triple were neither the highest nor the lowest proportions of abnormal eggs. It is apparent within particular abnormal patterns that there are saltatory increases in mortality with intermittent stable periods. Initial egg mortality rates up to about day 8 span the cleavage, blastula, and gastrula periods as defined by Hall et al. (2004), after accounting for differences in rearing temperatures. During the segmentation period, mortality becomes asymptotic, but that is not a consistent trend across patterns. Some patterns reach an asymptote at day 3 during the germ ring stage or 10%–25% epiboly within the blastula or gastrula period, respectively. Solemdal et al. (1998) show similar trends using 1 L glass jars as incubation chambers with a marked mortality rate (slope) until the blastula stage, and asymptotic thereafter. Their trend, however, was not present with parallel individual egg incubations using NUNC well plates in the same study.

The cumulative egg mortality for normal eggs of Atlantic cod was less than the mortality found by Makhotin et al. (2001) (23% and 31%) for normal eggs sampled from the wild, and similar to cod eggs collected from Skagerrak (a relatively stable oceanic environment compared with brackish waters of eggs from Baltic cod) and grown in large-scale rearing tanks (20.5%±6.41%) found by Pickova et al. (1997). Resolving Pickova et al. (1997) into their symmetric egg mortality (10.8%±4.99%) is quite similar to abnormal herein, but their asymmetric mortality (32.6%±10.8%) was greater. Solemdal et al. (1995) had markedly greater egg mortality (72% of batches were above 50%) for normal eggs of cod carefully selected from 10 individual females, but they showed similar high variability among pooled batches from one repeat-spawning (successive years), captive female. In a related study, Solemdal et al. (1998) found cumulative egg mortality for normal Arcto-Norwegian cod eggs reared in 1 L glass jars at pre-hatch of ~56% and ~42% during successive years using the same females, and only ~20% when rearing normal eggs in NUNC well plates (increasing to ~38% during the post-hatch phase). The high variability found among patterns herein emphasizes that attention to scale when comparing among studies is warranted. Some differences between this and other studies are undoubtedly because of differences in rearing conditions, additives to rearing water (antimicrobials, etc.), and handling. On a broader scale, other marine species such as gilthead seabream (~80%) and seabass (~93%) have similar cumulative normal egg mortality, but egg mortality curves showing low mortality early in development followed by saltatory increases nearer to hatch (Solemdal et al., 1998; Panini et al., 2001).

Avery and Brown (2005) suggested that egg rearing should be abandoned if egg mortality exceeded the mean "normal" or baseline mortality from previous rearing trials at the point where the mortality rate curve becomes asymptotic (or nearly so). Although not as clearly defined or consistent as for yellowtail flounder, where abnormal egg mortality rates were highest from days 1 to 3 (~27 degree-days) thereafter reaching an asymptote (Avery and Brown, 2005), a suggested time to abort egg incubation for Atlantic cod based on egg mortality rates would be day 7 or 8 (~45 to 50 degree-days) or when eggs reached the end of gastrulation. Comparing stages of embryogenesis to the characteristics of the mortality curves for various species is an obvious next step to determine if mortality is related to development stage and at what stages mortality rates change or stabilize. Vallin and Nissling (1998) suggested a similar approach. One possibility is that blastomere abnormalities occurring later in development may cause more damage than earlier abnormalities of undifferentiated blastomeres. In contrast, abnormalities occurring within a highly developed embryo or larva cause only local, limited damage and are replaced quickly by other germ cells, whereas abnormalities occurring at the 4-cell stage and persisting to 16 or 32 cells have a greater chance to inflict problems because a higher portion of the total embryo is affected. The blastodisc is simply much less developed than a larva and, as the evidence suggests, more susceptible to damage. However, the critical periods remain to be determined with more rigorous studies in accordance with the ideas of Vladimirov (1975). Studies describing types of abnormalities occurring at stages of development for Arcto–Norwegian cod (Makhotin et al., 2001) and detailed descriptions of Atlantic cod embryogenesis and early life stages by Hall et al. (2004) are significant steps. However, for some species such as gilthead or white seabream, hatching occurs after 2 days (Panini et al., 2001), preventing a meaningful analysis of egg mortality rates and likely obscuring saltatory changes unless rearing trials were observed at higher resolution time periods.

Though often ignored, the distinction between survival and hatching success is important in early development, as there is no guarantee that an egg which survives until the expected hatching time will, in fact, hatch. The collective knowledge of the drivers that produce egg-quality differences is growing, but descriptions and empirical evidence of specific mechanisms are rather limited. We cannot speculate why there is an increase in egg mortality at time of hatch (day 15). Eggs that are ready to hatch do not, but it was clear that eggs did not sink to the bottom of the incubation chamber because of a burst chorion leading to buoyancy loss, nor were larvae partially free of the egg but unable to completely hatch (see Thorsen et al., 2003). Similarly, Makhotin et al. (2001) and Solemdal et al. (1998; for NUNC trays) clearly show increased egg mortality concentrated around the time of hatch or shortly thereafter, and similarly low egg mortality through the gastrula and segmentation periods, although both show high variability between successive year samples and, for Solemdal et al., batches. The mechanisms for hatching *per se* are largely unknown, but the results herein suggest that there is some merit in considering hatching success separate from egg mortality (survival) as an egg quality criterion, because no correlation exists between the two criteria. As the ultimate goal of examining egg quality is to predict viable larvae (e.g. Kjørsvik et al., 1990; Brooks et al., 1997), finding the links between egg-quality criteria and their causative agents and mechanisms is paramount. Herein we show that hatching success is uncorrelated with egg mortality at the scale of abnormal patterns within batches.

Kjørsvik et al. (2003) found lower mean hatching rates for turbot (39%±26%) across six females, but their values were based on all floating eggs (unfertilized and fertilized) within batches. Our results are much more similar to Penney et al. (2006) who found hatching success of 75%–83% in three separate broodstock groups. The methods of Penney et al. (2006) were similar to the methods used herein, but their hatching success was based on all eggs originally stocked into incubators. Shields et al. (1997) found a maximum of ~80% hatching success for each of their abnormal patterns using an individual egg rearing technique. When eggs are not raised individually, hatching success is difficult to determine and survival is used as a best estimate. Kjørsvik et al. (2003) also showed that hatching success increased with increasing proportion of normal blastomeres, an affect that was not repeated herein. Again, a difference in their calculation of hatching success is likely the cause of the discrepancy in outcomes.

Relatively few larvae were deformed, so limited quantitative insights can be made. Vallin and Nissling (1998) found viable hatching rates (proportion of straight larvae to all larvae hatched) of 14%–97% (mean 64%±28%). In comparison, "viable hatch" herein was 72%±13% (abnormal) and 70%±14% (normal). Kjørsvik et al. (2003) suggested that larval mortality so soon after hatch may be too crude of a criterion under which to assess larval viability and that larvae should be assessed over longer periods and later in development. Parental studies on yellowtail flounder showed no difference in larval quality from 2 to 4 days after hatch based on up to 5 larval quality measures (Avery, 2001). Two points that are relevant to this issue are (1) at what point does embryogenesis no longer affect larval viability and (2) what effect will blastomere corrections have during ontogeny after embryogenesis?

4.2. Marine fish egg quality: towards standardized criteria?

Categorizing abnormal patterns as more or less severe, or relating abnormal patterns to environmental factors, would be useful for aquaculture operations and recruitment models. A standard classification system for abnormal cleavage patterns would be beneficial if patterns were common among species, but evidence suggests that although parameters may be ubiquitous after accounting for differences in terms,[2] specific blastodisc patterns are species specific. For example, abnormal eggs of turbot can resemble a clover pattern (Kjørsvik et al., 2003), a pattern that was not seen in eggs of Atlantic cod. Shields et al. (1997) outlined five basic parameters, however, two instances herein show that an expansion of those basic parameters is warranted based on observational scale. Margin errors are apparent for blastomeres and for blastodiscs, and asymmetry, or symmetry, can be assessed as (*i*) symmetric in all planes, (*ii*) symmetric in at least one plane, or (*iii*) radially symmetric. For example, eggs with the Triple pattern show bilateral symmetry, but that symmetry bisects a blastomere. Again, observational scale comes into question, as bilateral symmetry that bisects a blastomere is possible but probably not intended as a symmetry criterion. Penney et al. (2006) provided additional characteristics when assessing egg normality including clarity of blastomere cytoplasm and blastomere number (during the cleavage and blastula periods, normal eggs have a even number of blastomeres). The former is a well-known, general characteristic first suggested as a fish egg quality characteristic by Kjørsvik et al. (1990), and the latter is partly implicit in margin errors (cells that do not divide). Cameron and Berg (1992; Fig. 2) and Cameron et al. (1996; Fig. 2g) showed "blisters" in abnormally cleaving eggs of either cod, flounder, or plaice (species not identified), and Rowe and Eckhert (1999) link blebbing (blisters) to boron deficiency in zebrafish, *Danio rerio* (Hamilton, 1822). Subtle differences may also be important. Kjørsvik et al. (2003) found decreased contact surface area between blastomeres in their clover pattern, a variant of marginal errors. There is considerable merit in dissecting and constructing new characteristics, as they may prove to be better metrics of egg quality than the standard criteria described in the often cited key works by Kjørsvik et al. (1990), Shields et al. (1997), and Brooks et al. (1997).

If severity is indeed ordered as asymmetry<adhesions<margins≤inclusions<blastomere size, further emphasis and focus should be placed on determining causation, reducing confounded factors, and observing egg development on an individual egg basis. Greater sample sizes and more rigorous trials would be beneficial especially in those cases where scales are confounded within sample size (e.g. blastomere or blastodisc margin errors were grouped together in the marginal pattern). For example, inclusions may only be important as errors before the blastula period, since the blastocoel is created by first creating spaces between blastomeres. Some abnormal patterns cause increased mortality, decreased hatching rates, increased larval deformations, and (or) increased larval mortality, although sample sizes were lower than necessary for the observed variability within and among patterns to produce useful statistical comparisons. Asymmetry was the least severe abnormality by all metrics, and if partitioned as suggested above, some asymmetries may prove insignificant. There was no clear difference between adhesion and blastomere size errors. The cause and effect is unknown, but the trends were not apparent until eggs were sorted according to patterns and observed at a higher resolution. The practical implications for aquaculture could focus on particular abnormal patterns as non-plastic indicators of egg quality much like the observations noted for Jumbled eggs. The Jumbled pattern contained several errors and its association with Batch 1, assessed as poor quality based on historical metrics, supports our severity hypothesis.

[2] Westernhagen et al. (1988) refer to margin and adhesion errors as "loose cell aggregations".

The specific mechanisms underlying the manifestation of abnormal eggs are, for the most part, unknown, but egg abnormalities are widespread, appear consistent within species, and generally decrease egg quality. Indeed, Solemdal et al. (1998) state that "mortality always was the result of some kind of malformation". Some broad relationships to causative factors have been shown such as eggs from Baltic cod in highly variable, high salinity environments produce increased abnormalities over eggs from areas of constant salinity (Pickova et al., 1997). More comparative studies of this type are required on both wild and reared eggs. Most previous studies provide assessments based on the proportion of abnormal eggs within a batch, or on groups of eggs taken from captive spawners, or from ocean sampling, and too few studies have investigated individual eggs across too few species to make meaningful comparisons or perform meta-analysis. To illustrate this point, egg mortality for Atlantic cod was found to be significantly higher in abnormal than normal eggs, but no single abnormal pattern was significantly different when compared with normal eggs. If any of the developmental patterns that are currently considered abnormal produce similar egg mortality to normal eggs, current criteria for egg-quality evaluation would result in underestimating the number of viable larvae for aquaculture and recruitment.

Vallin and Nissling (1998) show errors occurring at several stages, but it is unclear whether the images are of the same blastodisc and, thus, persistent. In severe cases, such as eggs displaying the Jumbled or Marginal pattern, some blastomeres remained on the periphery of the blastodisc into the 32- and 64-cell stage, although blastomere outcroppings were seen in eggs classified as normal in these stages as well. The evidence suggests that some errors persist and are not easily corrected, and (or) that some errors apparently can occur rather spontaneously. We suggest that error corrections occur during later stages of embryogenesis (gastrula period onward), since low hatching success was not prevalent. in any one pattern and egg mortality stabilized by about the segmentation period. Perhaps, as suggested by Westernhagen et al. (1988), the worst eggs die-off early, and then the rest are able to course-correct during later development. Early egg mortality rates support this idea, and were not significantly different between normal and abnormal eggs, indicating that a high number of abnormal eggs in a batch may not be as detrimental as once thought at least from a development perspective. Errors spontaneously occurring later in development, as suggested by peripheral blastomeres occurring in normal eggs after the 64-cell stage, appear to have little effect on subsequent quality and support the course-correction hypothesis. Obviously, if clear differences persist between normal and abnormal eggs, such as the case with egg mortality at hatch, egg mortality rate *per se* would be a moot metric for egg quality.

Clearly, the saltatory and stabilized periods of egg mortality at specific developmental periods and the increased egg mortality over days 1–8 (as determined by a significantly greater *y* intercept for abnormal eggs than normal eggs) coupled with low cumulative mortality of abnormal eggs, and insignificant differences in hatching success, larval deformations, and larval mortality of abnormal eggs when compared with normal eggs, indicates a connection between development periods and mortality aside from egg abnormalities. For instance, the egg mortality rate was the same for normal and abnormal eggs, yet more abnormal eggs died earlier than normal eggs. The same rates suggest that some eggs die within similar periods regardless of cleavage pattern, and the differences in absolute numbers suggest that abnormal eggs are more susceptible to death at specific times. Vallin and Nissling (1998) and Kjørsvik (1994) are likely both correct in that some eggs may die early regardless of whether they are abnormal, and some die later owing to abnormalities either manifested early (Kjørsvik, 1994) or later (Vallin and Nissling, 1998) in embryogenesis. No doubt that parental genetics play an important role that is, as yet, not understood (e.g. Brooks et al., 1997; Avery, 2001; Kjørsvik et al., 2003; Rideout et al., 2004b; Probst et al., 2006), and nutrition is a factor both from maternal effects and post-yolk–sac feeding (e.g. Vladimirov,

1973; Pickova et al., 1997; Mazorra et al., 2003; Penney et al., 2006). Interestingly, water quality and elemental analysis is probably given the least attention, but the effects of boron deficiencies in zebrafish embryogenesis by Rowe and Eckhert (1999) provide a clear indication of one causative agent that produces abnormal eggs.

Some discrepancies with our findings and other studies can be attributed to the scale at which we observed the eggs, and (or) to the statistical analyses we employed. Often researchers use parametric analyses on raw data scores (proportions), or arcsine and (or) square-root transform data without considering the implications or necessity of doing so. Comparisons are difficult under these circumstances, as a statistically significant effect may not translate to a biological effect. A nonparametric approach is often of more use for practical application, as one is generally interested in groups that produce more or less or are greater or reduced in some capacity; the absolute values are less important especially when underlying factors are impossible to control (e.g., water-quality differences) or where high variability obfuscates parametric statistical analysis. Still, the findings herein relate well with previous studies and provide insights at an empirical resolution attempted only in a few previous cases.

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