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The morphological time of fixation of the total number of vertebrae in *Fundulus majalis* (Walbaum)

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Three lots of embryos with epiboly about one-third complete were reared continuously in different fluctuating temperature regimes (16°–20°, 22°–26° and 28°–32°C). At certain somite-number stages (3–6, 9–12, 15–23, 24–28 and 29–32) paired lots of embryos were transferred from the intermediate temperatures to the warmer and cooler regimes simultaneously. Mortalities were low (7·0%) to moderate (28·6%) in the 13 treatments. Embryos reared continuously in the three temperature regimes developed mean vertebral numbers showing a linear inverse relationship between temperature and mean vertebral count; on the average decreasing temperature induced an increased number of vertebrae. Among the transferred embryos only the 3–6 and 9–12 somite lots transferred to the cold regime responded to temperature changes. The response of presumptive vertebral tissue to cold temperature begins to decrease during the formation of the fifth and sixth somites and continues gradually until fixation occurs during formation of the tenth through twelfth somites. Cold temperatures influenced vertebral number more than warm and it is suggested that vertebral number in fishes spawning on rising water temperatures may be influenced more by cold water than by warm; conversely, fishes spawning on falling water temperatures may be influenced more by warmer than by colder water.

Introduction

In studies concerning the influence of environmental factors upon developing vertebrae in fishes the time of fixation of vertebral number is but imperfectly known. To express time during development different methods have been used: 1) measurement of elapsed time after fertilisation in hours or days at a given temperature; 2) accumulation of equal numbers of thermal units (day-degrees); and 3) noting the appearance of successive morphological stages. The latter method is used in this study.

Tåning (1944, 1946, 1952) with *Salmo t. trutta* found a resistance to response already existing in presumptive vertebral tissue during gastrulation and yet an especially sensitive period later during formation of the most posterior mesodermal segments, and still later, a capability for alteration in vertebral number with first appearance of pigment in the eye. Orska (1962), working with another salmonid, *Salmo irideus*, described three similar sensitive periods. Gabriel (1944) using *Fundulus heteroclitus* showed that fixation had not yet occurred at the four-or-five-somite stage. Ali (1962) found that the total vertebral number was fixed in *Oryzias latipes* when eye pigmentation began and when the pectoral fin buds were apparent. From some reports mentioning fixation times for vertebral number morphological stages could not be identified (*Clupea harengus*, Hempel and Blaxter (1961); *Oryzias latipes*, Lindsey and Ali (1965); *Oncorhynchus nerka*, Canagaratnam (1959); *Oncorhynchus tshawytscha*, Seymour (1959); *Pleuronectes platessa*, Molander and Molander-Swedmark (1957)). Results indicate only that fixation time for vertebrae occurs sometime during an early embryonic stage that varies from gastrulation to the appearance of pigment in the eye, a state of knowledge which led Garside (1966) to remark that the time of fixation for vertebral number "has not yet been determined with even slight precision".

The question considered in this study was the time during embryonic development of striped killifish, *Fundulus majalis*, when presumptive vertebral tissue no longer responds to temperature changes.

Materials and methods

Detailed descriptions for the operation of the incubation apparatus, for treatment of the natural sea water used in the fertilisation of ova, and rearing of embryos and larvae are given in Fahy (1964). General procedures for fertilising ova, handling fertilised eggs, rearing embryos and larvae, examining and cleaning containers, maintaining salinity and pH, and preparing specimens for study are described by Fahy (1972).

Eight physiologically ripe females and three males served as parent fish; these were captured by hand at night (29 May 1971) along the Morehead City-Beaufort Causeway (U.S. Highway 70), Carteret County, North Carolina, U.S.A. Ova were fertilised between 0125–0135 h EST and placed in a dark room at 21·5°C. A total of 1034 viable eggs resulted from 1355 ova stripped.

For controls three lots of 100 embryos each were reared continuously in separate fluctuating temperature regimes (Fahy, 1972); an intermediate range (22°–26°C), a cooler (16°–20°C) and a warmer (28°–32°C). To determine the morphological time when presumptive vertebral tissue no longer responds to temperature change five pairs of one-way transfers of embryos at different somite-number stages (3–6, 9–12, 15–23, 24–28, 29–32) were made from the intermediate to cooler and warmer regimes; each pair of transfers was made simultaneously. The range in development from the 3–6-somite stage through the 29–32-somite stage extended from late gastrula with yolk plug apparent (Fig. 1,A) to forming of the brain, flexure of embryo and visible vitelline circulation (Fig. 1,B). The salinity of the water in the apparatus varied from 33·3 to 33·4‰ and pH was 8·04. To ensure that embryos were at the same morphological stage at the moment of transfer, a transfer lot consisting of more embryos than required was prepared a few hours before transfer time. This pre-transfer examination of embryos permitted careful selection. Immediately before transfer, embryos in each lot were examined for the desired morphological stage. Embryos showing a stage different than the rest were removed but a sufficient number remained to preserve the original design of the experiment. About 42 h after fertilisation, with the blastoderm covering one-third of the yolk mass and the germ ring with a rudimentary embryonic shield apparent, embryos were placed without acclimation into the incubation chambers. When introduced into warm and cool regimes from 21·5°C water, the warm regime was operating at 28°C and the cool at 16°C. Thus a temperature shock of +6·5°C and −5·5°C, respectively, was experienced by embryos; the 0·5°C difference between table top and the intermediate regime operating at 22°C was probably negligible. All transfers were made between temperature regimes without acclimation.

Because embryos experienced difficulty in hatching, especially at higher and lower temperatures, it was necessary to release them surgically. In some experimental lots less than 50% of the embryos had hatched when surgical release was indicated. Thus, time of hatching when measured by 50% hatch was a somewhat subjective period.

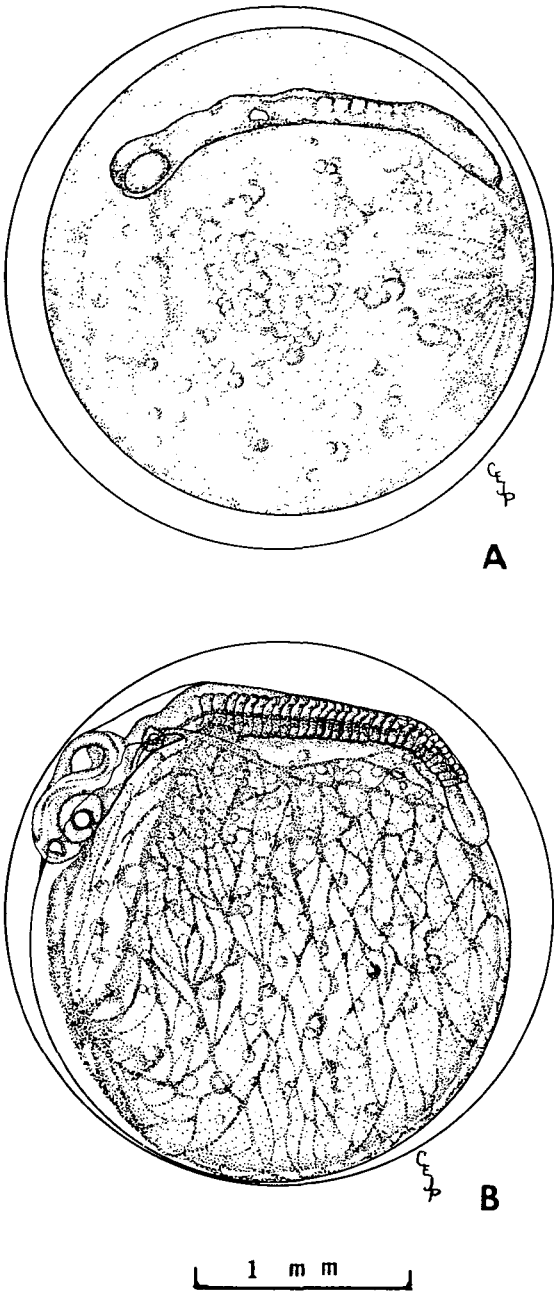

Figure 1. Embryos of *Fundulus majalis* (Walbaum) showing developmental range covered in transfer experiments. A, three to six somites. B, twenty-nine to 32 somites (actually 33 somites in figured specimen).

In all experiments the fish were killed when scale formation was complete. All meristic series are complete at this time and the experiment can be used

to provide information for designs of future experiments concerning other structures. Vertebral counts did not include the urostyle; complex vertebrae were counted as two. For statistical treatment an analysis of variance was applied to the data. When the *F*-test indicated significant differences to occur among the means, Student's *t*-test was used to compare differences among all pairs of means.

Results

Table 1 shows the relationship of temperature to number of days required for first hatching, for 50% hatching or surgical release, and for duration of an experiment. The periods involved formed a pattern one would expect, except for the days-to-first-hatch with the 3–6-somite lot transferred to the 16°–20°C regime. These embryos required considerably more time to first hatch (40 d) than those reared continuously in the 16°–20°C regime (34 d). Surgical release affected the 50% hatching time; among embryos reared continuously in the 22°–26°C temperature regime 15% were released but in the cool and warm regimes, 46 and 48%, respectively, required surgery. Among the embryos transferred, release rates were higher still, ranging from 58 to 92%.

Table 1. Hatching times of embryos and duration of experiments with *Fundulus majalis*, including three lots reared continuously in fluctuating temperature regimes and five pairs of transfers at different somite stages to cooler and warmer regimes

Experiment number and treatment		Embryos *n*	Days first hatch	Days 50% hatch or release	Days duration of experiment
Controls, steady temperature					
	temp. °C				
1	16–20	100	34	41	80
2	22–26	100	14	17	64
3	28–32	100	10	12	52
Experimental, transferred from 22°–26° to 16°–20°C					
	number of somites				
4	3–6	70	40	40	80
5	9–12	60	35	39	80
6	15–23	67	31	31	80
7	24–28	65	27	30	80
8	29–32	67	24	24	80
Experimental, transferred from 22°–26° to 28°–32°C					
	number of somites				
9	3–6	70	10	13	52
10	9–12	60	11	13	52
11	15–23	67	11	13	52
12	24–28	65	13	14	53
13	29–32	67	11	14	54

Table 2. Mortality of *Fundulus majalis* embryos reared continuously in three fluctuating temperature regimes and others transferred at different somite stages from an intermediate temperature regime to a warmer and a cooler

Experiment number and treatment		Number of embryos	Mortality number	Mortality %
Controls, steady temperature				
	temp. °C			
1	16–20	100	13*	13·0
2	22–26	100	7*	7·0
3	28–32	100	23+	23·0
	Total	300	43	14·3
Experimental, transferred from 22°–26° to 16°–20°C				
	number of somites			
4	3–6	70	5*	7·0
5	9–12	60	3*	5·0
6	15–23	67	13	19·4
7	24–28	65	12*	18·5
8	29–32	67	16**+	23·9
	Total	329	49	14·9
Experimental, transferred from 22°–26° to 28°–32°C				
	number of somites			
9	3–6	70	20*	28·6
10	9–12	60	10	16·7
11	15–23	67	14	20·9
12	24–28	65	13*	20·0
13	29–32	67	14+	20·9
	Total	329	71	21·6
Experiment Totals		958	163	17·0

* One embryo punctured during release
** Two embryos punctured during release
\+ Larvae accidentally lost

Mortalities for the experiment are presented in Table 2 and it is apparent that more embryos died at colder and warmer temperatures, especially the warmer. Although 163 embryos are listed for total mortality, 18 (11%) were accidentally lost to the experiment by careless handling (actually nine individuals were lost as postlarvae) and 126 (77·3%) died at time of hatching (not detected in time to release surgically). Only 19 individuals (11·7%) died "naturally" between epiboly and a late embryonic stage.

Frequency distributions for vertebrae formed in

Table 3. VS frequency distributions with percentage distributions in parentheses, number of individuals (n), mean, and variance (s^2) for embryos reared continuously in three fluctuating temperature regimes and five pairs of transfers at different somite stages to cooler and warmer regimes with a table of analysis of variance and an F-test

Experiment number and treatment		VS distribution 33	34	35	36	n	Mean	s^2
Controls, steady temperature								
	temp. °C							
1.....	16–20	1 (1·1)	38 (43·7)	42 (48·3)	6 (6·9)	87	34·609	0·4036
2.....	22–26	6 (6·5)	65 (69·9)	22 (23·7)	0 (0·0)	93	34·172	0·2745
3.....	28–32	7 (9·1)	59 (76·6)	11 (14·3)	0 (0·0)	77	34·052	0·2341
Experimental, transferred from 22°–26° to 16°–20°C								
	number of somites							
4.....	3–6	3 (4·6)	37 (56·9)	23 (35·4)	2 (3·1)	65	34·369	0·3928
5.....	9–12	3 (5·3)	32 (56·1)	22 (38·6)	0 (0·0)	57	34·333	0·3334
6.....	15–23	4 (7·4)	39 (72·2)	11 (20·4)	0 (0·0)	54	34·130	0·2658
7.....	24–28	5 (9·4)	38 (71·7)	10 (18·9)	0 (0·0)	53	34·094	0·2794
8.....	29–32	8 (15·7)	35 (68·6)	8 (15·7)	0 (0·0)	51	34·000	0·3200
Experimental, transferred from 22°–26° to 28°–32°C								
	number of somites							
9.....	3–6	4 (8·0)	40 (80·0)	6 (12·0)	0 (0·0)	50	34·040	0·2024
10.....	9–12	3 (6·0)	36 (72·0)	11 (22·0)	0 (0·0)	50	34·160	0·2596
11.....	15–23	5 (9·4)	35 (66·0)	13 (24·5)	0 (0·0)	53	34·151	0·3229
12.....	24–28	2 (3·8)	40 (76·9)	10 (19·2)	0 (0·0)	52	34·154	0·2112
13.....	29–32	3 (5·7)	37 (69·8)	13 (24·5)	0 (0·0)	53	34·189	0·2713

Table of Analysis of Variance

Source of variation	d.f.	sum of squares	variance estimates
Between treatments	12	23·68	1·973
Within treatments	782	230·49	0·295
Total	794	254·17	

Between treatments: $F = \frac{1{\cdot}973}{0{\cdot}295} = 6{\cdot}68^{**}$ ($F_{0{\cdot}01} = 2{\cdot}20$)

embryos exposed to the 13 different temperature treatments are given in Table 3 with an analysis of variance and F-test results indicating statistically significant differences occurring between the means ($F = 6{\cdot}68$; $F_{0{\cdot}01} = 2{\cdot}20$). Mean vertebral counts for the three lots reared continuously in different fluctuating temperature regimes showed an inverse relationship with temperature, decreasing average number of vertebrae with increasing temperature. These results agree with those of an earlier work (Fahy, 1972).

In Table 4 comparisons of differences between all pairs of mean vertebral counts are given and t-values are listed when statistically significant differences occur. Numerals in parentheses in the discussion below refer to experiment numbers in the table. Among the controls, differences between mean vertebral counts for embryos reared at 16°–20°C (1) and for those of both the 22°–26°C (2) and 28°–32°C (3) regimes were highly significant ($P = 0{\cdot}001$); no significant difference was apparent between mean vertebral counts of embryos reared continously in the 28°–32°C (3) and the 22°–26°C (2) regimes. Of the ten transfers only the 3–6 somite embryos (4) placed in cold temperatures developed a mean vertebral count significantly different from that of embryos reared at the intermediate temperatures; the mean vertebral count of this lot (4) was also significantly different from the mean count of embryos reared continuously in the cold regime (1). Although embryos of the 9–12 somite transfer to cold temperatures (5) did not develop a mean vertebral count significantly different from embryos reared continuously in the 22°–26°C regime (2), they did develop a mean verte-

Table 4. Comparison of differences between all pairs of mean vertebral counts for 13 temperature treatments of embryos of *Fundulus majalis*. A value of t is given when a significant difference occurs at the 5% (*), 1% (**) or 0·1‰ (***) level; NS denotes no significant difference occurring in a comparison; experiment numbers are identified in Table 3

Experiment number	mean VS	1 34·609	2 34·172	3 34·052	4 34·369	5 34·333	6 34·130	7 34·094	8 34·000	9 34·040	10 34·160	11 34·151	12 34·154	13 34·189
1.....	34·609	–												
2.....	34·172	5·02***	–											
3.....	34·052	6·36***	NS	–										
4.....	34·369	2·32*	2·08*	3·33**	–									
5.....	34·333	2·70**	NS	2·98**	NS	–								
6.....	34·130	4·90***	NS	NS	2·26*	NS	–							
7.....	34·094	5·17***	NS	NS	2·58*	2·27*	NS	–						
8.....	34·000	5·83***	NS	NS	3·32**	3·02*	NS	NS	–					
9.....	34·040	6·11***	NS	NS	3·27**	2·95*	NS	NS	NS	–				
10.....	34·160	4·53***	NS	NS	NS	NS	NS	NS	NS	NS	–			
11.....	34·151	4·42***	NS	NS	1·98*	NS	NS	NS	NS	NS	NS	–		
12.....	34·154	4·91***	NS	NS	2·14*	NS	NS	NS	NS	NS	NS	NS	–	
13.....	34·189	4·25***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	–

bral count that statistically was not significantly different from the 3–6 somite transfer lot (4). A significant difference in mean vertebral count occurred between the 3–6 somite transfer (4) and all other transfers from intermediate to cold temperatures, (6), (7) and (8). Similarly, the 9–12 somite embryos developed a mean vertebral count significantly different from those of (7) and (8); in regard to (6) Table 4 shows no significant difference occurring but significance was closely approached ($t = 1{\cdot}96$; $t_{0{\cdot}05} = 1{\cdot}98$).

Not only was there a tendency for decreasing temperature to increase the mean number of vertebrae developing in embryos, but lower temperatures

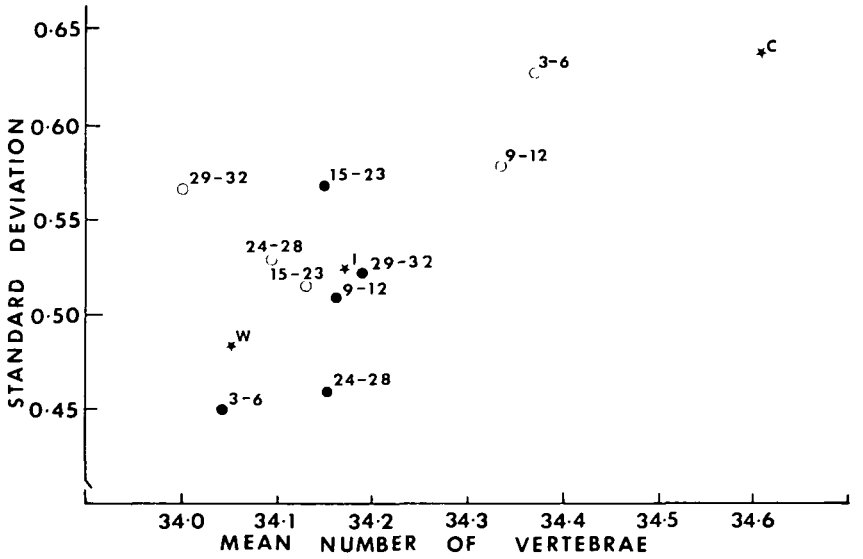

Figure 2. Relationship of standard deviation of samples to mean number of vertebrae developed in 13 temperature treatments of embryonic *Fundulus majalis* (Walbaum): stars represent points for embryos reared continuously at 16°–20°C (C), at 22°–26°C (I) and 28°–32°C (W); open circles are points for embryo lots transferred from 22°–26°C to 16°–20°C; solid circles are points for embryo lots transferred from 22°–26°C to 28°–32°C; numerals indicate range of somite number of embryos at time of transfer.

also increased the variability within a sample. This phenomenon is illustrated in Figure 2 with the standard deviation for each of the 13 lots plotted against the mean number of vertebrae developed in each. The starred points for embryos of the control lots, 28°–32°C (W), 22°–26°C (I) and 16°–20°C (C) suggest a linear relationship between increase in mean vertebral number and increase in the value for standard deviation. Embryos reared continuously in the 16°–20°C regime (C) developed the highest mean number of vertebrae and the greatest numerical value for standard deviation, the transfer lot from the intermediate regime to cool at 3–6 somites the second highest mean number of vertebrae and the second highest standard deviation, and the transfer lot from the intermediate regime to cool at 9–12 somites the third highest mean number of vertebrae and the third highest standard deviation. The points for these three lots are clearly separated from all others.

The mean vertebral count for parent fish was 34·273.

Discussion

Response of presumptive vertebral tissue of embryos of the three control lots reared continuously in different fluctuating temperature regimes confirmed earlier results (Fahy, 1972) by showing an inverse relationship between mean vertebral counts and temperature. Also, these lots demonstrated that presumptive vertebral tissue in embryos of this experiment could respond to the influence of temperature thus showing that embryos of the transfer lots had the capability of responding to temperature changes.

Embryos of experiment 4 (Table 4), the 3–6 somite transfer to cold water, developed a mean vertebral count (34·369) significantly different from that (34·172) of control embryos of experiment number 2 reared continuously at 22°–26°C. Embryos of experiment number 5 transferred at 9–12 somites developed a mean vertebral count of 34·333 that was not significantly different from that of control embryos in experiment number 2. However, there was also no significant difference between the mean vertebral counts of embryos transferred at 9–12 somites and those transferred at 3–6 somites. Embryos transferred later at 15 or more somites did not respond to temperature change. In experiment number 4 when the 3–6 somite stages were transferred, at least four somites had developed and the fifth or sixth were forming: at this stage it is difficult to count somites because the embryo rotates within the chorion as soon as it is positioned for viewing; as the number of somites increases, the embryo rolls less and counting becomes less difficult. In regard to the morphological time of fixation of vertebrae, experiment 4 clearly indicates that fixation had not yet occurred when the fifth and sixth somites were forming; experiments 6, 7 and 8, the transfers of embryos of 15 or more somites, indicate that fixation had already occurred. Thus the morphological time of fixation apparently occurred between formation of the seventh and fourteenth somites. Experiment number 5 provides information that may allow more precise determination of the time of fixation. Although statistically there was no significant difference between the mean vertebral counts for embryos transferred at 9–12 somites and those controls reared continuously in the 22°–26°C regime, these embryos developed the third highest mean vertebral count (34·333) of the experiment (Fig. 2 and Table 4). Statistically the mean vertebral counts for embryos of the 9–12 somite transfer and those of the 3–6 somite transfer belong to the same universe of mean vertebral counts (experiments 5 and 4, Table 4). In a biological sense, the relatively high mean vertebral count and the high degree of variability induced within the sample (Fig. 2) indicate that these embryos of the 9–12 somite transfer lot did respond to cold temperature change but not to the extent shown by embryos of experiment 4. One can speculate that the response occurred during the earliest part only of this morphological period, that is, during formation of the ninth somite. Morphological time of fixation in *Fundulus majalis* under the conditions of these experiments may be considered to have occurred during formation of the tenth through the twelfth somites. Although the times of formation of the 13th and 14th somites were not tested, it is unlikely that any response occurred then. These results indicate that the morphological time of fixation occurs earlier in development than was expected. No evidence was found to support Gabriel's (1944) suggestion that fixation time might occur during formation of the last posterior mesodermal body segments. There was also no support evident for sensitive periods of response after gastrulation as found in some salmonids by Tåning (1944; 1946; 1952) and Orska (1962).

From Tåning's and Orska's work it is suggested that the number of vertebrae developing can be influenced by temperature change during an early embryonic period, such as gastrulation, and then later at certain sensitive periods. The pattern of response is thus considered to occur during a series of discrete developmental stages. The present study indicates that the response period to cold temperature in *F. majalis* is a continuous one with the fixation period

being approached gradually with successive developmental stages showing a decreasing capability for response. Embryos reared continuously in the 16°–20°C regime were first exposed to colder temperatures during early gastrulation and showed a relatively high mean vertebral count of 34·609. The 3–6 somite transfer embryos were first influenced by 16°–20°C water during late gastrulation and these developed a mean count of 34·369 vertebrae. The 9–12 somite embryos had completed gastrulation and developed a lower mean count, 34·333. Embryos transferred to colder temperatures beyond the 9–12 somite stage did not respond. Apparently capability for response to colder temperatures began to decrease at least during formation of the fifth and sixth somites and this decreasing capability continued until it was lost during formation of the tenth through the twelfth somites.

In earlier work (Fahy, 1972) presumptive vertebral tissue did not respond to warmer temperatures (28°–32°C) to the extent that it did to colder temperatures. In the present experiment, embryos reared continuously in the 28°–32°C regime developed a lower mean vertebral count (34·052) than those in the 22°–26°C regime (34·172) but the difference was not statistically significant. Similarly, none of the embryo lots transferred to the warmer regime developed mean vertebral counts any different than that of embryos reared at 22°–26°C. When the difference in magnitude between responses of presumptive vertebral tissue to warm or cool temperature change is considered, the information presented in Figure 2 can be of value. Cold-treated embryos that responded to temperature change showed a direct relationship between increasing number of vertebrae developed on the average and increasing value for standard deviation of the sample means. Higher values for standard deviation indicate an increased amount of variability within a sample. The longer that embryos were exposed to colder temperatures during the response period the greater was the mean vertebral count and the greater the amount of variability induced in a sample.

In nature *Fundulus majalis* spawns during warming water temperature in late spring and summer along the eastern coast of the United States of America. During the embryonic period when presumptive vertebral tissue responds to temperature changes, experiments show that colder temperatures, induce a greater response than warmer temperatures as measured by mean vertebral number. I suggest that this cooling influence induces a stronger response because it is an interruption, or even a reversal, of the ordinary or expected temperature regime; a warming influence is the ordinary or expected condition and it may induce a lesser response. Conversely, when concerned with fishes that spawn on falling water temperature, a warming influence could be expected to induce a stronger response than a cooling influence.

In experimental studies involving the rearing of fish, massive mortalities usually occur, especially during embryonic stages; selective mortality is immediately suggested as a factor that can invalidate results. Of 163 mortalities in the experiment 88·3% were due to investigator error (11·0% were lost during handling, or punctured during surgical release and 77·3% died because they were not released soon enough). Only 11·7% of mortalities died from unknown causes. It is unlikely that selective mortality was of any consequence in this experiment.

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