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12. It is possible that the slight (nonsignificant) tendency towards tolerance shown by the animals given ethanol each day after practice might have resulted in tolerance if the experiment had been extended beyond 24 days. However, even if such tolerance were to have been obtained, it would not necessarily be attributable to ethanol exposure per se. There are at least two ways in which learning could mediate such tolerance. One is that the animals learn incidentally to tolerate ethanol while intoxicated in their home cages. A second possibility is some form of classical conditioning. All the injections of ethanol were given in the test room. The cues associated with this room reliably predicted the presence of ethanol and may have elicited compensatory responses through classical conditioning.
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Environmental Sex Determination: Interaction of Temperature and Genotype in a Fish

Abstract. *Sex determination in an atherinid fish, the Atlantic silverside (*Menidia menidia*), is under the control of both genotype and temperature during a specific period of larval development. The sex ratios of the progeny of different females are variable and differ in their responsiveness to temperature. This demonstrates that sex ratio in fishes that normally have separate sexes can be influenced by the environment.*

The evolution of genetic mechanisms that determine sex and the operation of natural selection on the sex ratio have long been of interest to population biologists (*1*). In many animals, the sex of offspring is determined at conception, and primary sex ratios of progeny approximate 1:1. Determination of sex by environmental factors, after conception, is a relatively rare phenomenon among gonochoristic species (those having separate sexes); this phenomenon has been found in a few invertebrates (*2*), one family of turtles, and an alligator (*3*). Although fishes probably have the most diverse array of sex-determining mechanisms and modes of sexuality of any vertebrate group (*4*), naturally occurring environmental determination of sex by factors such as temperature has not been found in any gonochoristic fishes. We now present data demonstrating that (i) sex determination in an atherinid fish, the Atlantic silverside (*Menidia menidia*), is under genetic and temperature-dependent environmental control during a critical phase of larval development, and (ii) sex ratios of progeny from different females are highly skewed, highly variable, and differ in their responsiveness to temperature.

The Atlantic silverside is a common estuarine fish of the eastern North American coast that completes its entire life cycle in 1 year (*5*). Breeding occurs on a semilunar cycle over a 2- to 3-month period during the spring, with each female producing four to five successive clutches of 200 to 2000 eggs (*6*). Our study of *Menidia* in Essex Bay and Salem Harbor, Massachusetts, revealed a consistent pattern of seasonal fluctuations in sex ratios (Fig. 1) (*5*). As juveniles of a new year class were recruited to the population in early July, proportions of females significantly exceeded 0.5 ($P < .01$); as recruitment continued, the excess of females rapidly declined. By completion of recruitment in September, the number of males either slightly (1977, 1978) or greatly (1976) exceeded that of females, and the mean lengths and weights of females were significantly greater than those of males (*5*). However, ranges in size were nearly equal, and experiments in 1978 and 1979 proved that males and females actually grow at equal rates when reared from eggs in laboratory aquariums (*7*). Furthermore, clutches of eggs taken from 6 to 10 females, fertilized by 10 to 25 males from the early, middle, and late portions of the spawning season, and reared under the prevailing photoperiod and constant warm temperatures (20° ± 1°C), produced similar proportions of females: 0.268 ($N = 123$), 0.297 ($N = 111$), and 0.273 ($N = 99$), respectively (*7*). These male-biased sex ratios focused our attention on the effect of environmental factors, specifically temperature.

In 1980, we conducted three experiments in which eggs and larvae were incubated in environmental chambers under two temperature regimes: cold fluctuating temperatures (CFT) of 11° to 19°C and warm fluctuating temperatures (WFT) of 17° to 25°C. These temperature regimes, based on data from a major spawning site in Salem Harbor, correspond to the average minimum and maximum temperatures experienced by eggs during the first 2 weeks of May (CFT) and the first 2 weeks of July (WFT). Since silversides deposit their eggs in the upper intertidal zone among vegetation,

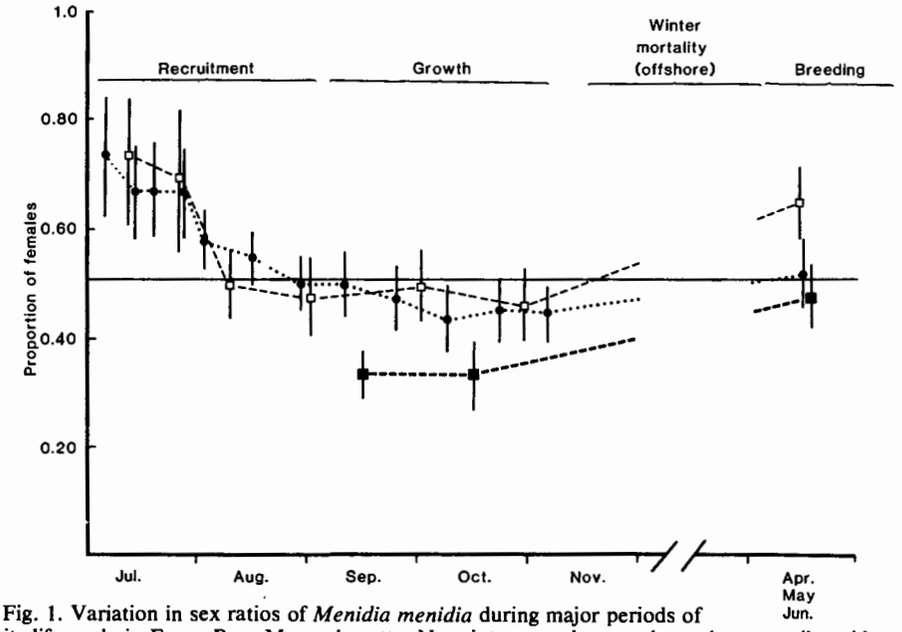

Fig. 1. Variation in sex ratios of *Menidia menidia* during major periods of its life cycle in Essex Bay, Massachusetts. No winter samples are shown because silversides winter offshore and are unavailable for capture in near-shore areas. Samples obtained in the spring are pooled because silversides suffer high winter mortality and are much less abundant afterward. Since a life cycle is completed in 1 year, each year class represents a distinct generation: (■) 1976; (●) 1977; (□) 1978. Vertical lines indicate 95 percent confidence limits based on exact probabilities (*9*). Sample sizes range from 55 to 442 (mean, 255). The horizontal solid line represents a 1:1 sex ratio.

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Table 1. Variations in sex ratios of progeny from six different females fertilized by the same male and reared to the juvenile stage at WFT (17° to 25°C) or CFT (11° to 19°C). Natural mortality is that occurring for any reason other than random removal of excess fish from the aquarium by the investigator; N is the size of the sample.

Female	Temperature regime					
	WFT			CFT		
	Proportion of females	N	Natural mortality (%)	Proportion of females	N	Natural mortality (%)
B	0.518	141	44.5	0.633*	169	41.1
C	0	138	26.2	0.040*	126	18.7
D	0.025	159	19.2	0.337†	86	30.8
E	0.503	161	22.8	0.520	123	49.7
F	0	135	27.6	0.536†	125	18.9
I	0.014	143	22.4	0.320†	169	21.9

*WFT and CFT treatments significantly different ($\chi^2_{1\ d.f.}$), $P < .05$. †$P < .005$.

developing embryos are often subjected to temperature shocks—for example, when a rising tide of cold water covers spawning beds that have been warmed by the sun. To simulate field conditions during experiments, developing embryos were subjected to a temperature cycle of 12 hours at each extreme of either CFT or WFT, with an abrupt temperature shift to the opposite extreme. This was achieved by rearing eggs in a thin film (1 mm) of seawater in 20.8-liter glass aquariums placed in the environmental chamber. After the eggs hatched, the aquariums were filled to capacity, thereby subjecting larvae to a fluctuating temperature cycle of 4 hours at each extreme, with a gradual linear temperature change over the intervening 8 hours. In all 1980 experiments, we maintained a constant photoperiod of 14.5 hours of light and 9.5 hours of darkness and salinities of 25 to 28 per mille. We fed the larvae nauplii of *Artemia salina* to excess and changed the seawater in each tank every 3 to 6 days. We removed and counted dead *Menidia* larvae from each aquarium daily. A treatment was terminated when all fish had reached a size at which they could easily be sexed by dissection and inspection of gonads (mean length, 25 to 30 mm attained 40 days after hatching at WFT, 75 days at CFT) (*8*). Chi-square analysis was used in all tests of independence between treatments (*9*).

In experiment 1, we tested the effects of temperature on sex ratio and assessed the influence of maternal characteristics, such as size, clutch size, and genotype, on sex ratios. We dip-netted *Menidia* from large spawning schools at Salem (*7*), stripped a clutch of ripe eggs from each of six females onto nylon screens (*10*), and fertilized each with an equal proportion of sperm from a single male. Hence, all progeny in experiment 1 had the same father. After fertilization, we placed half of each set of sibling eggs in CFT and half in WFT treatments. All eggs were counted regardless of viability. After the early larval phase, densities of fish were equalized to 150 to 200 per aquarium by randomly removing excess fish.

The results of experiment 1 show that (i) sex ratios of *Menidia* are influenced by temperature during development and (ii) sex ratios of progeny from different maternal sources vary greatly and differ in their response to temperature (Table 1). At WFT, the ratio of male to female progeny was either 1:0, or nearly so, or approximated 1:1. The sibling set of progeny reared under CFT consistently produced a higher proportion of females, but this proportion varied among the progeny of different mothers. The proportion of females in the progeny of some mothers seemed to be unaffected by temperature, whereas in the progeny of others it increased by 13 to 54 percent. There was no apparent correlation of these sex ratios with maternal size, clutch size, or level of natural mortality. The discontinuous nature of these variations suggests they are genotypic rather than the result of differential mortality (see experiments 2 and 3).

In experiment 2, we tested the effect of temperature on sex ratios during the larval phase alone to eliminate egg mortality as a factor. Naturally fertilized eggs were removed from the field and allowed to hatch under CFT conditions. From this common stock, 137 and 132 1-day-old larvae were individually counted and randomly assigned to aquariums in the CFT and WFT chambers, respectively. These two treatments resulted in 65 females, 49 males, and 23 unknowns (due to mortality) under CFT conditions and 28 females, 93 males, and 11 unknowns under WFT conditions. Even if all unknowns are assumed to be of the minority sex (23 males in CFT, 11 females in WFT), the resulting adjusted sex ratios still differ significantly ($\chi^2_{1\ d.f.} = 9.08$; $P < .005$). The numbers of CFT and WFT progeny of female F in experiment 1 likewise differed significantly when a similar adjustment for mortality was made, even with egg mortality included (*11*).

Table 2. Critical period for sex determination as demonstrated by shifting larvae from CFT to WFT periodically as larval development progressed; N is the size of the sample.

Days after hatching	N	Proportion of females
0	95	0.021
18	111	0.063
32	117	0.077
46*	121	0.215
60	81	0.309
75	107	0.318

*All sex ratios of progeny moved from CFT to WFT before 46 days are significantly different from all sex ratios of progeny moved later, $P < .01$.

In experiment 3, we hoped to identify the developmental stage during which sex ratios were most sensitive to temperature. We combined the ripe egg clutches from two females, fertilized them with the sperm of the same male as in experiment 1, and reared them under CFT simultaneously with experiment 1. Upon hatching, two groups, consisting of 125 larvae each, were removed and randomly assigned to aquariums under CFT and WFT conditions. The remaining larvae were maintained under CFT, and periodically, additional groups of 85 to 125 larvae were moved to WFT until termination of the experiment. Our results indicated that sex ratios were sensitive to temperature only during a specific stage late in larval development, just before completion of metamorphosis (Table 2). During this critical period, natural mortality was very low; it averaged 2.7 percent during days 32 to 60. Therefore, at least in some individuals, temperature directly affected sexual differentiation long after conception, rather than causing differential mortality of the sexes.

In natural populations, females are produced in excess early in the breeding season and hence are generally older and larger than males. During our study, population sex ratios varied significantly between successive generations during both the juvenile growth and breeding seasons (Fig. 1), as might be expected when fluctuating environmental factors have the potential to influence the sex ratio. The adaptive significance of this unusual mode of sex determination is related to the resulting sexual dimorphism in size (*12*).

We have not yet tested for paternal

variation in sex ratios of progeny, and we do not know whether temperature has a linear or a "threshold" relation to sex ratios of progeny. The high degree of variation in the sex ratios of progeny from different females may suggest polygenic sex determination, as has been hypothesized for other fishes (*13*). Although we have not proved that offspring maintain the same sexual expression until maturity, we consider sex reversals unlikely because we have never discovered any intersex individuals in culture or in the wild; cultures of *Menidia* allowed to grow well beyond the critical phase had the same sex ratio as identical cultures terminated earlier (*8*). Furthermore, sequential hermaphrodites are unknown among atherinids. Hence, in *M. menidia*, once sex is determined, it appears to be irreversible.

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7. These fish were raised in 60-liter aquariums and were fed *Artemia salina* nauplii to excess. In 1979 we conducted experiments involving removal of naturally spawned and reared eggs from the field just prior to hatching, but learned only that the critical period of sex determination was during the larval phase.
8. Sex cannot be determined externally. When *Menidia* had attained a minimum length of 20 mm, they were easily sexed by examination of gross morphology of gonads under a dissecting microscope and, when rarely necessary (frequency, < 1 percent), by the presence of numerous oocytes in slide preparations at higher magnification. Our criteria were nearly identical to those described for a poeciliid of similar size [F. F. Snelson, Jr., and J. D. Wetherington, *Evolution* **34**, 308 (1980)]. We validated our sexing technique by verifying that sex ratios of progeny allowed to grow to a much larger size (40 to 50 mm) were identical to the ratios in sibling sets of progeny terminated earlier (20 to 30 mm).
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The AF64A-Treated Mouse: Possible Model for Central Cholinergic Hypofunction

Abstract. *A loss in the number of functional, sodium ion–dependent, high-affinity choline transport sites was observed in the cortex and hippocampus of mice given an intracerebroventricular injection of 65 nanomoles of AF64A (ethylcholine mustard aziridinium ion) 3 days earlier. Such an effect was not observed in the striatum. This effect of AF64A represents a long-term neurochemical deficit at cholinergic nerve terminals in some brain regions which can lead to a persistent deficiency in central cholinergic transmission. The AF64A-treated animal may thus be a model for certain psychiatric or neurological disorders that appear to involve central cholinergic hypofunction.*

We recently proposed that choline mustard analogs may be toxic to cholinergic nerve terminals and could be used in animals to model the psychobiology and pharmacology of disorders characterized by an underactivity of central cholinergic neurotransmission (*1*). We now report that intracerebroventricular injection of one of these compounds, ethylcholine mustard aziridinium ion (AF64A), into mice produces a long-lasting reduction in the number of functional, Na^+-dependent, high-affinity choline transport (HAChT) sites. The HAChT system is a rather specific marker for cholinergic nerve terminals (*2*); moreover, HAChT activity may regulate acetylcholine synthesis (*2*). Thus, our results demonstrate that AF64A causes a persistent neurochemical deficit at cholinergic nerve terminals which may lead to chronic central cholinergic hypofunction in animals.

To test whether a low dose of AF64A could affect cholinergic nerve terminals in situ, mice were lightly anesthetized with ether, and 65 nmole (25 μl) of AF64A or a vehicle solution (*3*) was unilaterally injected into the lateral ventricle with a 3/8-inch, 27-gauge Hamilton hypodermic needle attached to a 0.5-ml glass syringe (*4*). This dose of AF64A was not immediately lethal. The mice were then housed in groups for 3 days. During this period, the AF64A-injected animals developed neurological disturbances (such as hypokinesia) and various ataxic syndromes with variable frequency, and typically lost 10 to 20 percent of their original body weight. Vehicle-injected mice incurred no morbidity and gained weight at a natural rate. On the third day after treatment the mice were decapitated and their brains were dissected. Dissection was performed at room temperature to permit postmortem reversal of adaptive changes in the HAChT system (*5*). The tissue was then kept on ice until homogenization. A crude synaptosomal pellet (P_2) was pre-

Table 1. Kinetic constants of Na^+-dependent HAChT in synaptosomes from mice 3 days after AF64A or vehicle was administered into the brain, as calculated by Eadie-Scatchard analysis of transport data. Values are means ± standard errors for independent matched experiments.

Brain area	Treatment	N	V_{max}*	Percent of vehicle V_{max}	K_T† (μM)
Cortex	Vehicle	3	19.8 ± 1.9		0.39 ± 0.04
	AF64A	3	6.3 ± 0.7	32‡	0.48 ± 0.12
Hippocampus	Vehicle	3	38.8 ± 11.5		0.33 ± 0.08
	AF64A	3	20.1 ± 8.4	52‡	0.29 ± 0.05
Striatum	Vehicle	3	107.5 ± 11.8		0.46 ± 0.08
	AF64A	3	123.3 ± 25.7	114	0.56 ± 0.16

*Maximum velocity of enzyme reaction, measured as picomoles per milligram of protein per 4 minutes. †Apparent Michaelis constant for transport. ‡$P < .05$, two-tailed paired Student's t-test.

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