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Evolution of a Balanced Sex Ratio by Frequency-Dependent Selection in a Fish

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Balanced (1 to 1) sex ratios are thought to evolve by a process known as frequency-dependent selection of the minority sex. Five populations of a fish with genetically based variation in temperature-dependent sex determination were maintained for 5 to 6 years in artificial constant-temperature environments that initially caused the sex ratio to be highly skewed. Increases in the proportion of the minority sex occurred in subsequent generations until a balanced sex ratio was established, thus confirming a central premise underlying the theory of sex-ratio evolution.

FISHER WAS THE FIRST TO EXPLAIN why many species tend to invest equally in the production of sons and daughters (*1*). Because every offspring has one mother and father, the contribution of genes from each sex to succeeding generations must be equal. If one sex is less numerous, its per capita contribution is higher and genes that overproduce the minority sex should therefore increase until the primary sex ratio is balanced. Fisher's model hinges on frequency-dependent selection: an evolutionary process where the fitness of a phenotype is dependent on the relative frequency of other phenotypes in the population. Fisher's theory has gained wide acceptance: it potentially accounts for the widespread occurrence of sex-determining mechanisms, such as heterogamety, that ensure the production of 1:1 sex ratios and it is the foundation for virtually all adaptive sex ratio theory (*2*).

Serious doubts have arisen because balanced sex ratios could also simply be a nonadaptive consequence of Mendelian segregation of sex chromosomes (*3*). Moreover, the only direct evidence is the same observation that originally generated the model: the ubiquity of species that have balanced sex ratios in nature (*4*). The dilemma has been that the sex-determining mechanisms of many of these species contain little (if any) genetic variation and, therefore, may not be capable of evolving. Is frequency-dependent selection such a potent evolutionary process that dioecious species capable of producing unbalanced primary sex ratios are rare?

A species ideally suited for directly testing Fisher's theory is the Atlantic silverside, *Menidia menidia*. In this fish, sex is determined by the joint effects of temperature and major sex-determining genes during a specific period of larval development (*5*). The Atlantic silverside is an annual fish that breeds during the spring and summer in bays and estuaries along the east coast of North America. Offspring produced early in the breeding season experience low temperatures that cause most larvae to differentiate into females; high temperatures that prevail during the late breeding season cause most offspring produced then to become male. Previous experiments have conclusively shown that temperature exerts a direct influence on primary sex differentiation rather than causing sex-specific mortality (*5*). The influence of major genes on sex determination is indicated by large nonadditive effects of parentage on the response of sex ratio to temperature within families (*6*). Moreover, the degree of genetic control differs greatly with latitude (*7*). In South Carolina fish, the sex ratio changes by as much as 70% with temperature. The sex ratio of Nova Scotia fish, however, is insensitive to temperature, suggesting complete genetic control. Fish from New York show an intermediate sex ratio response to temperature.

In nature, the normal pattern of seasonal change in temperature usually results in a sex ratio close to 0.5 (*5*). When progeny from a natural population are transferred to a constant-temperature environment, however, the resulting sex ratio can be highly skewed. We established five separate laboratory populations of silversides that constantly experienced either a high or low temperature environment during development each generation. Two populations were started in 1984 by randomly subdividing a common stock of several thousand newly hatched larvae that were collected as embryos from South Carolina (*8*). One such population was reared during the temperature-sensitive period at 28°C (SC-H) and the other at 17°C (SC-L). This produced sex ratios (F/F+M) that were skewed in the initial generation toward opposite extremes: 0.18 in SC-H, 0.70 in SC-L. Fish in each line were reared to maturity and allowed to spawn en masse in laboratory tanks (*9*). Their progeny were reared at the same high or low temperature during the sensitive period of larval development as were their parents. After the sensitive period, juvenile fish were subsampled to estimate the sex ratio (*10*) and about 100 remaining fish were reared to sexual maturity (*11*). Subsequent generations were treated to a like manner. Two other populations were established in 1985 with field-collected New York embryos and maintained as above, but in these lines the initial sex ratios at 28°C (NY-H) and 17°C (NY-L) were 0.05 and 0.29, respectively. The fifth population was founded in 1985 with embryos from Nova Scotia, where the sex ratio is close to 0.5 at all temperatures. This laboratory population (NS-H) was reared at 28°C each generation (*12*).

There are five possible general outcomes to this experiment. (i) No changes in sex ratio among generations. (ii) Random changes in sex ratio as might be caused by the effects of genetic drift. (iii) Shifts in sex ratio depending directly on thermal environment (that is, all high temperature lines change in a like manner, all low lines in another). (iv) Increases in the proportion of the majority sex, perhaps leading to the loss of one sex. (v) Increases in the proportion of the minority sex, with convergence of the sex ratio to 0.5. Only alternative (v) would support Fisher's theory. Note that we can distinguish between alternatives (iii) and (v) because the SC-L line begins with a female excess while the NY-L line begins with a male excess. Moreover, the NS-H population serves as a control because its sex ratio starts at 0.5 and is therefore not expected to change.

Changes in sex ratio closely followed the predictions of Fisher's theory. Increases in the minority sex occurred in each of the four populations that started with skewed sex

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ratios (Fig. 1). In three populations (SC-H, NY-H, and NY-L), the sex ratio leveled off at 0.5. More generations are needed to verify that the sex ratio in SC-L is going to stabilize at 0.5. As expected, the control population, NS-H, maintained a sex ratio near 0.5 throughout the experiment.

The trajectory by which the sex ratio of each population approached 0.5 differed greatly among the populations (Fig. 1). One population reached 0.5 in generation one (NY-H), another approached 0.5 more gradually (SC-H), and still another initially overshot 0.5 by a considerable margin (SC-L), and then gradually approached 0.5. These differences in trajectory deserve further study, but they are not particularly surprising given that the genetic control of sex determination in fish from South Carolina, New York, and Nova Scotia differs substantially (*7*).

Significant deviations away from 0.5 occurred in several cases after a balanced sex ratio had become established (for example, generations two, five, and seven of the NS-H line, Fig. 1E). This result is not unexpected, nor is it at odds with Fisher's theory. The sex ratio is selectively neutral at the equilibrium level of 0.5 (*2*). Given that our laboratory populations are small (*11*) and that sex determination within populations is genetically variable (*6*), shifts away from 0.5 can occur when the sex ratio is balanced, due to the random effects of genetic drift. Whenever departures from 0.5 occur, however, frequency-dependent selection has an opportunity to operate.

An alternative way of elucidating the effect of frequency-dependent selection on the laboratory populations is to examine the direction of sex ratio change following each generation where the sex ratio was skewed. Summed over all five populations, there were 19 generations where the sex ratio differed significantly from 0.5. In 18 of these instances, the proportion of the minority sex increased in the next generation (the exception was over generations two to three in the SC-L line). Genetic drift acting alone would have produced random changes in sex ratio. Our results, therefore, provided persuasive evidence of the effect of frequency-dependent selection on sex ratio evolution.

We have also explored whether frequency-dependent selection has altered the level of environmental (ESD) as opposed to genetic sex determination (GSD) within each of the laboratory populations. Several theoretical analyses have shown that ESD is adaptive if, and only if, the environment that offspring enter varies in a manner that affects the fitness of males and females differently (*13*). Otherwise, the skewed sex ratios caused by environmental variability would result in frequency-dependent selection for strict GSD (*14*). Because, in our experiment, ESD cannot be adaptive (all offspring within a population enter the same constant environment) and because the sex ratio is initially skewed, we would expect the level of ESD within each line to decline across generations. We measured changes in the level of ESD by rearing some offspring from each generation of each population at the temperature extreme opposite to that of their parental line. Results so far have been promising: the level of ESD has declined initially in three of the four lab populations with ESD (*15*), but additional generations are required to confirm this conclusion.

This study provides, to our knowledge, the first direct confirmation of Fisher's sex ratio principle (*1*). Because our model system involves a species with ESD, these findings also have implications for the debate over the impact (if any) of rapid changes in climate on species with ESD (*16*). Our results show that given genetic variation, sex-determining mechanisms are capable of evolving so as to ensure continued production of a balanced sex ratio, even after substantial perturbations.

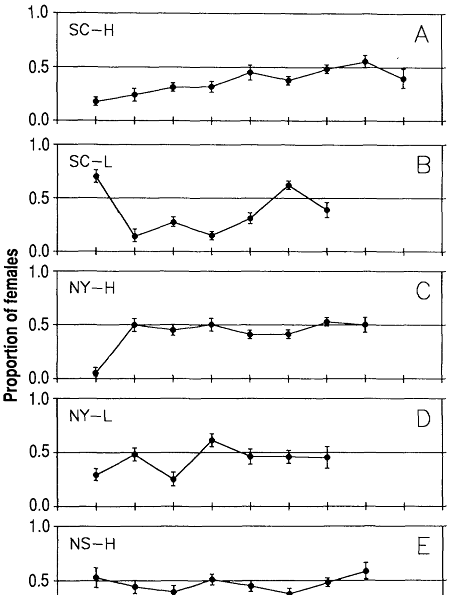

Fig. 1. Sex ratio (F/F+M) among successive generations of five laboratory populations of the Atlantic silverside that were reared each generation at a constant temperature during the temperature-sensitive period of sex determination. (**A**) Founded with embryos collected from South Carolina and reared at 28°C (SC-H). (**B**) Founded with a subsample of the same group of embroys as in (A) but reared at 17°C (SC-L). (**C**) Founded with a group of embryos from New York and reared at 28°C (NY-H). (**D**) Founded with a subsample of the same group of embryos as in (C) but reared at 17°C (NY-L). (**E**)Founded with embryos from Nova Scotia and reared at 28°C (NS-H). The zero generation represents the sex ratio observed from the original field-collected embryos after rearing at the specified temperature in the laboratory. Vertical lines represent 95% binomial confidence limits. Sample size per datum (juveniles and adults combined) ranged from 108 to 677 (mean, 322). Where the vertical lines overlap with the horizontal line at 0.5, the sex ratio does not differ significantly from 1:1.

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8. Egg collection procedures and specific localities are as described in (*7*). Rearing techniques are described in (*6, 7, 10*).
9. Maturity was induced by photoperiod manipulations. Silversides spawn in large groups and do so daily in laboratory tanks [D. P. Middaugh, *Copeia* **1981**, 766 (1981); D. O. Conover and B. E. Kynard, *Environ. Biol. Fishes* **11**, 161 (1984)]. Eggs are deposited on filamentous substrates. We placed small mops of yarn in the spawning tanks and removed these every few days after several hundred embryos had accumulated. Embryos were reared in temperature-controlled seawater baths until beyond the temperature-sensitive period of sex determination. To ensure that the fish in each generation were a representative sample of progeny produced by the parental generation, at least 3 to 5 such batches of offspring were combined. Afterward, juveniles were transferred to the spawning tanks and reared at 20° to 25°C to maturity.
10. All fish examined for sex were either juveniles (≥20 to 25 mm in total length) or adults, both of which can be sexed by dissection and direct examination of the gross morphology of gonads under a microscope with techniques described by D. O. Conover and M. Fleischer [*Can. J. Fish. Aquat. Sci.* **43**, 514 (1986)]. Conover and Fleisher also show that once sex is determined it is irreversible and that the sex ratio of juveniles is not affected by temperature. Sex ratios of juveniles and adults within each generation of each line generally reported here agreed closely. No hermaphrodites were found.
11. The mean number (and range) of breeding adults per generation of each population was: SC-H, 129 (60-271); SC-L, 108 (48-220); NY-H, 131 (41-179); NY-L, 125 (42-198); and NS-H, 139 (50-247). However, the effective population sizes (n_e) were generally less because some populations started with highly unbalanced sex ratios. Harmonic mean n_e for each population over the course of the experiment was: SC-H, 69; SC-L, 53; NY-H, 39; NY-L, 80; and NS-H, 100. These estimates of n_e assume that all fish exposed to photoperiod manipulations (9) become mature. Direct observations of groups spawning in the tanks and of the enlarged size of gonads in preserved specimens examined after spawning suggest that this assumption is correct.
12. There was one exception. Because of an insufficient number of fish available at 28°C, the initial generation of the NS-H line came from fish reared at 19°C. This should have little bearing on the outcome of the experiment because the sex ratio at all tempera-

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Acetylcholine Binding by a Synthetic Receptor: Implications for Biological Recognition

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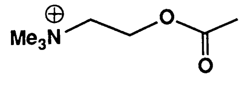
The neurotransmitter acetylcholine (ACh) is bound with 50-micromolar affinity by a completely synthetic receptor (host) comprising primarily aromatic rings. The host provided an overall hydrophobic binding site, but one that could recognize the positive charge of the quaternary ammonium group of ACh through a stabilizing interaction with the electron-rich π systems of the aromatic rings (cation-π interaction). Similar interactions may be involved in biological recognition of ACh and other choline derivatives.

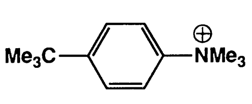
ACETYLCHOLINE (ACH) AND RELATED quaternary ammonium compounds (R_4N^+, where R is an alkyl or aryl group) interact with a wide range of biological binding sites, including those in acetylcholine esterases (AChE), ACh receptors (AChR), and voltage-gated potassium channels. We report that the synthetic, designed receptor (host) **1** (Fig. 1) shows a strong affinity for ACh. At 295 K in aqueous buffer, **1** binds ACh with a dissociation constant K_d = 50 μM, a value comparable to those of the biological recognition sites (*1–5*). Previous studies (*6, 7*) have established that **1** is, in fact, a general receptor for quaternary ammonium compounds. The primary binding force is a cation-π interaction (*8*)—the stabilizing attraction between a quaternary ammonium group and the π electrons of electron-rich aromatic systems. These findings lead us to propose a novel model for biological binding sites for choline and its derivatives, in which aromatic amino acids (Phe and especially the electron-rich Tyr and Trp) are critical for ACh binding through the cation-π interaction, with anionic residues playing a secondary role.



ACh

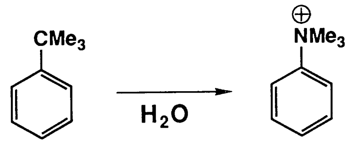
The tight binding of ACh was presaged by our extensive studies of **1** and related hosts with a variety of substrates (guests) (*6*). The binding region of **1** (Fig. 1) is quite hydrophobic, and in aqueous media, nonpolar organic molecules are driven into the cavity by the hydrophobic effect. Superimposed on this is a specific attraction (*9*) between **1** and organic cations, including ammonium and immonium (such as alkylated pyridines and quinolines) (*6*), sulfonium (R_3S^+) (*10*), and alkylated guanidinium (*11*) compounds. It has been established that aromatic π systems in the host, but not analogous aliphatic structures, are important for cation recognition. In organic media, the neutral host **2** also shows a significant preference for cationic guests (*6*). This finding and others (*6*) establish that the remote anionic groups of **1**, which are included to induce water solubility, are at best a secondary factor in cation binding. Other synthetic receptors bind quaternary ammonium compounds (*12, 13*), but charge-charge interactions between anionic groups on the host and cationic guests are much more important in these systems. For example, one system (*12*) binds ACh (K_d = 2 mM), but RNH_3^+ structures are bound more effectively than $RNMe_3^+$ (Me, methyl), in direct contrast to hosts **1** and **2** and to the biological receptor sites. In another case (*13*) *tert*-butyl analogs do not bind at all, establishing the dominant role of charge interactions.

To appreciate the potential magnitude of the cation-π attraction, consider guest **3**.



3

Previous nuclear magnetic resonance (NMR) studies (*6*) established that the ammonium group ($-NMe_3^+$) of **3** is bound in the cavity of **1**, in preference to the *tert*-butyl ($-CMe_3$). This effect occurs even though the ionic ammonium group must be much better solvated by water than the neutral *tert*-butyl, and binding must involve a considerable desolvation. We have evaluated the potential magnitude of this effect by calculating the differential solvation energy (ΔDG_{sol}) in water for the species in Eq. 1.



(1)

$\Delta\Delta G^{\circ}_{sol} = -65$ kcal/mol

The calculations involved statistical perturbation theory with full Monte Carlo simulations, following a standard protocol (*14–19*). Similar results were obtained for the conversion of CMe_4 to NMe_4^+. Clearly, the ammonium ion is not completely desolvated on binding in the cavity of **1**, but these results do suggest that the magnitude of the cation-π interaction can be quite substantial in aqueous media.

We propose that similar forces are important in a variety of biological choline binding sites. Detailed structural data are sparse, and so evidence concerning important binding interactions is mostly circumstantial. Still, many observations can be rationalized by invoking cation-π interactions between the ammonium ion of ACh and π systems at the binding site. We present here a necessarily selective overview of this broad area to provide support for the model. We emphasize from the start that our goal is not to rule out any possible role for anionic groups at the binding sites, but simply to demonstrate that cation-π forces are likely to be important in the binding and that, in some cases, all available data can be accounted for without invoking an anionic site.

The assumption that AChEs contain an anionic site that is responsible for binding the quaternary ammonium ion is widespread (*20*). However, neutral analogs (that is, $RCMe_3$ versus $RNMe_3^+$) are effective inhibitors of AChE, and they can bind at the same site as ACh (*2*). This result is certainly inconsistent with a model invoking a charge-charge interaction as the primary

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