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Post-insemination signalling systems and reinforcement

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SUMMARY

Theory indicates that the conditions necessary for the operation of reinforcement are very restrictive, yet two recent surveys of the literature report the widespread occurrence of patterns predicted by the reinforcement hypothesis. We begin a reconciliation of theory and data by focusing on one of the most troublesome restrictions placed on reinforcement: the need for strong selection against hybridization. Laboratory hybridization studies often do not find evidence of the required selection. We point out that the design of most laboratory hybridization studies precludes the detection of barriers to fertilization. Recent work on the ground crickets *Allonemobius fasciatus* and *A. socius*, which we summarize here, suggests that barriers to fertilization evolve quickly. If mating is a risky business, such barriers can promote the evolution of premating isolation. Post-insemination signalling systems are little understood by evolutionists and worthy of much greater attention.

1. INTRODUCTION

Biologists agree about the existence of mate recognition signalling systems in animal species. They also agree that one of the consequences of divergence in mate recognition signalling systems is reproductive isolation among closely related species. But begin to discuss the processes by which mate recognition signalling systems diverge and watch the fur fly. Perhaps no potential process of divergence is more controversial than reinforcement. By reinforcement, we mean the evolution of prezygotic isolating barriers in zones of overlap and/or hybridization as a response to selection against hybridization (Howard 1993). This is a broader definition of reinforcement than that of Butlin (1989), who considers the process to be limited to situations in which fertile hybrids are actually formed and selected against.

The first detailed description of reinforcement was given by Dobzhansky (1940), who took his inspiration from his work with Koller on *Drosophila pseudoobscura* and *D. miranda* (Dobzhansky & Koller 1938). Although the initial reception the hypothesis received was positive (e.g. Mayr 1942), theoretical objections were eventually voiced (J. Moore 1957). At first, the criticisms were hardly noticed by biologists, but as new theoretical objections were published (e.g. Bigelow 1965; Paterson 1978; Barton & Hewitt 1981) the drumbeat against reinforcement became quicker and louder. The drumbeat reached a deafening level as the perception grew that the predicted outcome of reinforcement, reproductive character displacement, is an exceedingly rare phenomenon in natural populations (see, for example, Littlejohn (1981); Phelan & Baker (1987)). By the end of the 1980s, many evolutionists regarded reinforcement as a largely rejected hypothesis.

Into this landscape have come two recent surveys of the literature contending that evidence from nature is consistent with an important role for reinforcement in the evolution of prezygotic barriers to gene exchange. Coyne & Orr (1989) reported, after a survey of 119 pairs of closely related *Drosophila*, that prezygotic isolation evolves more rapidly than postzygotic isolation in sympatric species pairs, but not in allopatric pairs. This is a pattern predicted by the reinforcement hypothesis. After an extensive review of the literature on reproductive character displacement and hybrid zones, Howard (1993) concluded that reproductive character displacement is a common phenomenon in nature as are deviations from random mating expectations in hybrid zones, another prediction of the reinforcement hypothesis. He attributed the widespread impression that reproductive character displacement is rare to an over-reliance on a single study, that of Walker (1974). Coyne & Orr (1989) and Howard (1993) noted that it is difficult to infer process from pattern and called for further studies to clarify the role of reinforcement in individual cases of mate recognition divergence in sympatric species pairs.

Although both sets of authors were careful to point out that the patterns they reported could be explained by processes other than reinforcement, one is left with the nagging feeling that there is something wrong with the picture before us. Broad patterns suggest that reinforcement occurs, yet theory indicates that the conditions necessary for reinforcement are very restrictive (Templeton 1981; Barton & Hewitt 1985). In this paper, we will try to bring the picture into sharper focus not by going over the arguments that are trotted out for and against reinforcement by everyone, including one of us (Howard 1993), but by establishing some common ground and then applying lessons learned through in-depth studies of the *Allonemobius fasciatus*–

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A. socius hybrid zone. We will finish with a description of recent work on gamete recognition loci and an appeal for much more intensive study of post-insemination signalling systems.

2. COMMON GROUND

By common ground we mean the conditions necessary for reinforcement agreed to by both sides in the debate over the importance of the process. An area of little argument is the need for strong selection against hybridization. The hallmark of successful selection for reproductive isolation in the laboratory has been intense selection against hybrids (e.g. Koopman 1950; Knight *et al.* 1956; Paterniani 1969). Various models and simulations also indicate that for reinforcement to work selection against hybrids must be quite strong (Crosby 1970; Sawyer & Hartl 1981), or weaker selection must be combined with non-random mating (Felsenstein 1981).

Another area of agreement is the need for some initial positive assortment between the two interacting taxa (Howard 1993). We know of no biologist arguing that reproductive isolation will crystallize out of a hybrid swarm. Other factors generally agreed to enhance the possibility of reinforcement are: independent regulation of the sizes of the two populations in areas of sympatry (Maynard Smith 1966; Rand & Harrison 1989); broad zones of interaction which diminish the influence of parental population gene flow and bolster the amount of genetic variation available (Barton & Hewitt 1981: Howard 1986), and control of premating isolation by one or a few genes (Maynard Smith 1966; W. Moore 1981).

The need for strong selection against hybridization is perhaps the most troublesome of the above conditions for the hypothesis of reinforcement. The necessary selection pressures often do not appear to exist. For example, Coyne & Orr (1989) pointed out that in their comparative study only five of the 12 closely related, sympatric pairs of *Drosophila* with strong prezygotic isolation showed any postzygotic isolation in the laboratory, casting doubt on reinforcement as an explanation for the prezygotic isolation. However, fundamental problems may exist with the way laboratory hybridization studies are typically carried out, problems that we will illustrate with our work on the ground crickets *Allonemobius fasciatus* and *A. socius*.

3. A CASE STUDY

The sister species, *A. fasciatus* and *A. socius* are small, ground-inhabiting crickets in the subfamily Nemobiinae. The former species occurs in the northeastern and north-central United States; the latter is found in the southeastern and south-central United States (Howard 1986; Howard & Waring 1991). They meet and hybridize along an extensive, mosaic contact zone stretching from New Jersey at least as far west as Illinois. One of the most interesting aspects of the hybrid zone is the lack of hybrids. Pure species individuals are abundant in all mixed populations, predominating in most (Howard & Waring 1991). Most individuals classified as hybrid are not highly intermediate in genotype, rather they possess genotypes more characteristic of backcrosses (Howard & Waring 1991). Thus, successful hybridization between pure species individuals appears to be a relatively rare event in most mixed populations throughout the zone. Howard (1986) suggested that slight male calling song differences may isolate the two taxa, and Benedix & Howard (1991) reported evidence of calling song displacement in some mixed populations.

The intimation that isolating barriers may be stronger inside than outside the zone led to studies of hybrid fitness. We wanted to know whether selection against hybridization could be driving the evolution of stronger reproductive isolation within the zone of contact.

Our first approach to the problem was a cohort analysis (Howard *et al.* 1993). In cohort analysis, the same populations are sampled several times during the course of the life cycle and the proportion of hybrids in each sample is measured. This sampling scheme permits monitoring of the proportion of hybrids in a population over time. If hybrids are not selected against, their frequency in the population would remain constant. A selective elimination of hybrids would be reflected in significant differences among the proportions of hybrids in younger and older age classes from the same cohort (Dowling & Moore 1985; Kocher & Sage 1986).

Five mixed populations from the hybrid zone were each sampled three times from June 1987 (early instar crickets) to August 1987 (adult crickets). Individuals were assigned to one of three categories based on their genotypes at four protein loci: *A. socius* (individuals harboring only alleles unique to *A. socius*), hybrid (individuals with alleles unique to both species), and *A. fasciatus* (individuals harboring only alleles unique to *A. fasciatus*). The results were fairly consistent. The relative survival of *A. socius* during the summer of 1987 was significantly greater than the relative survival of members of the other two groups in four of the five populations. The relative viability of *A. fasciatus* was significantly greater than that of hybrids in one population, did not differ significantly from that of hybrids in two populations, and was less than that of hybrids in two populations (see Howard *et al.* (1993) for details).

Based on the cohort analysis, individuals of *A. socius* appear to be under strong selection pressure to avoid mating with *A. fasciatus*. The reverse is not always true. Paradoxically, it was males of *A. fasciatus* that were reported to display reproductive character displacement in calling songs within mixed populations from the hybrid zone (Benedix & Howard 1991). Evidently, hybrid inviability from hatching to adulthood will not account for the high proportion of pure species individuals in most mixed populations, nor will it account for the selection pressures that may have led to the displacement of song in *A. fasciatus*.

Although cohort analysis is powerful, it does have its limitations. In particular, it does not detect differential mortality at the embryonic stage of develop-

Table 1. *Mean number of offspring produced by crosses when the number of matings was not controlled*

cross (male × female)	fraction of crosses producing offspring	mean number of offspring per cross
A. fasciatus × *A. fasciatus*	28/30	56.53
A. socius × *A. fasciatus*	53/62	32.76
A. socius × *A. socius*	25/28	100.11
A. fasciatus × *A. socius*	50/60	62.00

Table 2. *Patterns of fertilization when females were allowed to mate once with two males*

female male male	number of females mated	mean proportion of offspring fathered by first male	mean proportion of offspring fathered by second male
two conspecific males			
f[a] × f × f	20	0.364	0.637
s[b] × s × s	12	0.518	0.482
conspecific male followed by heterospecific male			
f × f × s	8	0.981	0.019
s × s × f	7	0.996	0.004
heterospecific male followed by conspecific male			
f × s × f	11	0.000	1.000
s × f × s	7	0.046	0.954

[a] f = *A. fasciatus*
[b] s = *A. socius*

ment. To look at this aspect of hybrid fitness, we moved into the laboratory.

Laboratory crosses were set up with crickets collected from four localities (two *A. fasciatus* sites, two *A. socius* sites) outside or within the zone of contact. The parental crosses consisted of all possible mating combinations among the four field collected populations, a total of 16 different cross classes. In setting up the crosses we followed a procedure that is standard in animal hybridization studies, we placed individual males and females together for an extended period of time (in this case four days), which allowed ample opportunity for multiple matings (Howard, personal observation).

Table 1 shows the mean number of offspring produced by heterospecific crosses and conspecific interpopulation crosses. *Allonemobius fasciatus* females mated to a heterospecific male produced 58% the number of offspring yielded by *A. fasciatus* females mated to a conspecific male. *Allonemobius socius* females mated to a heterospecific male produced 62% the number of offspring generated by *A. socius* females mated to a conspecific male. The F_1 hybrids developed normally in the laboratory and were fertile. Furthermore, they produced about the same number of offspring as F_1 conspecific crosses, whether involved in hybrid or backcross matings (Gregory & Howard 1993).

We found the laboratory hybridization results puzzling. Heterospecific matings were successful, produced many offspring, and the offspring were fertile. At the same time, preliminary phonotaxis experiments by Doherty suggested that females of *A. fasciatus* and *A. socius* from some mixed populations were not preferentially attracted to conspecific songs, at least in the laboratory. Yet, in virtually all mixed populations the two species remained distinct (Howard & Waring 1991). What could account for the strong reproductive isolation between the taxa and why were the barriers not breaking down in mixed populations? Other work had eliminated the possibility that habitat and phenological differences isolate *A. fasciatus* and *A. socius* (Howard *et al.* 1993).

Answers to these questions emerged from a surprising source: sperm competition experiments (Gregory & Howard 1993). In the experiments, an individual female of *A. fasciatus* or *A. socius* was mated once to each of two males: two conspecifics, a conspecific followed by a heterospecific, a heterospecific followed by a conspecific, or two heterospecifics. Matings were 24 hours apart. Males mated to the same female were diagnostically different at one or more protein loci. By electrophoretic analysis of offspring, we could unambiguously identify the male parent and thereby document patterns of fertilization.

The results are summarized in table 2. When females were mated to two conspecifics, the sperm of both males fertilized eggs. The pattern of fertilization was quite different when one of the mating partners was a heterospecific male. Regardless of the order of matings and regardless of whether the female was from a pure population or a mixed population, heterospecific sperm rarely fertilized eggs. The results were especially compelling for females of *A. fasciatus*. Of 19 trials, the heterospecific male fathered offspring in only two, and even in these two, the heterospecific male accounted for less than 12% of the offspring. In the case of *A. socius* females, the heterospecific male fathered offspring in six of 14 trials, but never accounted for more than 18% of the offspring.

Matings to two heterospecific males provided further insight into fertilization (table 3). Only two of 13 such trials produced any offspring when the female was *A. fasciatus* (mean for all 13 trials = 1.77 offspring).

Table 3. *Mean number of offspring produced by females mated once to each of two males*

female male male	fraction of crosses producing offspring	mean number of offspring per cross
two conspecific males		
f[a] × f × f	17/17	33.88
s[b] × s × s	9/9	74.11
two heterospecific males		
f × s × s	2/13	1.77
s × f × f	7/7	51.00

[a] f = *A. fasciatus*
[b] s = *A. socius*

On the other hand, *A. fasciatus* females mated to two conspecifics all produced offspring ($n=17$, mean = 33.88 offspring). Matings to two heterospecific males were more successful when the female was *A. socius*. All seven trials yielded offspring and the mean number of offspring (51.0) was 69% of the mean number of offspring obtained when *A. socius* females were mated to two conspecifics.

Thus, barriers to fertilization exist between *A. fasciatus* and *A. socius*, barriers that appear to depend on the number of spermatophore transfers, at least in the case of *A. socius* males and *A. fasciatus* females. The barriers were not detected in our earlier crossing experiments, in large measure because we did not control the number of matings, which allowed males to inseminate females repeatedly and overcome the barriers. Barriers to fertilization of the sort reported here would rarely be perceived in animal hybridization studies because, typically, numbers of matings are not controlled and sperm competition experiments are not performed.

4. FERTILIZATION BARRIERS AND REINFORCEMENT

Barriers to fertilization should lead to positive assortment even between taxa that mate at random. But will such barriers establish a selection pressure for the evolution of trait differences that isolate taxa earlier in the mating sequence? The answer to this question depends on the impact of the fertility barriers on the fecundity of individuals that participate in heterospecific matings. If mating activities are not risky and males and females mate repeatedly, females are likely to mate with at least one male capable of fertilizing her eggs and heterospecific matings may not severely diminish the fitness of females or males. Under these conditions, reinforcing selection will be weak or nonexistent (A. R. Templeton, unpublished results). On the other hand, if a successful mating entails great risk, is costly in terms of energy expenditure, or does not occur frequently for intrinsic reasons, a female mating with a male that will not fertilize her eggs, or a male mating with a female who will not utilize his sperm, may suffer a significant loss of fecundity. Mating activities, in particular sexual signalling by males and mate searching by females, frequently enhance the risk of predation and/or engender considerable energetic costs (MacNally & Young 1981; Burk 1982; Robinson & Doyle 1985; Ryan 1985). Thus, fertilization barriers may often promote the evolution of premating isolation.

In the case of *A. fasciatus* and *A. socius*, we lack the data necessary to evaluate the strength of reinforcing selection, but we suspect that it is quite high, at least for males. A male who mates with a heterospecific has engaged in a relatively lengthy sequence of calling, courtship, nuptial feeding, and spermatophore transfer to inseminate a female who will not use his sperm if she mates with a conspecific. Electrophoretic analysis of the offspring of field-collected adult females demonstrates that females typically mate with more than one male in nature (P. G. Gregory & D. J. Howard, unpublished data).

5. MOLECULAR SIGNALLING AND FERTILIZATION

After insemination, successful fertilization depends on a complex sequence of interactions between male gametes and female gametes. Garbers (1989) recently categorized the interactions in the following way: specific activation of sperm motility, attraction of spermatozoa to the egg, adhesion of sperm cells to the egg, induction of an acrosome reaction, membrane fusion between the gametes, and subsequent egg activation. As Garbers points out, current evidence suggests that there is some species-specificity in all of these interactions, although the barriers are often not absolute and can be overcome by higher concentrations of an effector molecule. In organisms with internal fertilization, successful fertilization also depends on sperm transport to the region of the egg.

In many marine animals, such as corals, bivalves, and echinoderms, males and females do not interact behaviourally prior to gamete release. In these taxa, interactions between gametes are the most important components of successful fertilization and reproductive isolation is typically due to the failure of sperm and eggs from different species to bind and fuse (Palumbi 1992). One might expect the evolution of reproductive isolation to be a slow process in such animals, not only because of the simplicity of their spawning behaviour, but because their planktonic eggs and larvae are capable of travelling great distances (Mayr 1963).

Recent work with sea urchins and abalones suggests otherwise. Palumbi & Metz (1991) reported that in the sea urchin *Echinometra mathaei* (an array of morphological types), strong reproductive isolation can evolve with only slight genetic differentiation. The reproductive isolation occurs, at least in part, because attachment and fusion of eggs and sperm is greatly reduced between different types of *E. mathaei*. Palumbi & Metz (1991) suspect that the cause of the attachment failure is rapid divergence of the sperm protein, bindin, among types. Bindin, a molecule on the surface of the sperm acrosomal process, is the major sperm surface protein mediating attachment and fusion to the egg. The middle third of this molecule appears to be highly conserved among sea urchin species, but the flanking sequences have repeated sequences that vary in number between species as well as short areas of species-specific sequence (Minor *et al.* 1991). A peptide derived from a unique region of *Strongylocentrotus franciscanus* bindin inhibited fertilization in a species-specific manner (Minor *et al.* 1991) supporting the idea that the variable region of the molecule is the binding domain.

Lysin is a protein in the acrosome granule of the sperm of abalone, that recognizes and dissolves the egg vitelline envelope. In the first extensive comparison of a gamete recognition protein in congeneric species, Lee & Vacquier (1992) found that lysin has a hypervariable, species-specific domain, between amino acid positions 2 and 12. Current evidence suggests that these are the positions involved in the binding of the vitelline envelope ligand. As surprising as the hypervariability, was the finding that in the vast majority of pairwise comparisons of the seven DNA sequences available, non-synonymous base pair substitutions (amino acid altering) outnumbered synonymous substitutions (silent), suggesting that natural selection promotes the divergence of lysin sequences in abalone (Lee & Vacquier 1992). Thus, accumulating evidence indicates that gamete recognition loci diverge rapidly in marine organisms.

The potential for rapid evolution of gamete interactions or interactions between sperm and the female reproductive tract exists in terrestrial organisms that undergo internal fertilization, but such signalling systems have received little attention from evolutionists. Instead we have concentrated on animal behaviour, presuming that animal behaviour evolves more quickly than post-insemination signalling systems. This presumption has been supported by a lesson evolutionists learn early in their training; namely, that many taxa that do not hybridize in nature will mate and produce fertile hybrids in the laboratory. Traditionally, this result is attributed to the breakdown of behavioural differences under artificial conditions and is taken to indicate that barriers to fertilization often do not exist between closely related taxa. However, the repeated matings typical of laboratory hybridization studies will overcome concentration dependent barriers to fertilization. So very few laboratory hybridization studies provide information about the presence or absence of fertilization barriers. The results of the work on *A. fasciatus* and *A. socius* as well as recent work on the grasshoppers *Podisma pedestris* (Hewitt *et al.* 1989) and *Chorthippus parallelus* (Bella *et al.* 1992), in which numbers of matings were controlled and sperm utilization patterns were monitored, indicate that interactions between sperm and the female reproductive tract or sperm and eggs can evolve as quickly as behavioural interactions. These post-insemination interactions are worthy of much greater scrutiny by evolutionists.

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