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The Evolution of Cooperation and Conflict, Experimental Model Systems and Theory.

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The Evolution of Cooperation and Conflict,

Experimental Model Systems and Theory.

by

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Dedication

For my father, Martin Gordon Sachs, who passed away midway through my Ph. D. Although he was not a scientist by vocation, he had an abiding interest in science and he shared that with me from a very early age. Thanks, Dad.

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The Evolution of Cooperation and Conflict,
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I present three different studies in three chapters. In chapter 1, I describe a general theoretical framework for the evolution of cooperation both within and between species. Three general models are distinguished by which cooperation can evolve and be maintained: (i) directed reciprocation—cooperation with individuals who give in return; (ii) shared genes—cooperation with relatives; and (iii) byproduct benefits—cooperation as an incidental consequence of selfish action.

In chapter 2, I investigate the origins of cooperation at the genotypic and phenotypic levels. While theory and empirical work enlighten the maintenance of cooperation, few studies explore its origins. Here, I examine the origins of cooperation by experimentally evolving two antagonistic bacteriophages. I experimentally enforced the two bacteriophages, f1 and IKe, to undergo fifty iterated cycles of co-infection, paired vertical transmission, and infectious transmission in *Escherichia coli* cells. Phenotypic and genomic analysis then characterized the outcome. Strikingly, the two bacteriophages evolved to co-

package their genomes into one symbiotic unit, ensuring co-transmission during the infectious stage. Furthermore, one bacteriophage evolved a minimal genome with the *inability* to infect cells independently, becoming an obligate viral symbiont. These results parallel a wide variety of natural systems: evolution of reduced genomes, co-transmission of partners, and obligate coexistence between cooperating species.

In chapter 3, I examine a puzzling example of cooperation between species, the symbiotic interaction that occurs in corals, hydras, and jellyfish and their dinoflagellate algae. These algae are mostly acquired infectiously, and according to models of virulence evolution should be selected to exploit the host. However, symbiont cheating is virtually unknown. I experimentally manipulated transmission mode of algal symbionts in jellyfish hosts to determine if altering symbiont transmission mode selects for cheating within symbiont populations. Cheating symbionts evolved under experimentally enforced horizontal transmission. Fitness estimates revealed that cheater algae had faster within-host growth, higher dispersal rates, and caused lower host growth compared to algae which underwent repeated vertical transmission. A trade-off was detected between harm caused to hosts and symbiont fitness. Such trade-offs have been modeled for pathogen evolution and may be critical in stabilizing 'infectious' symbioses.

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CHAPTER I:

The evolution of cooperation

CHAPTER ABSTRACT

Darwin recognized that natural selection could not favor a trait in one species solely for the benefit of another species. The modern, selfish-gene view of the world suggests that cooperation between individuals, whether of the same species or different species, should be especially vulnerable to the evolution of noncooperators. Yet, cooperation is prevalent in nature both within and between species. What special circumstances or mechanisms thus favor cooperation? Currently, evolutionary biology offers a set of disparate explanations, and a general framework for this breadth of models has not emerged. Here, we offer a tripartite structure that links previously disconnected views of cooperation. We distinguish three general models by which cooperation can evolve and be maintained: (i) directed reciprocation— cooperation with individuals who give in return; (ii) shared genes—cooperation with relatives (e.g., kin selection); and (iii) byproduct benefits—cooperation as an incidental consequence of selfish action. Each general model is further subdivided. Several renowned examples of cooperation that have lacked explanation until recently—plant-rhizobium symbioses and bacteria-squid light organs—fit squarely within this framework. Natural systems of cooperation often involve more than one model, and a fruitful direction for future research is to understand how these models interact to maintain cooperation in the long term.

INTRODUCTION

A few key studies in the 1960s led to a radical change in the way biologists viewed the evolution of cooperative interactions. Challenging the nearly pervasive and casual attitude of most biologists that held that interactions evolve for the good of the species, Hamilton (1964a,b) and Williams (1966) explained how natural

selection was intrinsically selfish, and that cooperative acts were likely to evolve only under restrictive conditions. This shift in paradigm then set the stage for a major focus on the evolution of cooperation in the last two decades. The evolution of cooperation contrasts with the evolution of traits that directly and solely benefit the individual possessing them, such as sharp teeth, visual acuity, or crypsis. Cooperation by definition involves an interaction between individuals that benefits the recipient but not necessarily the donor. At face value, therefore, cooperation presents an evolutionary dilemma, one that underlies a famous quote of Darwin (1859): "Natural selection cannot possibly produce any modification in any one species exclusively for the good of another species" (p 228). Darwin realized that the bearers of a trait must themselves benefit if the trait is to be favored under natural selection. The modern version of Darwin's criterion is that the genes underlying a cooperative trait must themselves benefit disproportionately if they are to increase in frequency. How then do cooperative traits overcome this evolutionary hurdle?

Hamilton (1964a, 1964b) proposed one solution that can operate within species: the genes for cooperation tend to benefit copies of themselves in others, and thus are favored by a process of "kin selection" (Maynard Smith 1964). While kin selection has provided the conceptual framework for understanding cooperation between relatives, a vast number of cooperative traits are not explained by Hamilton's solution (cooperation between species, for example). Today, a pluralistic approach has emerged, in which multiple models attempt to explain various idiosyncratic examples of selfless behavior. The full account of models for cooperation includes not only kin selection, but the iterated prisoner's dilemma of reciprocal altruism (Trivers 1971; Axelrod and Hamilton 1981), synergism (Queller 1985), indirect reciprocity (Alexander 1987; Nowak and Sigmund 1998), partner choice (Eshel and Cavalli- Sforza 1982; Noë 1990; Bull and Rice 1991), policing (Frank 1995, 2003), pseudoreciprocity and parceling (Connor 1995b), to name a

few. However, this panoply of models does not offer obvious themes that underlie our modern understanding of the evolution of cooperation.

This paper offers a hierarchical framework in which the principal models of cooperative evolution are readily compared, and in which known examples can be accommodated. Alternative frameworks have been proposed: inclusive fitness theory (Queller 1985), trait group selection (Wilson 1975; Wilson and Dugatkin 1997), and repression of competition/policing (Frank 1995, 2003). We think that the framework offered here is the most comprehensive and provides a more natural accommodation of the diverse biological examples of cooperation. We suggest that multiple frameworks are desirable, however, and are motivated by the belief that enlightenment emerges out of the contrast between different conceptual frameworks.

The structure for this paper is as follows. We define cooperation and then proceed to explain our framework using traditional examples and models. Since none of the models presented are original to us, it is the organization of models that distinguishes this paper from others. Next, we apply this framework to diverse biological systems, ranging from well-studied and well-understood examples to cases that are complex and enigmatic. Finally, we suggest avenues of further study.

What is Cooperation?

All cooperation involves acts by one individual (X) that benefit one or more other individuals (Y). Beyond this deceptively simple core, there is an intricacy that complicates attempts to unite different models under a single approach. The greatest focus in the field has been on "costly" acts by individual X that benefit Y, because the challenge in such cases is to understand how natural selection can tolerate the maintenance of acts by X that potentially lower X's fitness. More recently, however, the field has included cases in which acts by individual X benefit both X and Y. These cases, known as byproducts, can be understood by

relatively straightforward selective mechanisms.

Cooperation is usually considered a two-way interaction, such as a mutualism or symbiosis. For the sake of deconstructing the evolution of cooperation, we adopt a one-sided perspective that considers the costs and benefits accruing to one partner at a time. This one-sided perspective is essential for addressing the evolution of cooperation between species, because the evolutionary process leading to and maintaining cooperation is operating separately in each species. The critical mechanisms stabilizing cooperation can be different in two interacting species.

This one-sided perspective also expands the realm of examples that are relevant to the evolution of cooperation to include exploitation and parasitism. This generalization can be realized by considering a one-dimensional continuum of possible actions of individual X on individual Y, with cooperation at the left end and antagonistic/exploitative interactions at the right. Evolution in X that shifts its location on the continuum toward the left has, by definition, evolved to be more cooperative, regardless of where it sits on that continuum. Thus, the framework applies beyond interactions that are strictly cooperative.

THE FRAMEWORK

Our classification divides types of cooperation into three general models: i) *directed reciprocation*—cooperation with individuals that return benefits; ii) *shared genes*—cooperation with relatives (e.g., kin selection); and iii) *byproduct benefits*—cooperation with others as a coincident of selfish actions (Table 1). Each general model can be further subdivided. Directed reciprocation is divided into *partner choice* (benefits are returned by specifically chosen partners) and *partner fidelity feedback* (benefits are returned by partners that are coupled in fitness). Shared genes is divided into *kin choice* (cooperation with relatives based on phenotypic recognition of those relatives) and *kin fidelity* (cooperation with

relatives based on a social context of spatial association). Finally, byproducts is divided into: *one-way byproducts*—one individual receives incidental benefits from another individual; *two-way byproducts*—two or more individuals receive incidental benefits from each other; and *byproduct reciprocity*—where an individual maximizes incidental benefits it receives from another by actively helping that individual.

Each of these models have been proposed before (Hamilton 1964a, 1964b; Eshel and Cavalli-Sforza 1982; Queller 1985; Bull and Rice 1991; Noë and Hammerstein 1994, 1995; Connor 1995a, 1995b; Frank 1995, 2003; Noë 2001; Wilkinson and Sherratt 2001; Simms and Taylor 2002), but without the overall structure presented here. We attempt to provide a comprehensive hierarchy of models in which each model invokes the fewest assumptions required to evolve (or maintain) cooperation and does not invoke any of the other models. This emphasis on "minimal" models stands in contrast to some other approaches. In particular, Noë (whose approach shares similarities with ours) has developed a framework around "biological markets" (Noë and Hammerstein 1994, 1995; Noë 2001). Markets combine several models present in our framework and thus can be dissected with our approach. Frank (1995, 2003) has developed a framework around policing (repression of competition), which we dissect and reclassify under several models below. Likewise, as we will explain below, the collection of examples that have been lumped under the well-known "iterated prisoner's dilemma" model are subdivided here into partner choice and partner fidelity feedback. We first introduce the models for the evolution of cooperation, then apply that framework to specific examples of cooperation to identify the mechanisms driving cooperation in each case.

Directed Reciprocation

In directed reciprocation, an individual accepts a cost to benefit a specific

partner, and the partner in turn compensates or reciprocates that benefit back to the donating individual (hence the reciprocation is "directed" to the partner). To anticipate the other models in our framework, directed reciprocation is distinguished from shared genes because it can operate between nonrelatives and between species, and it is distinguished from byproduct benefits because the cooperative traits are potentially costly, not directly benefiting the individual expressing them.

Of these three classes of models, directed reciprocation best epitomizes the Darwinian dilemma, because most examples involve adaptations in one species that benefit another species. Furthermore, the mere fact that directed reciprocation has been established does not ensure its persistence—these systems are potentially vulnerable to exploitation, in which an individual receives the benefit from its partner and then enjoys a further benefit by not reciprocating (also known as "cheating"). Models that account for the evolution of directed reciprocation must thus account for the stability of cooperation against cheating.

The iterated prisoner's dilemma (IPD) game is the most celebrated model of directed reciprocation. The mechanism driving the evolution of cooperation under this model was first explained by Trivers (1971) but was later developed by Axelrod and Hamilton (1981). This model has two main requirements: (i) an extended series of interactions within a pair of individuals, and (ii) the ability of each individual to vary its behavior in each interaction according to a partner's previous action. The conclusion from the Axelrod-Hamilton paper was that the simple strategy of "tit-for-tat" evolved under a wide range of conditions if the likelihood of future interactions between the same partners was high. (The tit-for-tat strategy is the rule of "cooperate when your partner has cooperated in the previous iteration but refuse to cooperate if your partner did not cooperate in the previous iteration.") The Axelrod-Hamilton paper inspired a surge of theoretical and empirical studies on cooperation, mostly supporting the generality of the

original conclusions.

Many empirical examples of cooperation were initially interpreted as fitting this model, including intracellular symbionts, parasite virulence (Axelrod and Hamilton 1981), the cooperative behavior of fish (Dugatkin 1997), and even dynamics of WWI trench warfare (Trivers 1985). While there is no question that the IPD model describes conditions that can favor cooperation, there are few examples that are now thought to adequately satisfy its assumptions. Many between-species examples clearly do not fit, such as two-species cooperative systems that lack long-term interactions between the same partners.

The IPD has two main components: repeated interactions of partners and the ability of interactants to alter their behavior in response to the other's action. As has been realized previously, either component alone can drive the evolution of cooperation. Our framework for directed reciprocation thus separates these two components of the IPD into *partner fidelity feedback* (involving repeated interactions between partners) and *partner choice* (differential response to partners). Connor (1995b) has also partitioned and reclassified examples formerly interpreted as IPD, but along different lines than ours.

Partner Fidelity Feedback

Two partners (X, Y) are associated for an extended series of either discrete or continuous exchanges (Figure 1). The association lasts long enough that a feedback operates: changes to the fitness of individual Y affect the fitness of its partner X. Thus, by failing to cooperate, individual X ultimately curtails its own fitness because its partner's fitness loss feeds back as a fitness loss to X (merely because its partner cannot provide as much benefit). This feedback is automatic and, unlike tit-for-tat, does not require recognition or conditional response. Biological examples to be described below include vertically transmitted symbionts, commensals and parasites (e.g., mitochondria), and ant-acacia

symbioses.

It has also been suggested on theoretical grounds that partner fidelity feedback operates in viscous populations, where spatial structure enforces the long-term association of different lineages living in proximity to each other (Nowak and May 1992; Frank 1994; Doebeli and Knowlton 1998). However, theoretical work in the field of kin-selected cooperation has noted that competition between neighbors may override selection for cooperation (Taylor and Wilson 1988; West et al. 2001, 2002a). This latter work, however, studies competition between neighbors of the same species, and competition may be weaker when cooperative partners are of different species.

Partner fidelity feedback is not merely an extended series of contests. Rather it is *a coupling of fitness* between two individuals through repeated interactions (Bull and Rice 1991); the fitness effects may extend across generations, as in vertically transmitted symbionts, or not, as is described below for ant acacia symbioses. The stability of partner fidelity feedback is strongly dependent upon the strength of fitness feedback between partners. In theory, fitness feedback is strongest under uniparental vertical transmission of symbionts, and this is the application of the model for which there is the greatest empirical support (Axelrod and Hamilton 1981). However, a number of other factors can facilitate strong fitness feedback (fitness coupling) between partners. Factors that limit the dispersal abilities of partners, including high population viscosity, are frequently discussed mechanisms to facilitate cooperation under partner fidelity feedback (Nowak and May 1992; Doebeli and Knowlton 1998; but see Taylor and Wilson 1988; West et al. 2001, 2002a). Partner fidelity feedback is also facilitated if small short-term fitness gains by cheating result in a large fitness loss to the partner. Thus, the negative effect of cheating on partner fitness increases disproportionately with the degree of exploitation. This is a common assumption in models of virulence evolution (discussed below).

Partner fidelity feedback differs from the explicit IPD in two basic ways. First, under partner fidelity feedback, interaction between partners involves automatic fitness feedback. Under the rules of the IPD, a cheater paired with a cooperator achieves the highest fitness attainable. In partner fidelity feedback a cheater's fitness declines by failing to maintain its cooperative partner's fitness. Second, in partner fidelity feedback no choice of partners is required to stabilize cooperation, and cheaters fail to receive benefits solely because of the automatic fitness feedback. The IPD, however, leads to maintenance of cooperation only with a conditional strategy for reciprocation (e.g., tit for- tat), which involves an element of partner choice manifested as termination of the cooperation.

Partner Choice

An individual X interacts with and rewards a specific cooperative partner Y and avoids rewarding less cooperative partners (Figure 2). By choosing a cooperative partner Y, individual X not only enhances its own fitness but it promotes the evolution of cooperation in species Y. This latter effect occurs because X selectively benefits cooperative individuals of Y through its cooperation. (However, it should be emphasized that X is selected to choose a cooperative partner only because of the immediate benefit to itself and not through the effect it has on Y's fitness.) Choice may take several forms, ranging from establishing cooperation with only one of several potential partners, to altering the duration of cooperation with a partner according to its actions, to actually reducing the fitness of selfish partners. Thus, partner choice differs from the IPD in one critical respect: partners need not interact repeatedly for cooperation to be maintained by partner choice. Cooperation can evolve by partner choice even if individuals interact only once.

Partner choice is easy to contemplate as a mechanism for the evolution of cooperation, but several quantitative factors determine whether it is sufficient to

maintain cooperation (Bull and Rice 1991; Noë and Hammerstein 1994; Batali and Kitcher 1995; Noë 2001). For example, there is an inherent density dependence in which choices become more limited when few partners are available than when many are available (Noë and Hammerstein 1994; Noë 2001). That is, the costs of rejecting a potential partner are lower if many alternative partners are available.

Partner choice involves both an *assessment* of how cooperative a partner is and a *decision rule* about whether to accept exchange with that partner (and how much to exchange). Decision rules may be relative, "accept the most cooperative individuals," or absolute "accept any partner above some value" (West et al. 2002b). Decision rules may be behavioral, as with partner choice in baboons (Noë 1990), or chemical, as is in the yucca-yucca moth symbiosis discussed below. "Tit-for-tat," for example, is a type of decision rule commonly modeled in the IPD.

Decision rules are often coupled with an assessment system. The assessment system is the biological arena in which one or more potential partners are observed for their cooperative tendencies, such that their level of cooperation in further interactions can be predicted. While a decision rule is the basis by which an individual chooses a partner, the assessment system allows an individual to gain information about which partners are cooperative and how cooperative they are. Three different assessment systems have been described in the empirical literature: parceling, distributing, and image scoring. In *parceling,* a single resource is presented to a partner incrementally, over time (Connor 1995b). A simple example of parceling involves grooming in impalas (Connor 1995b) in which individuals exchange short bouts of grooming in pairs. A cheating individual, in this case a nongrooming impala, can at best exploit a single grooming bout before its selfish tendencies are revealed. The choosing partner then avoids spending time grooming the selfish partner. Parceling is an integral part of the classic IPD model—it represents the iterations.

Yucca plants use a *distributing* assessment system to evaluate the

cooperative tendencies of their obligate pollinating moths. Distributing is a spatial portioning of the resource. Moths oviposit into the ovary before pollinating the flower, and the developing seeds are used as food by the larvae. Uncooperative moths lay more than the average number of eggs per flower, thus lowering plant fitness. Yucca assessment is thought to depend on the number of larvae within each ovary. In contrast to parceling, there is no sequential exchange of benefits over time: if the plant aborts the flower, then both the plant and the moth lose all offspring from that flower (Pellmyr and Huth 1994; Huth and Pellmyr 2000). In contrast to parceling, therefore, distributing divides a resource into spatial allotments rather than temporal allotments. Distributing further differs from parceling in that the decision rule may act separately in each allotment (e.g., ovary). While a decision in parceling is made one partner at a time, decisions in distributing can be made simultaneously upon many partners.

A third type of assessment system, *image scoring* (Nowak and Sigmund 1998), exists in reef fish that choose among "cleaner fish" (Bshary 2002). Some species of reef fish, termed clients, benefit from cleaner fish that remove and eat their mouth parasites or dead infected tissue (Grutter 1999). However, the cleaner may cheat the client by biting healthy tissue off the client instead of, or in addition to, the removal of parasites (Bshary and Grutter 2002). Some species of client fish choose cleaners after observing the cleaner's behavior with a previous client; clients choose cleaners that they observe to be cooperative (Bshary 2002). This form of cooperation has been termed image scoring (Nowak and Sigmund 1998; Riolo et al. 2001) or more generally "indirect reciprocity" (Alexander 1987); a benevolent act by X to Y increases the chance that X receives benefit from others.

A History of Partner Choice

Partner choice is clearly a widespread evolutionary mechanism for cooperation, yet it is neglected in many reviews. This omission seems to stem from

a widespread emphasis on the IPD as the model for the evolution of cooperation between unrelated individuals. Historically, however, partner choice underlies Darwin's contemplation on the evolution of nectaries in flowers (1859:139, see below), Dawkins's model of female choice of males differing in levels of paternal care of the brood (1976), the fig-wasp model described by Axelrod and Hamilton (1981), Eshel and Cavalli-Sforza's (1982) model of assortment of encounters, and Bell's model for the evolution of empty flowers (1986). Nonetheless, both Dawkins (1989) and Axelrod and Hamilton attempted to use the IPD to explain the evolution of cooperation where repeated interactions were absent, and Bull and Rice (1991) included one model involving partner choice under partner fidelity (feedback), illustrating the common difficulty and confusion over these mechanisms.

Noë (1990) proposed that certain types of choice-based games in baboons constituted an alternative to the IPD model as a way to maintain cooperation. Bull and Rice (1991) proposed the two basic models we recognize here, partner choice and partner fidelity (feedback). Noë and Hammerstein and their collaborators have since elaborated variations of partner choice and illustrated that the efficacy of choice increases with the number of partners in a density dependent manner (Noë and Hammerstein 1994, 1995; Noë 2001).

We view some group-level selection models of *active* assortative interactions as partner choice models (Eshel and Cavalli-Sforza 1982; Peck 1993; Wilson and Dugatkin 1997). Choice of partners by individuals can drive the assortative interactions of cooperators. Assorted interaction then leads to between group variance, and thus could allow selection to favor some groups over others. These models may be particularly predictive for within-species cooperation, though more empirical work is needed to test the importance of partner choice within species.

Shared genes

Cooperation by shared genes occurs when one individual benefits another individual with which it shares alleles through descent from a common ancestor. By definition, this mechanism operates only when the partners are members of the same species. A shared genes model for the evolution of cooperation (altruism) was first proposed and developed quantitatively in the classic papers of Hamilton (1964a, 1964b). In Hamilton's model, interactions occur among relatives, and evolved cooperative acts are directed toward other individuals, depending on the average degrees of relatedness of those individuals (Figure 3). Thus, genes that encode for (shared genes) cooperation tend to benefit copies of themselves in others (Dawkins 1976). Shared genes cooperation differs from all other models considered here in that the cooperative individual need not benefit from its act. This section is included in our review for completeness but is otherwise brief, since this subject already has several excellent reviews (Queller 2000; Alonso and Schuck-Paim 2002; West et al. 2002a).

We recognize two classes of mechanisms by which an individual preferentially gives benefits to others with shared genes: *kin fidelity* and *kin choice*. This distinction highlights the different mechanisms by which cooperative acts are directed to kin and the divergent ways that shared genes cooperation may be vulnerable to cheating. Kin fidelity versus kin choice have been variously described as passive versus active assortment (Eshel and Cavalli-Sforza 1982), spatial association versus kin recognition (Grosberg and Quinn 1986), spatial location mechanism versus phenotype matching (Reeve 1989), and phenotypic versus nonphenotypic kin recognition (Pfennig 1997), though the context of these various terms are not always completely overlapping. This structure is obviously parallel to that of directed reciprocation.

Kin Fidelity

With kin fidelity, benefits are given to relatives based on context-dependent

spatial association, as in offspring sharing a nest (Hamilton 1964a). By definition, no recognition of individuals per se is involved, because the act is performed to benefit individuals nearby. Although kin fidelity originally seemed to be an obvious mechanism for kin selection, it has recently been challenged as a sufficient mechanism to promote cooperation. The main problem is that relatives living in close proximity may also compete for common resources, and competition between close relatives can overwhelm selection for cooperation (Taylor and Wilson 1988; West et al. 2001, 2002a). Despite this problem, kin fidelity may be important in the evolution of cooperation, especially where kin recognition systems are unable to evolve (Crespi 2001).

Perhaps the first kin fidelity hypothesis was Fisher's model (1930) for the evolution of aposematism through the clustering of brightly colored sibling larvae. A predator tasting the first larva would learn to avoid the siblings because of their spatial proximity and similar appearance. This model has since received empirical support: in experiments, two predator species learned to avoid a conspicuous-distasteful species of aphid while continuing to eat a cryptic-tasteful species after limited experience with both (Malcolm 1986). The aposematic aphids live in large familial congregations, so the kin-selected benefits of aposematism are only received by nearby relatives, as in Fisher's model.

Another unambiguous example of kin fidelity is revealed in the experiment of Turner and Chao (1999), in which a bacteriophage evolved lower levels of selfishness when bacteria were infected with phage clone mates than when infected with nonclone mates. The level of kin fidelity is merely the extent that bacteria were coinfected by related phage genotypes versus unrelated genotypes, and the results showed that higher levels of kin fidelity selected higher levels of cooperation.

For many birds that invest significantly in their brood, parents often feed or incubate whichever young are in that parent's nest, even when those young are not

their offspring (as in cuckholdry or experimental crossfostering). This fundamentally involves kin fidelity rather than kin choice. In contrast to the two preceding examples, however, there is a certain level of recognition required: parents recognize their nest even if not their offspring in that nest. Hence this example has also been classified as nonphenotypic recognition (Pfennig 1997).

Kin fidelity is vulnerable to a specific kind of cheating because a nonkin individual can receive kin fidelity benefits simply by being present in the correct context or location, as in the brood parasitic birds just described. Kin fidelity is no doubt important in many contexts: even when proximity leads to competition, kin fidelity may moderate the competitive interactions of relatives.

Kin Choice

Individuals benefit others whose phenotypes indicate shared genes. Kin choice implies (kin) recognition, and the mechanisms of recognition are diverse. There are two dimensions to kin recognition: which phenotypes are used in recognition, and how the discrimination is acquired. The phenotypes include visual or auditory cues as well as odors, pheromones, and other diffusible chemicals (Greenberg 1979; Gamboa et al. 1986, 1996). Most commonly, the recognition is acquired by learning some type of environmental cue (Gamboa et al. 1986; Neff and Sherman 2002), and this learning often has elements of kin fidelity (because the individuals who are learned as kin are neighbors). One example of kin choice that does not involve kin fidelity comes from colonial tunicates that fuse to form colonies. The fusion systems typically exclude nonrelatives from colonies, and this "choice" appears to be based solely on heritable cues (Grosberg and Quinn 1986; Rinkevich and Weissman 1992; Bishop and Sommerfeldt 1999).

Although learned recognition may blur our distinction between kin choice and kin fidelity, it operates in most animals with kin recognition (Pfennig 2002). Learned discrimination often has elements of kin fidelity, because the individuals

learned as kin are those of the same nest or other immediate environment. Kin recognition is often learned simply by exposure, so that an individual's specific phenotype becomes familiar. Experiments involving exposure of naive social wasps to nonkin nests have shown that nonkin can become accepted and that quarantined kin can be forgotten and excluded (Pfennig et al. 1983). In wood frogs, naive individuals kept free of variable environmental cues prefer kin to nonkin, suggesting an intrinsic ability to discriminate. However, this effect is overcome by exposing nonkin groups to similar environmental cues, whence they now recognize each other as kin (Gamboa et al. 1991).

Although recognition can be based on a phenotype that reflects kinship per se (whole genome relatedness), it can also be based on specific genes that are the true targets of selection. A case in point is what has been described as "green beard" selection (Hamilton 1964a). Three properties are required for green-beard selection: (i) a gene which causes a phenotypic effect; (ii) recognition of the phenotype; and (iii) differential behavior by bearers of the gene to those with the phenotype (Hamilton 1964a). In this model, first proposed merely as a hypothetical principle, benefits are directed to individuals who are phenotypically recognized as carrying the cooperative gene(s). The interacting individuals need not be kin per se (may not share whole-genome relatedness), but the recognized phenotype enables the benefits to be bestowed directly on the genes affecting the cooperation. (We include the green-beard model in this section on kin choice for convenience, even though it does not require choice of *kin* per se.) Green-beard mechanisms may operate to specifically reward individuals carrying the proper genes, or to harm individuals that lack those genes, regardless of how many alleles individuals share throughout the rest of the genome. Empirical work matches the predictions of green-beard selection for the fire ant Gp-9 locus (Keller and Ross 1998), M-factors in flour beetles (Beeman et al. 1992), and cell adhesion genes in social amoebae (Queller et al. 2003). As far as we know, all the above examples of green beard are

pure examples of kin choice; they work irrespective of environmental or context dependent cues.

In contrast to kin fidelity, kin choice can be exploited by nonkin that imposter as of mimic relatives (Alexander and Borgia 1978). We are unaware of exploitation of this specific type, but it may well exist. One interesting line of research will be to study specifically how cooperation is stabilized against cheaters in each case, and whether recognition or proximity maintains cooperation between relatives.

Byproduct benefits

Byproduct models have only recently been emphasized in the cooperation literature. They are potentially confusing because they do not obviously qualify as cooperation in the classic sense, but they overlap with cases that clearly do qualify. Byproduct benefits are integral parts of some cooperation systems, and they likely formed the origins of many systems that evolved into more elaborate cooperative interactions. We distinguish three categories.

One-Way Byproduct Benefit: No Evolution of Cooperation

The benefit that Y receives is an automatic consequence of the otherwise selfish act in which individual X does something to benefit itself (West-Eberhard 1975; Brown 1983; Figure 4). For example, the feces from large ungulates are food for dung beetles; vultures and carrion-feeding insects benefit from abandoned lion kills. Following Connor (1995b), there has been no evolution of cooperation per se in these cases. That is, lion behavior has not been evolutionarily modified to benefit vultures or other carrion feeders, and vulture behavior has not evolved to increase the chance of a kill. Whatever evolves in the case of this byproducts model, it is not selected to offer a cooperative act. In byproduct models, there is no potential Darwinian dilemma, because the basic cooperative trait directly benefits its bearer

and only incidentally benefits others.

Two-Way Byproduct Benefits: Byproduct Mutualism

Byproduct benefits can be one sided, in which X performs an act that benefits itself and coincidentally benefits another, Y, but benefits may also go in both directions to give byproduct "mutualisms" (West-Eberhard 1975; Brown 1983; Figure 4). Two-way byproducts can be simple extensions of one-way examples, or can promote group behavior. One general class of byproduct mutualism is *synergism:* actions or coordinated behaviors that are automatically more profitable when performed in groups (Queller 1985), such as flocking, selfish herds (Hamilton 1971), and Mullerian mimicry (Connor 1995a). Synergism has the appealing and simple formulation that group behavior evolves via individual selection whenever benefits increase disproportionately with group size. As a specific example, empirical work on aquatic hemipterans, which congregate in large groups, suggests that per capita predation risk decreases with group size (Foster and Treherne 1981). Thus, an individual joining a group reduces its own per capita predation rate as well as reducing the per capita predation rate of the other group members through simple predator dilution (Foster and Treherne 1981). Everyone benefits, and no special mechanism for "cooperation" need be invoked.

Although controversial in the details, cooperative founding of colonies between unrelated ant queens also fits the requirements of byproduct mutualisms, because grouped queens automatically achieve higher mean (expected) fitness than solitary females (reviewed in Bernasconi and Strassmann 1999). Two-way byproduct cooperation also extends to examples of "helping at the nest" by unrelated individuals. At least 300 species of birds exhibit cooperative breeding, in which some individuals forgo independent nesting to act instead as helpers at a conspecific's nest (Arnold and Owens 1998). In some cooperatively breeding birds, the helpers are unrelated to the individuals they assist in raising young (Cockburn

1998). Recent work on cooperatively breeding warblers showed that unrelated helpers gained significantly more direct fitness benefits via breeding opportunities than through indirect fitness benefits (Richardson et al. 2002). Thus, the benefits that other birds receive from the unrelated helpers is a byproduct of the helpers' pursuit of direct fitness benefits.

Maximizing Byproduct Benefits Without Evolving Cooperation

When byproduct benefits exist, individuals may be selected to increase the benefit they can obtain. Evolution of byproducts may take the form of "harvesting" the byproduct benefits *without* benefiting the partner that produces the benefit. As an imaginative example, dung beetles might evolve to search for large mammals that provide dung, limiting their foraging to the vicinity of these animals. There would be no specific evolution of cooperation —promoting a benefit to another individual—but this evolution may increase the appearance of the cooperation because the "harvesting" individual has undergone evolutionary modification to increase its dependence on the byproduct.

Byproduct Reciprocity: Evolution of Cooperation from Byproducts

When one individual (X) receives automatic byproduct benefits from another individual (Y), natural selection can shape X to maximize these benefits by being cooperative toward Y. The greater cooperation *toward* Y yields greater byproduct benefits *from* Y (pseudoreciprocity: Connor 1986; Figure 5). For example, consider the remarkable case of the greater honeyguide, an African bird that guides humans to beehives for collection of honey (Hoesch 1937; Isack and Reyer 1989). In Africa, humans have foraged for beehives for many thousands of years. As a consequence of diminishing returns during hive destruction, there is generally honey left behind after human foraging (Dean et al. 1990). Upon destruction of the hive by the human, the bird forages on the discarded hive

remnants, and thus receives automatic benefits as a byproduct of the human foragers' selfish act. Presumably to maximize this benefit, the bird has evolved to call the humans and lead them to beehive locations. Although the coevolutionary history of this apparent human-bird mutualism is speculative (Dean et al. 1990), the inescapable conclusion is that this bird behavior evolved to benefit another species because the bird is incapable of attacking an intact hive by itself. The behavior of the other species in turn benefits the bird. No special mechanisms are needed to prevent exploitation of bird behavior, because the cooperator (human) automatically returns the benefit by unavoidably leaving scraps of the hive behind.

Why is byproduct reciprocity not prone to exploitation? For all examples of byproduct reciprocity known to us, the underlying feature is a common resource not totally monopolizable by either party: each interactant is assured adequate benefits. For example, the honeyguide requires only a small fraction of the hive, which is unavoidably left over during dismemberment of the hive by humans; honeyguiding behavior would presumably not have evolved if humans harvested entire hives without leaving scraps. A parallel case is food sharing in social cliff swallows, which alert conspecifics when insect swarms are found. Efficient group tracking of swarms can benefit the caller through increased foraging (Brown et al. 1991).

EMPIRICAL EXAMPLES OF COOPERATION

We now review examples of cooperation in nature, illustrating the application of this framework. Our examples focus heavily on partner fidelity feedback and partner choice because the other examples, listed in the tables below, do not present the difficulties in interpretation that directed reciprocation does. Some systems of interspecific cooperation involve multiple mechanisms. Furthermore, for a given cooperative interaction between species, the mechanism

maintaining cooperation in one species may differ from the mechanism maintaining cooperation in the other species.

Partner fidelity feedback: a diversity of contexts

Organelles and Maternally Inherited Microorganisms

Strong partner fidelity feedback (PFF) exists between eukaryotes and their vertically transmitted bacteria-derived symbionts, such as mitochondria and chloroplasts. The evolution of these symbionts has been sometimes accompanied by extreme reduction in gene content and genome size of the bacterial symbionts, and by tight interdependence of physiologies between the symbiont and host cell (Palmer 1997; Moran and Wernegreen 2000). Axelrod and Hamilton considered this case under the IPD game (1981), but in most cases there is no element of choice (e.g., mitochondria cannot be rejected) and cooperation is maintained entirely by PFF.

Parasite Virulence Evolution

One of the most prominent applications of the PFF principle has been to understand the evolution of virulence in infectious diseases. Beginning with Fine (1975), Axelrod and Hamilton (1981), Anderson and May (1982), and Ewald (1983), the standard model for the evolution of virulence invokes a strict negative correlation between the parasite's propensity to be transmitted and the harm it causes its host (the virulence). Thus, an increased ability to infect new hosts comes at the expense of a shorter life span and/or fecundity of the current host (higher virulence). The optimal virulence along this tradeoff depends on how long the parasite occupies its current host before it is transmitted to other hosts, the duration of the infection (limited by the longevity of the infected host and the speed of immune clearance), whether the populations of infected hosts are expanding or at a dynamic equilibrium (Lenski and May 1994), and whether the infection is

transmitted vertically or horizontally (Fine 1975; Axelrod and Hamilton 1981). All of these factors affect the PFF between the parasite and host, and in general, *the greater the PFF, the lower the optimal virulence*. For example, a strictly vertically transmitted parasite can afford but limited virulence (Ewald 1983). PFF is thus a central part of the framework for understanding the evolution of virulence, although the extent to which optimal virulence models are supported empirically is not clear (Ebert and Bull 2003). Note also that these examples focus on PFF from the perspective of the parasite, not the host, since the host does not benefit from the infection.

Fungal Endophytes

Various groups of fungi are specialized to invade plant tissues and exist inside living plants, for example, in the interstitium between leaf cells, or even inside of cells. Many of these endophytic fungi are parasitic and cause disease symptoms in the plant host, but others form mutualistic relationships with plants (Clay 1988; Saikkonen et al. 1998). The best-studied mutualist endophytes are in grasses, within which the fungi are vertically transmitted via the seeds (Schardl and Clay 1997). The fungus grows into the seed tissue during seed formation, subsequently infecting any developing seedling and ultimately the seeds of the next generation, thus spanning the fungus-host life cycle. The tight vertical transmission sets up conditions of PFF, and both partners are therefore expected to enhance each other's fitness. Indeed, grass endophytes produce secondary compounds (e.g., ergot alkaloids) that protect the grass host against herbivores; the grass host in turn provides the fungus with nutrients and facilitates fungal persistence. Investment by the fungus into secondary compounds thus feeds back via protection of the nourishing plant host (feedback returning to fungus). Likewise, nutritional provisioning of the fungus by the host feeds back via increased delivery of secondary compounds (feedback returning to grass host). Interestingly, horizontally

transmitted endophytes of plants generally have deleterious effects on their hosts, consistent with reduced or absent PFF between horizontally transmitted endophytes and their hosts.

Ants and Acacias

PFF exists in a short-term setting in the mutualism between bullhorn acacia plants (Mimosoideae) and ants in the genus *Pseudomyrmex*. The acacia plant grows chambers to house ants and provides protein and lipid rich "Beltian" bodies that nourish the ants (Belt 1874). In turn, the ants attack animals that contact the plant, preventing loss from herbivory. The ants also remove local vegetation in the immediate vicinity of the plant to reduce competition (Belt 1874; Janzen 1966). From the perspective of PFF, plant protection by the ants ensures the ants a future home and food supply that would not exist (or not be as extensive) if herbivores were allowed to reign freely on the plant. Likewise, the plant promotes positive feedback to its fitness by providing a home and food for the ants living on it. This PFF can only operate when plants are a limiting resource for the ants, so that ants cannot completely exhaust resources of the current plant and then move on to a new plant.

Although fitnesses in PFF are often coupled between partners across generations (as in the case of the endophytes), PFF does not operate across generations in the ant-acacia case. Each new plant starts from seed and must be colonized by ants, and those ants do not necessarily come from the parent plant producing the seeds. These short-term PFFs are less intrinsically stable than across-generation cases. For example, the ant-acacia system is ultimately maintained because plants attended by ants enjoy enhanced reproductive success. If ants evolved to consume flowers and all seeds of the plants they attended, the short-term PFF would continue to operate and benefit the growth of existing adult plants as well as the ants, but recruitment of new plants would decline until the system

collapsed when the acacia goes extinct. Plant castration occasionally occurs in a related ant-plant symbiosis, and the plant minimizes this cheating by restricting ant domatias (hollow structures that house ants) to certain parts of the plants (Izzo and Vasconcelos 2002). Finally, some plant-ants are effectively parasites on their plants (Janzen 1975) and recent work by Stanton et al. (1999) proposes that this parasitism is favored by a high density of ant trees. This supports the prediction that PFF can only occur in this system when plants are a limiting resource for ants. Thus, the success of PFF in maintaining cooperation must ultimately be assessed for its consequences across generations, even if the feedback operates on a shorter time scale.

Partner choice could also operate in this system, depending on the availability of empty plants. Ants whose plant "cheated" them and did not provide a home or food for them could potentially move out in search of a new home, rather than die with the current plant. Also, PFF would fail to operate if ant turnover was high, because ants that did not remain in their home for long would be unlikely to reap the return benefits of maintaining it (akin to arguments about the evolution of parasite virulence under high levels of horizontal transmission). Thus, depending on environment and relative abundances of the two partner species, the ant-acacia system could potentially exhibit a turnover of mechanisms from pure PFF, to a mix of PFF and partner choice, to a destabilization of cooperation.

Breakdown of Partner Fidelity Feedback

The automatic feedback of PFF can operate at different levels of organization and different time scales, and is correspondingly vulnerable to exploitation. Specifically, PFF may sporadically break down when one of the partners has a different generation time than the other. For example, and perhaps surprisingly, mitochondria are the cause of some profoundly deleterious phenotypes, such as male sterility in plants (Schnable and Wise 1998) and some

degenerative diseases of aging (Wallace 1999). The evolution of mitochondrial male sterility derives from the fact that the PFF between mitochondria and host is matrilineal, so sons do not contribute positively to the feedback loop of mitochondrial fitness. Moreover, mitochondrial diseases of aging may be due to within-cell evolution of the mitochondrial population. This is a consequence of the within-cell evolution of mitochondria operating faster than the between host evolution of cooperation (akin to cancer in this respect). PFF still operates and stabilizes host mitochondrion cooperation over the long run, but some invasion of cheater mitochondria can be expected, given their faster evolutionary rate and their resulting temporary liberation from PFF.

Partner choice: many enigmas resolved

In partner choice, individuals engage in one or more exchanges in which one partner can vary its response to accept or exclude the other partner. The strongest data for partner choice in a cooperative interaction is a variable and effective response to alternative partners. These data are not trivial to generate, but such responses are being worked out in elegant detail in two eukaryote-bacterial symbioses described below: the legume-rhizobium symbiosis and the bobtail squid-*Vibrio fischeri* symbiosis.

One of the biggest difficulties in exploring and understanding natural systems of cooperation is that partner choice, which is evidently rampant, is inherently density dependent and cannot operate effectively unless the preferred chosen partners are common (Noë and Hammerstein 1994; Noë 2001). Thus for systems in which the chosen partner is at least sporadically uncommon (Nuismer et al. 2000), cooperation may need to be supplemented by another mechanism, or otherwise the choosing partner may be exploited (see Bshary 2001 for this effect in cleaner fish). However, partner choice has the advantage over partner fidelity feedback in that, once established, it can work to the individual's benefit over short

time-scales (e.g., within generations). In contrast, many cases of partner fidelity feedback operate through differential reproductive success of the interacting lineages, hence across generations.

Yuccas and Yucca Moths

Yucca plants (Agavaceae), the plant family that includes Spanish daggers, have a highly specialized and largely obligate mutualism (Pellmyr and Thompson 1992). Yucca flowers require pollination by a yucca moth, and in return the developing yucca fruit provides an essential resource for the moth larvae. The larvae consume developing seeds and so reduce plant seed set directly. Thus, there is a potential evolutionary conflict in which moths try to maximize egg loads while the plant tries to maximize the number of developed seeds (Pellmyr and Huth 1994). Since the yucca system was first described, various intricacies have been discovered that paint a complicated picture for the maintenance of these systems (Pellmyr and Huth 1994; West and Herre 1994; Herre and West 1997; Huth and Pellmyr 1999, 2000; Marr et al. 2001).

In the "basic" mutualism, the moth gathers pollen from one or more flowers, typically flies to a new plant, oviposits into a flower, and then (often) pollinates that flower before moving to other flowers on the same plant (Huth and Pellmyr 1999). Moth species that exhibit this type of behavior could potentially violate the mutualism in two ways. First, they could oviposit but fail to pollinate. This is in fact a common but puzzling behavior, because unpollinated flowers do not develop and thus are dead ends for the offspring of the nonpollinating moths. However, at high moth density, an oviposited flower will sometimes be pollinated by another moth, which could save the eggs of a moth that did not pollinate. A second type of violation is to lay excessive numbers of eggs per flower, such that the plant produces few or no seeds (the same effect would be achieved by ovipositing in flowers with eggs deposited by another moth). Through selective

maturation of fruit with low moth egg loads and high pollen loads the plant has a partner choice mechanism to reward moths that do not overload plant ovaries with larvae (Pellmyr and Huth 1994; Huth and Pellmyr 2000). A high percentage of flowers are normally abscised early; floral abortion not only prevents seed development, it also kills all moth larvae in that flower (Marr et al. 2001). The "choice" is discriminatory in that pollinated flowers with many oviposition scars are more likely to be abscised than those with few scars (Pellmyr and Huth 1994; Huth and Pellmyr 2000). The plant is thus able to ensure that seeds are produced, although the final distribution of egg loads per ovary may vary with the density of moths. In order for the plant to exercise choice, one would expect that the plants have evolved to produce initially more ovaries than they can actually support, allowing the plant to eliminate the least desirable flowers and thus select against the most undesirable moths.

Virtually nothing is known about how the plant is prevented from cheating the moth, which could be any form of killing the larvae while retaining pollinated ovaries. An additional complication is that there are moths that do not exhibit the above form of mutualism. Nonpollinating "parasitic" moth species are known that lay eggs in developing ovaries, after the plant has made its choice of which ovaries to abort (West and Herre 1994; Pellmyr et al. 1996). These parasites can only be maintained in the presence of the mutualists. Yet other species pollinate flowers but lay eggs near the surface of the ovary (Pellmyr and Leebens-Mack 2000). These scars do not affect the plant's abscission decision, so it is not known how the fecundity of these (apparently mutualistic) moths is maintained at an acceptable level.

Squid Light Organs

The symbiosis between the bobtail squid, *Euprymna scolopes,* and the luminescent bacterium, *Vibrio fischeri,* is an elegantly studied example of partner

choice. The squid houses luminescent *V. fischeri* cells in a specialized light organ on its mantle. The bacteria benefit from maximal growth conditions in the light organ, conditions that can barely be improved upon in lab cultures (Boettcher and Ruby 1990). The nocturnal foraging squid (Berry 1912) probably uses the bacteria in a camouflaging behavior called counterillumination (McFall-Ngai 1990). Partner fidelity feedback via vertical transmission across generations is unlikely to occur because squids are born symbiont free and acquire their bacteria from the environment (Wei and Young 1989). There is no evidence that adults remain near their eggs (Singley 1983), nor that there are sufficient bacteria on the coating of the eggs to inoculate them (see Ruby and Lee 1998), thus there is no evidence for PFF. However, partner choice appears to occur at two steps in the interaction: initiation and maintenance of the symbiosis.

***Initiation*—**For the squid, initiation of the interaction is specific to the bacterial species level, and even between strains (McFall-Ngai and Ruby 1991). While the light organ tissues remain open to new strains after initial infection (Lee and Ruby 1994a), they are resistant to all other marine bacteria but *V. fischeri* (McFall-Ngai and Ruby 1991) and its congener *V. logei* (Ruby 1996). A surface peptide on the bacterium plays a critical role in its recognition by a squid host and the specificity of the interaction (Hensey and McFall-Ngai 1992).

Maintenance—Once *V. fischeri* infects the squids, the mechanisms of partner choice are both elegant and specific. Even if hosts are infected with a single strain, new strains could arise through mutation or superinfection, so partner choice must also occur after initial infection, particularly since the bacteria are evolving faster than the host. Each morning, squids expel 90% to 95% of their symbiont population into the environment (Lee and Ruby 1994b), the remaining symbionts being tightly bound to microvillus structures lining the light organ (Montgomery and McFall- Ngai 1994). Although differential retention may be a mechanism for partner choice, no work has specifically addressed this aspect. However, there is

intriguing evidence that the squid can select directly on luminescence as a bacterial trait. Visick et al. (2000) developed several mutant *V. fischeri* strains, defective for either the luciferase enzyme or a step in its regulation. These mutants were unable to completely colonize the light organs of the squids unless luciferase activity was replaced experimentally. An elegant mechanism has been hypothesized for how the host can choose specific partners based on their luciferase activity. The crypts of the squid light organs produce poisonous concentrations of peroxidase (McFall-Ngai and Hensey 1992), which may function to act specifically against nonluminous strains. Because the functioning bacterial luciferase has a higher binding affinity for oxygen than for the peroxidases, luminous strains may escape the effects of the deadly poison (Visick et al. 2000). Ruby (1996) pointed out that, of the thousands of *V. fischeri* strains isolated from bobtail squids, no nonluminous strain has been found. Thus, partner choice seems to be an effective mechanism selecting against light cheaters in the *V. fischeri* / *E. scolopes* symbiosis.

The Legume-Rhizobium Symbiosis

The legume-rhizobium symbiosis offers a near parallel to the squid-*Vibrio* system described above, with partner choice occurring at both initiation and maintenance of the symbiosis. Legumes form symbioses with rhizobial bacteria that fix atmospheric nitrogen into organic form. The rhizobia reside as differentiated bacteroids harbored within root swellings called nodules. Plants usually benefit from this interaction, as nitrogen is often a factor limiting their growth (Tamm 1991), but it is difficult to measure the benefits to rhizobia. Studies show that there are higher concentrations of rhizobia surrounding symbiotic legumes (Reyes and Schmidt 1979; Kuykendall 1989), but evidence is scant beyond this (reviewed in Denison 2000; Simms and Taylor 2002). Partner fidelity feedback is unlikely to be a force in this system: rhizobia are not transmitted directly from parent to offspring but are spread between plants in the soil, and most

plants are infected with several strains (Dowling and Broughton 1986). Experiments show much more bacterial genetic diversity within plants than between them (Hagen and Hamrick 1996).

***Initiation*—**Two factors contribute to legume choice of rhizobia at the initiation of the interaction (Simms and Taylor 2002). Host plants produce flavonoids that are specifically recognized and matched by some rhizobial strains, and transcriptional regulators (NodD factors) on rhizobia induce critical stages of infection (Perret et al. 2000). Though strain specificity at initiation is important, it is unlikely to be immune to cheating (Denison 2000; Simms and Taylor 2002; West et al. 2002b).

***Maintenance*—**Recent work has suggested that cooperation is maintained via postinfection legume sanctions of nonsymbiotic rhizobial strains (Denison 2000; Simms and Taylor 2002; West et al. 2002b; Kiers et al. 2003). Experimental evidence suggests that legumes punish nonfixing strains through limiting oxygen supply (Uvardi and Kahn 1993; Kiers et al. 2003). It seems likely that the elegant research on this system will soon unravel the mechanistic basis underlying rhizobial cooperation.

A Rule About Partner Choice?

Partner choice between species often operates on just one side of a mutualism. In particular, if there is an asymmetry in population size and/or generation time, the chosen partner is typically the one with the more rapid generation time and larger population size. There may be a meaningful generality in this pattern: that choice is a mechanism that the more slowly evolving species can use against the more rapid evolutionary changes of the partner. At present, we can offer no more than speculation of the possible existence or significance of such a pattern.

Multiple mechanisms and potential puzzles

The application of our framework to even a modest number of examples from nature leads quickly to the realization that multiple mechanisms operate in many systems. In many cases, for example, one species uses partner choice to prevent exploitation, but the other partner species relies on a different mechanism. A system may also involve multiple mechanisms within one of the partner species. For some of the systems we analyze below, the evolution of cooperation is not well understood. We apply our framework to illustrate what kind of data need to be gathered to identify the mechanisms maintaining cooperation in each system.

Generalized Animal Pollinators of Nectar-Producing Flowers

An example of cooperation that is familiar to everyone is the use of insects or vertebrates as pollen vectors for flowering plants. The flower offers the pollinator nectar or other reward, and the pollinator deposits pollen to fertilize the flower and/or carries pollen off from that flower in search of other flower rewards. The degrees of sophistication and specialization in this relationship vary widely across plant species, from largely nonspecific pollinators of sunflowers to the highly coevolved systems of euglossine bees and orchids. In most cases, the delivery and dispensing of pollen by the animal is inadvertent, a byproduct of the fact that pollen sticks to the pollinator and that the animal cannot easily remove it. To attract the pollinator, the plant offers a reward in the form of nectar. However, the pollinator is vulnerable to being cheated (Bell 1986; Gilbert et al. 1991), because some pollen will already have been deposited before the insect can determine whether there is a reward present in that flower. Partner choice is at work in at least some cases: insects (Chittka et al. 1999) and hummingbirds (Waser and Price 1981; Meléndez-Ackerman et al. 1997; Schemske and Bradshaw 1999) remember plant characteristics that do and do not offer rewards, such that the selfish plant receives fewer visits (see Noë 2001 for a model of this effect). As far

as we know, no work has specifically tested partner choice mechanisms in pollinators, as work has focused on choice by pollinators between plant *species* and according to flower characteristics (Meléndez-Ackerman et al. 1997). It is therefore unclear how partner choice is operating on individual flowers. Perhaps insects visit few flowers on a plant if that plant has little nectar, thus potentially reducing plant fitness through its choice to leave quickly. Interestingly, Darwin (1859) recognized that partner choice acts in nectar producing flowers (though not identified as such):

> "Those individual flowers which had the largest glands or nectaries, and which excreted most nectar, would be oftenest visited by insects, and would be oftenest crossed; and so in the long-run would gain the upper hand. Those flowers, also, which had their stamens and pistils placed, in relation to the size and habits of the particular insects which visited them, so as to favour in any degree the transportal of their pollen from flower to flower, would likewise be favoured. . . " (p 139).

In the first sentence Darwin describes partner choice by pollinators; the second sentence, however, he describes maximization of the byproduct benefits received by the plants. This latter effect should not be confused with byproduct reciprocity, since the insect does not necessarily reap benefits from the plant's specialization.

Leaf-Cutter Ants that Cultivate Gardens

Fungus-growing ants require the cultivation of fungus for food. When associated with ants, the fungal cultivars are clonally propagated within ant nests, and also between ant generations through the transfer by foundress queens of clonal inocula from maternal to offspring nest. Cultivar clones are occasionally exchanged laterally between different ant nests (Mueller et al. 1998;Adams et al. 2000; Green

et al. 2002). Associations of ant and fungal lineages thus persist for prolonged evolutionary times through partner fidelity feedback, but are occasionally punctuated by novel fungal imports or lateral cultivar transfer.

Partner fidelity feedback is certainly one mechanism that will curb the spread of unproductive or exploitative "cheater" cultivars, but partner choice is a second *reinforcing mechanism* (Mueller 2002). For example, ants may be able to pick between productive and unproductive cultivars that coexist in a given nest, using indicators of cultivar productivity(e.g., nutrient level, growth rate). Behavioral assays in which ants were presented with genetically differentiated cultivars indicates that attine ants are indeed capable of exerting "symbiont choice" necessary for the operation of partner choice (Mueller et al.2004). Moreover, cultivar substitution involving lateral transfer from other nests is inherently based on partner choice of cultivars selecting for cultivar productivity because: i)the substituting ants may screen against cultivars that appear suboptimal; and ii) cultivars are most likely to be picked up from ant lineages with large productive nests (nests that have nonexploitative cultivars, which are mutualisms that persist because of partner fidelity feedback). Both partner fidelity feedback and partner choice thus interact, but both can also operate independently and modulate the evolution of cooperation between ants and their fungi.

Algal-Invertebrate Symbioses

A wide variety of symbioses are known among tropical marine invertebrates in which large populations of photosynthetic unicellular algae live within the tissues of the host (Trench 1993). The majority of the algal symbionts are dinoflagellates; the hosts include sponges, cnidarians, mollusks, flatworms, and foraminiferans (Trench 1993). In some species there is evidence that the algae provide the host with carbohydrates derived from photosynthesis (Balderston and Claus 1969). The algae, in turn, presumably have access to the rich store of

nitrogen present in the host tissue, which enables them to reproduce in a protected environment (Muscatine 1990).

Approximately 85% of corals and other invertebrate host species acquire their complement of symbionts horizontally, from the external environment rather than from their parents (Fadlallah 1983; Babcock and Heyward 1986; Harrison and Wallace 1990). Symbionts available to colonize new hosts likely arise from neighboring conspecific hosts. Within-host symbiont growth rates are generally in excess of host growth rates, and some fraction of the excess symbiont population is expelled into the environment. Expelled symbionts are viable, and are presumably available to infect additional hosts. Thus, in systems with horizontal transmission, symbiont within-host fitness can translate into among host fitness. Invertebrate hosts can harbor one or more species of algal symbiont, with the number of algal partners varying among host species (Rowan and Knowlton 1995; Baker and Rowan 1997; Belda-Baille et al. 2001). Changes in the relative abundances of different symbiont species have been noted for hosts that can simultaneously harbor multiple symbiont types, particularly when the host is stressed (Rowan and Knowlton 1995; Baker 2001). However, dynamic symbiont populations are not found in all hosts (Goulet 1999), and generally little is known about how much turnover occurs within that intracellular population, either via further colonization or via competition within the host.

The horizontal transmission and large algal populations within the host suggests that partner choice may be the mechanism required to maintain cooperative algae. Variants of algae are known that infect and kill the host or otherwise retard host growth (Wilcox, personal communication), so a byproduct benefit seems unlikely as a universal mechanism. The turnover that can occur within hosts questions whether partner fidelity feedback operates across host lifetimes, although it may operate early in the critical stages of the host life history (Wilcox, personal communication). By analogy with the squid-*Vibrio* and plant-

rhizobium systems, we should expect that partner choice plays an essential role in maintaining these dinoflagellate symbioses, but there has been scant investigation of this possibility. Several experiments have shown that hosts infected with multiple strains of dinoflagellates ultimately resolve to a single strain, but whether this resolution is due to the host (choice) or simply competition among dinoflagellates is not clear (Belda-Baille et al. 2001; Coffroth et al. 2001). Thus, the forces maintaining symbiont cooperation remain unresolved in these systems.

Policing

As a final example of multiple mechanisms, we consider how our framework relates to a concept (policing) that has been presented in a different framework. Frank (1995, 2003) considered policing to be one of the two major classes of models for the evolution of cooperation. By analogy to human societies, policing is the imposition of costs by one individual on another in response to their uncooperative behavior (Frank 1995). Models of policing overlap with several parts of our framework. We neither defend nor challenge the biological evidence that policing evolves in ways consistent with Frank's models, rather we merely illustrate how the two frameworks overlap.

(i) Partner choice. Virtually all policing models involve some form of partner choice within species, because one individual imposes a cost/punishment on specific individuals who are behaving noncooperatively. Models of policing thus differ in the nature of partner choice and in how the benefits from partner choice are distributed to others, as described next.

(ii) Shared genes combined with byproduct benefits. In one model that applies to social Hymenoptera, policing is the consumption of worker-derived eggs by other workers. It is favored as a worker behavior because it results in queen-laid eggs automatically replacing worker-laid eggs—the policing individual shares more genes with queen-laid eggs than with worker-laid eggs (Ratnieks and

Visscher 1989). The policer thus benefits via shared genes. The main difference between this policing model and our kin-choice model is that the policing act is not cooperative between the two interactants. Instead, policing is cooperative to other workers in the colony (because they too share more genes with queen-laid eggs than with eggs laid by other workers).

(iii) Byproduct benefits only. In yet another model, group benefit occurs when the policing action reduces selfish interactions, enhancing group productivity. Although this mechanism is typically thought to apply in groups with related members, in principle it can operate when group members are unrelated: by policing others, an individual directly improves its own fitness through its fair share of the improved group productivity (Frank, 2003). Noncheating group members benefit as a byproduct of the selfish action of the policer, and their byproduct benefit helps maintain the policing.

CONCLUSIONS AND FUTURE DIRECTIONS

The study of cooperation has progressed greatly in the past thirty years, and there are now many evolutionary models to explain a wide array of empirical systems. Our goal has been to consolidate the models and examples into a framework of relatively few evolutionary mechanisms. This framework allows the recognition of parallels between seemingly disparate systems (e.g., rhizobium-legume mutualisms and squid-bacterial mutualisms), and also suggests studies of empirical mechanisms to identify the detailed ways that mutualisms are maintained against exploitation (cheating). Discoveries of new systems are also easily classified in this system, and those additions may lead to the recognition of new mechanisms.

Our framework recognizes: (i) directed reciprocation; (ii) shared genes; and (iii) byproduct benefits as three classes of models for the evolutionary maintenance

of cooperation. The perspective of this framework is individual selection (why cooperating individuals are favored over noncooperators), but most or all of the underlying mechanisms can be modeled with no loss of generality in various frameworks (e.g., trait-group selection or policing, as detailed above). Thus, we suggest that the mechanisms at work here transcend the specific formulation of the model.

The framework is also a starting point that opens many avenues for further study, some of which could lead to discoveries that expand the framework or even change its perceived relevance. We discuss a few unexplored problems that seem worthy of further attention.

Incorporating other models.

The framework here attempts to organize the known empirical examples. Several models have been proposed in which cooperation can evolve, but for which there is scant empirical evidence, and those models have not necessarily been accommodated here. It would be useful to know whether new models can be incorporated into this framework; if not, then the search for examples that satisfy those models could be intensified, and a new framework proposed if examples are found.

Embedding ecological factors

The framework attempts to isolate the minimal elements that allow the maintenance of cooperation within a species or between two species. Yet nearly all natural examples are embedded in complex ecologies involving multispecies interactions. How do these ecological dynamics impinge on the evolution of cooperation? A mild parasite may become a mutualist in the presence of a more severe parasite, if the mild parasite can prevent infection by the severe one or reduce its harmful effect. How do the dynamics of the two parasites affect the

evolution of cooperation? Alternatively, can a third species interact with a mutualist to prevent the evolution of cheating via some mechanism that we have not identified? Investigators who conduct field studies of mutualisms certainly convey a suspicion that ecological dynamics may provide key insights into the maintenance of cooperation in ways that have not been anticipated (A Herre and O Pellmyr, personal communication).

Origins of cooperation and the evolution of parameters

The maintenance of cooperation in our framework requires many conditions that are treated as invariant in our mechanisms. For example, partner fidelity feedback requires that partners are associated for an appropriate duration, possibly across generations. Partner choice typically assumes an asymmetry in which the chosen individual is forced to accept the consequences of being chosen or rejected; there is an appropriate level of "control" for the persistence of cooperation, whereby choice operates effectively but cannot enslave an individual. A broader perspective for the evolution of cooperation would consider the evolution of these parameters, ultimately addressing the origins of cooperation.

CHAPTER II:

Evolution of conflict mediation between genomes

CHAPTER ABSTRACT

A central challenge in evolutionary biology is to explain the origins of novel levels of biological complexity. Evolutionary transitions in complexity often require cooperation among component individuals, however selection of individuals acts as a counteracting force promoting conflict. Contemporary systems of cooperation similarly provide avenues of conflict that can lead to the evolutionary decay of cooperation, hence the evolutionary outcomes are often indeterminate and defy prediction from first principles. Theory describes mechanisms of conflict mediation, which limit selection among individuals and promote fitness variation at the higher level of organization, however empirical work on this topic has been scarce. Here, we experimentally investigate the evolution of cooperation and conflict between two divergent bacteriophages (f1, IKe). We used an iterated tripartite protocol: i) co-infection of the bacteriophages in *Escherichia coli*, ii) enforcement of paired vertical transmission, iii) and production of infectious bacteriophage progeny for the next cycle. The life cycle had episodes favoring cooperation (i,ii), other episodes with conflict (iii), and the phages were propagated to observe how the system would resolve. Remarkably, f1 and IKe evolved a system in which opportunity for conflict nearly vanished: they evolved to co-package their genomes into one protein-coat, ensuring co-transmission during the infectious stage. Furthermore, IKe evolved to become dependent upon f1 by evolving a minimal genome and the inability to infect cells independently. These results parallel a variety conflict mediation mechanisms in nature -- evolution of reduced genomes in symbionts, co-transmission of partners, and obligate coexistence between cooperating species.

INTRODUCTION

Organisms compete to procreate, and much of natural selection consequently stems from the conflict ensuing from this competition, be it among replicating molecules, unicellular organisms, or complex eukaryotes. However, cooperation overcoming such conflict is a requirement for key features of life, including (in theory) its origin (Eigen 1971; Eigen and Schuster 1977; Michod 1983). A rich body of theory and empirical work exists to explain the evolutionary maintenance of cooperation (Hamilton 1964 a,b; Trivers 1971; Axelrod and Hamilton 1981; Queller 1985; Bull and Rice 1991; Connor 1995; Sachs et al. 2004), but our understanding of the origins of cooperation and the specific pathways that evolution can take to and from cooperation is relatively shallow.

Conflict mediation is the evolution of features in an individual or group that minimize selection among component parts while promoting selection at the higher (group) level (Michod 2003). Conflict is defined as divergent evolutionary pressures favoring selfish or antagonistic outcomes. We specifically distinguish forces of selection (i.e. cooperation, conflict) from their potential evolutionary outcomes (i.e. benevolence, antagonism). Mediation of conflict is thought to be critically important to major transitions in evolution. This is because cooperation among once independent individuals is a prerequisite to their integration into a higher level of biological complexity (Maynard-Smith and Szathmary 1995). For example proto-replicator cooperation is a predicted condition for the origin of the gene networks necessary for cellular life (Michod 1983; Szathmary 1986; Szathmary and Demeter 1987), as well as the origin of chromosomes (Maynard-Smith and Szathmary 1993, 1995). Similarly, cooperation between once autonomous cells permitted the origins of eukaryotes (Margulis 1981) and multicellularity (Michod 1997, 2003).

Conflict mediation may also be vital to contemporary systems of

cooperation where avenues of conflict can lead to their breakdown. For example, mitochondria are obligate symbionts of eukaryotic cells but can evolve to kill sons because they are not transmitted through sons. In lichens, ancient symbioses between algae and fungi, the partners can also evolve antagonism when routes to selfishness exist (Richardson 1999). Correspondingly, algal symbionts of jellyfish which are transmitted infectiously between hosts can evolve to selfishly exploit those hosts (Sachs and Wilcox *submitted*). Proposed mechanisms of conflict mediation are diverse, including specialization by individuals, partner fidelity feedback (shared fate or coupled fitness between individuals, Bull and Rice 1991; Sachs et al. 2004), and policing of uncooperative individuals to name a few (Frank 1995). However, there is little understanding of the genotypic or phenotypic details inherent conflict mediation, nor whether common themes govern the evolutionary pathways to cooperation.

Here, we experimentally investigated the evolution of cooperation and conflict between two divergent filamentous bacteriophages (phages) of *Escherichia coli,* f1 and IKe. Unlike most phages, f1 and IKe establish permanent non-lethal infections in bacteria, and reproduce by extrusion through the cell wall without lysis (Model & Russel 1988). Phenotypically, they act as highly transmissible plasmids, however these phages experience antagonism when they co-infect cells (Russel 1992). The experimental evolution protocol was a life history cycle of three steps: encounter, growth and reproduction, iterated 50 times in series sequentially. i) Encounter: coinfection of f1 and IKe in naïve *E. coli*, ii) Growth of the coinfected cells for 18 hours, and iii) Reproduction: isolation of phage progeny produced after step ii for the next cycle (Fig. 6; see methods). Each phage was engineered to contain a distinct antibiotic resistance gene to enforce this protocol. By manipulating vertical and infectious transmission of phages, our experimental system creates a life history in which pairs of phage genomes are periodically selected for cooperation (steps i and ii), interrupted by episodes in which each

phage genome was potentially selected for selfish reproduction at the expense of its partner (step iii: infectious transmission). The system provides pathways between conflict and cooperation and thus can evolve in multiple directions.

MATERIALS AND METHODS

Bacteriophages and cell lines

The filamentous coliphages f1 and IKe are composed of circular, single-stranded DNA encased in flexible protein capsids. These phages are known to negatively interact with each other within cells: each suffers severe fitness losses during co-infection relative to single infection (Russel 1992). This discord is likely driven by competition for host resources, and via non-productive binding interactions between inter-specific DNAs and gene products (Peeters et al. 1986, 1987; Russel 1992). The f1 genome is 6407 nucleotides (nt) (Gen Bank accession #V00606; Beck and Zink 1981) and the IKe genome is 6883 nt (Gen Bank accession #X02139; Peeters et. al. 1985). Capsid length is determined by genome size, allowing easy genetic manipulations including insertion of non-phage DNA (Messing 1979). While f1 and IKe share only 55% nucleotide identity, they contain the same 10 genes in synteny and share features indicating common ancestry (Peeters et. al. 1985; Model & Russel 1988). Infection by f1 requires hosts with the F-episome (expressing the F pilus) while infection by IKe requires the IncN episome (expressing the N3 pilus; Bradley 1979). Both f1 and IKe halt super-infection by their own species through retraction of those particular pili (Dotto et al. 1981; Model and Russel 1988; Messenger et al. 1999). Hence, each phage uses different means to enter cells and conflict during cell entry is unlikely.

The ten genes of f1 are numbered I-X, and the IKe homologues are numbered similarly. Genes III, VI, VII, VIII, and IX encode structural proteins: VIII encodes the major coat protein, III and VI encode minor coat proteins at the pilus attachment end of the phage and VII and IX are at the opposing end. Genes II

and X regulate DNA replication (X is encoded in-frame within II). V is a single-stranded DNA binding protein also involved in replication regulation, and genes I and IV encode proteins that assist in phage assembly and export from the cell (Model and Russel 1988; Russel 1993; Feng et al. 1999). Two intergenic regions also exist with regulatory DNA rich in secondary structure.

Three *E. coli* K12 cell lines were employed (received from M. Russel). Strain A527 contains the tetracycline resistant IncN conjugative plasmid encoding N-pili, K19 is an HfrC strain that encodes F pili, and K1037 is both HfrC and contains the IncN plasmid, expressing both N and F pili (see Russel 1992). Only K1037 can be infected by both phages.

Genetic manipulations of phages

Each phage was engineered to include gene inserts encoding antibiotic resistance. The Tn903 kanamycin resistance gene (Gen Bank accession #X06404; Taylor and Rose 1988) was inserted into the IKe genome, and the Tn9 chloramphenicol resistance gene (Gen Bank accession #J01841; Alton and Vapnek 1979) was inserted into the f1 genome (received from M. Russel). Inserts were cloned into the intergenic regions of f1 and IKe between genes II and IV (f1 insert site 5614, IKe insert at site 0). Engineered phage were passaged individually prior to the experiment so that the mutations we scored were unlikely to be compensatory mutations to the insert DNA. We refer to these engineered and singly passaged f1 and IKe as 'ancestral' genomes in this study, not to suggest that they are wild-type.

Passaging protocol

i) **Encounter** (i.e. infection)—Cells were incubated in shaking water-baths at 200 rpm, 37^{o}C, and grown in standard LB medium (10gm/L NaCl, 10gm/L Bacto-tryptone, 5gm/L yeast extract, 2μM $CaCl^2$). Phage were added to 10 ml

cultures at cell densities ≈$2 \cdot 10^8$/ml, during logarithmic growth at low multiplicities (MOI <0.1). Infection proceeded for sixteen minutes before kanamycin (37.5 μg/ml) and chloramphenicol (25.0 μg/ml) were added. We set antibiotic concentrations at twice the empirically determined minimal inhibitory concentration to minimize copy number selection between the phage genomes. Phage used for the initial coinfection were derived from stocks of the individual phages. Whereas subsequent infections used phage mixtures from the previous cycle.

ii) **Growth** of coinfected cells—Once antibiotics were added, only coinfected cells could grow (enforcing paired vertical transmission). Coinfected cells grew for 18 hours in the original media.

iii) **Reproduction** of phage progeny—Cells were twice centrifuged and washed with fresh, pre-warmed medium containing both antibiotics. After the second suspension, the culture was grown for one hour to produce phage, at which point the cells were pelleted and killed by a 20 minute exposure to 65°C. The phage supernatant was diluted and used to infect naïve cells for the next passage. Only phage could evolve under this protocol, since cells were completely replaced at the beginning of each cycle. Coinfected cells and phage supernatant of every passage were archived. Cells were stored in LB (25% Glycerol) at –80°C, and phage supernatants were filter sterilized and stored at 4°C. This procedure was iterated for fifty cycles.

Fitness measures

i) **Vertical transmission fitness**—To assess the level of cooperation (between phages and with the host) that existed during growth of coinfected cells, populations of cells infected with evolved phages were competed with populations of cells infected with ancestral phages. Competitions took place in one flask, with one of the competing cell lines marked with nalidixic acid resistance. Competing

cell lines were mixed at low concentrations in pre-warmed media with both antibiotics present and grown for 18 hours. Relative frequency of marked and unmarked cells was estimated before and after growth by replica plating. The change in frequency of the cells coinfected with evolved phages was used to derive relative fitness measures, with ancestors arbitrarily assigned a fitness of one. The nalidixic resistant cells differed slightly (≈10%) in fitness from wild type k1037. Consequently, assays were done reciprocally (ancestral versus evolved phages in nalidixic resistant cells) to correct for fitness differences in the marked cell lines.

ii) **Infectious transmission fitness**—Fecundity of each of the phages was estimated from the supernatant titer after the hour long phage production step. Serially diluted supernatants were infected into k1037 cells at very low MOI (<0.01) and plated separately on kanamycin and chloramphenicol to measure densities of each of the phage types.

Sequencing and nomenclature

Both phage genomes were sequenced completely from time points before and after selection with at minimum 2X coverage, and regions with mutations were sequenced over multiple passages to estimate when the mutations ascended. Sequences were obtained from PCR products of phage genomes from supernatant phage populations and from clones (when results were ambiguous). Multiple peaks on the sequence electropherogram (Seqman 3.6.0, DNASTAR Inc.) at one nucleotide position were assumed to represent polymorphism, and fixation was assumed once multiple peaks resolved to one. However, using consensus sequences in this way roughly approximates mutant frequencies and fixation times (Badgett et al. 2002). Nucleotide positions of mutations follow Gen Bank designations.

Protein coat packaging assays

The frequency of cross-packaging (one genome packaged in the heterologous coat) and co-packaging (both genomes packaged into one coat) were measured because they had important consequences to evolution in this system. The phage's protein coat determines the type of pilus that it uses to infect cells. By using different combinations of host pili (F, N3 only) and antibiotic resistance selection, we could discern these packaging variants from the wild type phages (fig. 7). Cross-packaging rates were estimated as the proportion of all phage infected cells (with one pilus type) that contained the heterologous genome. A proportion of these phages had co-packaged genomes (i.e. also packaged their native genome). Co-packaging rates were calculated in two ways: i) as the proportion of total phages infecting a cell with one pilus type that were resistant to both antibiotics. This was estimated by replica plating sub-samples of the above colonies on the alternate antibiotic. For example, sub-samples of colonies that grew on kanamycin in K19 cells (IKe genomes in f1 coats) were replica plated on chloramphenicol to reveal the proportion of these phage infected cells that *also* contained the f1 genome. ii) Infections were done on k1037 cells (both pilus types) at very low MOIs ($<10^{-4}$ each phage type), and the proportion of phage containing both genomes was estimated by the density of plated colonies that grew on both antibiotics. Since these infections were done at such a low MOI, errors introduced by coinfections should have been negligible.

RESULTS

Chronicle of f1 and IKe evolution

Over the course of the evolution the two phages evolved molecular changes which allowed both f1 and IKe genomes to be packaged into the same protein coat, thus ensuring their co-transmission during the infectious stage. Subsequently, IKe evolved a minimal genome unable to infect cells independently. The evolution occurred more or less in two steps. i) Initially IKe's fecundity was feeble relative to

f1 (≈2%), and at the outset the rarity of IKe may have favored evolution of co-packaged genomes as a mechanism to ensure coinfection. The frequency of co-packaged genomes (mostly in f1 coats) increased significantly from the ancestral condition in which they were rare (see below). ii) IKe evolved a minimal genome, dispensing all but three of its genes (≈ passage 41; fig. 8), and completely relied on f1 for all coat proteins and morphogenetic processes. The substantial gene loss in IKe required it to be coinfected with f1 to produce infectious particles. The evolution co-packaging rate continued to increase and after IKe minimization all genomes were packaged in f1 coats. Both the genome minimization in IKe, and the evolution of co-packaging apparently enhanced overall fitness benefit to both phages (see below).

Co-packaging and cross-packaging evolution

i) Co-packaging. The ancestral genomes were rarely co-packaged, the initial rate was 0.1% (±0.02%). By passage 20 co-packaging had evolved a twenty-fold increase to 2.1% (±0.01%) with >99.9% of these in f1 coats. At the end of the evolution the co-packaging rate reached 15% (±1.2%), a ≈150 fold increase from the ancestral condition (fig. 9).

ii) Cross-packaging. Initially both genomes were cross-packaged at relatively low rates: f1 = 2.1% (±01.2%), IKe = 4.5% (±03.1%). Cross-packaging rate evolved in both phages such that each genome increasingly became packaged in f1 coats (fig. 9). Whereas IKe experienced a dramatic increase in cross-packaging by passage twenty to 78.3% (±12.5%), in the same period the proportion of f1 genomes in IKe coats rate decreased to 0.05% (±0.002%). Thereafter, IKe's cross-packaging rate continued to increase reaching 99.2% (±0.007%) by passage 40, whereas f1 did not change significantly in this interval. After the full genome of IKe went extinct (≈passage 43) all genomes of both phages were packaged in f1 coats. (see fig. 9).

Fitness evolution

i) Vertical transmission fitness. Fitness increased to 2.9 (±0.9) by passage 38 and to 8.6 (±3.0) by 50 (Kruskal-Wallis nonparametric 1-way test, $P = 0.01$; fig. 10). We used 38 as an intermediate point because it preceded the major genome reduction in IKe, hence we separately estimated the fitness effects of genetic change occurring before and after the genome reduction. Fitness increased ≈300% during each of these periods.

ii) Infectious transmission fitness. Fitness (titer of phages in supernatants) increased significantly in both phages: f1 titer increased from the ancestral $\approx 2.6 \bullet 10^7$/ml to $\approx 1.4 \bullet 10^9$/ml at passage 50 (ANOVA, $p = 0.016$), and IKe increased its titer from initial $\approx 6.2 \bullet 10^5$/ml to $\approx 1.8 \bullet 10^9$/ml at passage 50 (ANOVA, $p < 0.001$; fig. 11). Baseline rates of infectious fitness, in which the titer of each of the ancestral phages was measured unaccompanied, were consistent with significant, asymmetric costs to co-infection: f1 $\approx 1.3 \bullet 10^8$/ml, IKe $\approx 1.4 \bullet 10^8$/ml. Thus, initial co-infection (passage 1) reduced phage output ≈5x in f1, and ≈225x in IKe compared to these baselines However, these single infections necessarily required the presence of only one of the antibiotics (as opposed to both) so are rough approximations.

Molecular evolution

Mutations

Over the course of the evolution the f1 genome accrued eight point mutations while IKe had nine point mutations (including two which reverted) and two large deletions (tables 2, 3). Each phage accrued three missense mutations. Other point mutations occurred at three non-coding loci in IKe and two of these affected DNA secondary structure. Five point mutations occurred in f1 non-coding DNA, four of which affected DNA secondary structure. We classify mutations into

putative functional classes: i) reduction of cost to host, ii) packaging, iii) anti-interference and reproduction control, and iv) compensatory to IKe minimization.

i) Reduction of cost to host. Two deletions occurred in IKe. These likely minimized costs to the host and thus enhanced fitness during vertical transmission (see table 3, fig. 10). The first, IKe (insert) sites 411-523, emerged in passage 12 and removed 212 nt of non-coding insert DNA between the kanamycin resistance gene and IKe II. The major deletion, IKe sites 1442-5983 removed 4541nt (all genes but II, X and Kn insert) and emerged in passage 41, though PCR evidence suggests that it was present in very low concentrations in passage 40. This deletion quickly spread to fixation (see fig. 8). While the first mutation streamlined the IKe genome by ≈2.5%, the larger removed ≈57% of the genome. We predict that savings in phage metabolism drove the three-fold increase in coinfected cell fitness which was temporally correlated with the larger deletion.

ii) Packaging. most co-packaging and cross-packaging evolution occurred in the first 20 passages (see fig. 9). Hence, we focus on the five mutations that fixed (or ascended) in this period. These include three point mutations in f1: f1 (insert) site 186, in non-coding DNA, f1 site 5674, in the minus-strand origin, and f1 site 957, a missense mutation in gene V. The two mutations in IKe included the 212 nt deletion (above), and a missense point mutation in gene III, IKe site 2173, which had ascended at passage 20, but without evidence of fixation until 30.

Packaging evolution in this period was likely driven by single missence mutations f1 gene V and IKe gene III. Gene V encodes the ssDNA binding protein which coats genomes and readies them for morphogenesis and export (Russel 1992), and thus could evolve to promote packaging of the heterologous genome. Gene III encodes a minor coat protein residing at the end of the phage where packaging is terminated, and mutations in this gene have been shown to promote packaging of multiple genomes (Lopez and Webster 1983; Model and Russel

1988). The three other mutations in this interval were non-coding, and unlikely affect packaging evolution (see tables 2,3). Two clustered missense mutations, which occurred in the overlapping genes II and X in this period, are discussed below.

iii) Anti-interference and reproduction control. Both phages initially experienced significant fitness costs to co-infection under our measures. Infectious transmission fitness was lower during initial co-infection than the individual (baseline) measures of infectious fitness. Under different protocols Russel (1992) also detected interference between f1 and IKe during co-infection with >50 fold depression of phage production in both f1 and IKe. Infectious transmission fitness increased during the evolution and reached levels that were significantly above baseline (see fig. 11). Thus, evolution minimized the costs of co-infection. Most of the change in infectious fitness occurred before passage 20 for f1 and passage 40 for IKe, and we concentrate on the mutations occurring in this period. In f1 three point mutations, all discussed above, fixed in this period: f1 insert site 186, and f1 sites 5674 and 957. In IKe five changes fixed, IKe insert site 411 and IKe site 2173, both discussed above, as well as three clustered mutations in the non-coding minus-strand origin, IKe sites 6377, 6379 and 6382. The two clustered missense mutations in the overlapping genes II and X (IKe sites 1116 and 1156) appeared to interact over this period.

The changes in the minus-strand origins of both phages are interesting, especially since these they occurred in apparently homologous stem and loop structures in both genomes. The minus strand origin is a non-coding region of defined secondary structure in the large intergenic region between II and IV (Beck and Zink 1981; Peeters et al. 1985), which serves as a double-stranded attachment point for the cell's RNA polymerase and initiates complementation of the single-stranded phage DNA upon its initial entry to the cell. However, these DNA structures act *in cis*, so it is unclear how they could be involved in phage

competition for host resources or in non-productive binding. Non-productive binding is much more likely to be minimized by the mutations in protein coding regions that act *in trans* such as the mutants IKe sites 1116 and 1156 that overlap IKe X and II (X is encoded in-phase within II). These mutants may ameliorate cross-genome termination, or other non-productive binding (Peeters et al. 1986, 1987), and are also implicated in replication control, as X acts as a negative regulator of II and catalyzes rolling-circle replication of the phage genome (Model and Russel 1988). Perplexingly, neither of these mutations ever fixed in the experiment. The two loci appear to alternate nucleotide states with each other (when one is predominately g the other is c and vice versa), so any fitness advantages to these mutations may be frequency dependent.

iv) Compensatory to IKe minimization. Over one third (seven) of the mutations that occurred in both phages arose immediately after the major genome reduction of IKe (see tables 2,3). These mutations included two missense mutations in f1 genes I and VI (f1 sites 4072 and 2988) and non-coding mutations in the morphogenetic signal of f1 (f1 sites 5521 and 5529), the minus-strand origin of IKe (IKe sites 6377 and 6379) and the intergenic region of IKe (IKe site 6681). We posit that this burst of mutations was driven by IKe genome minimization, since at no other point in the experiment are so many mutations clustered, however we can offer no more than speculation.

DISCUSSION

Indeterminacy in evolution and genetic details

The design attempted to mimic natural systems of cooperation with elements of conflict, whereby the evolutionary outcome was indeterminate. This study is therefore unusual among studies of experimental evolution, since there is a clear anticipated phenotypic outcome in most studies. Here, with selection for cooperation in some stages and routes to conflict in others, theory offered no

predictions of whether the system would resolve to increased antagonism, benevolence or some combination thereof.

The growth period of co-infected cells favored cooperation between the two phages, and if the entire life cycle was restricted to this vertical transmission phase, the predicted outcome is enhanced cooperation (e.g., Bull et al. 1991). The inclusion of a reproductive phase to the life cycle adds a dimension of conflict, and the direction of evolution then becomes unpredictable, depending on system biology. The reproductive phase favors high progeny output from each phage in the cell, independently of its partner, but mutations benefiting the reproductive phase may harm the growth phase (or vice versa) and evolve according to the balance of those opposing effects. If a mutant f1 arose that increased its progeny by co-opting the reproduction of its Ike partner, the mutant f1 would spread even if it ultimately caused the loss of Ike and extinction of the population (by analogy with selection for sex chromosome meiotic drive, Hamilton 1967). However, if the mutant f1 had the pleiotropic effect of failing to maintain its Ike partner in the cell during the growth phase, the mutant would die before it had an opportunity to produce progeny. Thus, the genetic details must be specified before the outcome can be predicted.

When our design is coupled with an understanding of f1 and Ike biology, the evolution of increased co-packaging is seen as a likely outcome. To appreciate this, consider f1 progeny (in f1 coats, hence which infect through the F pilus), some as "single" particles containing just f1 genomes, and others as "double" particles containing an f1 and Ike genome. If the probability of encountering and infecting a cell is P, and the fraction of those cells that will independently be infected by Ike is Q_{Ike}, the success of a single particle is PQ_{Ike}. For comparison, the success of a double-particle is P. Thus, a double particle is 1/Q times more likely to succeed than a single particle.

If Q is small, there is strong selection for co-packaging, even though there is

a likely tradeoff between double-particle and single-particle production. This result is simply that a double particle has a much higher probability of success than a single particle. The same conclusion applies to f1 and Ike, but an important difference is that Q_{f1} may be different than Q_{Ike}. Thus, the system may be more predisposed to evolve co-packaging by Ike than by f1, or vice versa, but as long as Q is small for both, both phages will benefit from co-packaging, regardless of which coat surrounds them. The passaging was conducted so that Q for the most abundant phage in the supernatant was 0.1 – 0.01 (data not shown). In the early passages, f1 dominated the supernatant, and Ike was much rarer (Fig. 4), so the system should have been most predisposed to evolve co-packaging of Ike by f1, which is what evolved.

Conflict mediation by the evolution of co-packaging

The two phages evolved an elegant system in which the opportunity for conflict between them was greatly minimized. Conflict mediation was achieved principally via molecular changes allowing both phage genomes to be packaged into the same protein coat, minimizing conflict during the phase of infectious transmission. During this step each phage is selected for maximal reproduction at the potential expense of the other. However, by packaging both genomes together their fitness interests become coupled and conflict reduces. Coupling of fitness interests is a common factor promoting cooperation between individuals, also known as partner fidelity feedback (Bull and Rice 1991; Sachs et al. 2004). Partner fidelity feedback stabilizes cooperation between longstanding neighbors (Nowak and May 1992; Doebeli and Knowlton 1998), in vertically transmitted symbionts and organelles (Fine 1975; Axelrod and Hamilton 1981; Bull and Rice 1991), and any case where repeated or long-term interactions couple fitness between interactants (Frank 1994; Sachs et al. 2004). Experimental work has shown the

efficacy of partner fidelity feedback (via vertical transmission) in selecting for benevolence (Bull et al. 1991; Bull and Molineux 1992; Messenger et al. 1999).

While the evolution of co-packaging reduced conflict between f1 and IKe it was probably not favored for this reason. As our model above shows, the initial rarity of IKe relative to f1 strongly favored packaging of both genomes into f1 coats, simply as a mechanism ensuring survival to the next stage in the life history. It is illuminating that the mechanism of conflict mediation (co-packaging) initially evolved for a different reason. This highlights the importance of the genetic details of a system in deciding its trajectory towards cooperation or conflict.

The evolution of co-packaging apparently provided an overall benefit for both phages. All our measures of phenotypic change were consistent with the evolution of cooperation between IKe and f1. Fitness during vertical transmission increased almost nine-fold over the experiment (see fig. 10), and both phages enjoyed significantly increased fitness during infectious transmission as well; (see fig. 11). Assays of phage genome packaging revealed the evolution of a symbiotic relationship between the phages: co-packaging of genomes evolved from an initial rare event to where a substantial proportion of phages packaged both genomes (see fig. 9).

Genome minimization and evolution of cross-packaging

The genome minimization in IKe potentially provided an overall benefit for both phages, probably because it reduced costs to the host as well as minimized competition and interference between the two phages. However, genome minimization in IKe was necessarily preceded by the evolution of cross-packaging in f1 coats, and the emergence and fixation of cross-packaging by IKe could be construed as the evolution of parasitism. Whereas co-packaging is a clear (though unexpected) route to cooperation in this system, cross-packaging offers a pathway to conflict. Cross-packaging of genomes allows one partner to selfishly sequester

key resources from its partner (coat proteins and morphogenetic proteins) at the potential cost to the other.

If cross packaging and genome minimization by IKe represent selfish evolution, this was not born out by the fitness data: f1 enjoyed increased fitness throughout most of the experiment, though appeared to level off between passages 40 and 50. However, the details of the molecular evolution offer the clearest evidence against a parasitic IKe model: we propose that the f1 gene V mutation drove the cross-packaging, whereas IKe was mainly passive. Under our model the gene V mutation in f1 is jointly responsible for the increase of cross-packaging rate in IKe *and* the decrease in f1. The IKe gene V protein binds single stranded genomes in preparation for packaging (Salstrom and Pratt 1971; Model and Russel 1988), and also acts as a translational repressor of II: production of pV promotes the shift from replication of double-stranded genomes to single-stranded genomes ready for packaging (Michel and Zinder 1989a). Under our model the f1 gene V mutation drives cross-binding of the f1 gene V protein (f1pV) to IKe genomes, and when f1pV is later exchanged for the proteins of the coat (Russel 1992) the f1pV bound genomes are preferentially packaged in f1 coats.

This conjecture is based on two main assumptions, that f1pV is genome specific in f1-IKe co-infections and that the type of pV bound on a genome leads causes transfer of the same type of coat proteins onto that genome. There is some support for pV specificity in co-infections: while V can be successfully interchanged between f1 and IKe genomes (Russel 1992), Michel and Zinder (1989b) showed specific binding of V to the gene II RNA-operator (which differ between f1 and IKe), and suggest that this may act as a nucleation site for binding of pV to single-stranded genomes.

Parallels to natural systems

The evolution of cooperation between f1 and IKe in our experiment shares key features with a variety of natural systems in which conflict mediation has evolved. Three main parallels are drawn: evolution of reduced genomes, obligate dependence in symbionts and organelles and the evolution of co-transmission of symbiotic partners.

i) Reduced genomes

Reduced genomes are a common feature in vertically transmitted symbionts. This phenomenon is particularly well demonstrated in bacteria that form ancient associations with eukaryotic cells (Palmer 1997; Moran and Wernegreen 2000) including organelles. Genome reduction can result from selection for streamlining, as we predict drove genome reduction in IKe, or via drift. Streamlining may evolve cooperatively, to minimize costs to hosts with which the symbiont shares fitness interests (Fine 1975; Axelrod and Hamilton 1981; Sachs et al. 2004) or may directly benefit the symbiont by removing metabolic pathways which are redundant with the host (Moran and Wernegreen 2000). Drift is also clearly implicated in the minimization of such 'resident' genomes, as they are exposed to repeated bottlenecks and replication in small populations (Andersson and Kurland 1998). However, as the genome reduction in IKe was associated with a three-fold fitness gain in coinfected cell fitness (see fig. 10), it was unlikely to be a drift process.

ii) Obligate coexistence

Obligate coexistence in symbionts may derive from such genome streamlining. Loss of genes in symbionts whose function are shared with the host may accumulate until independent existence becomes impossible. Gene loss in IKe rendered it unable to independently produce infectious progeny, as it lost all packaging and morphogenetic functions. IKe, however, retained the ability to be

vertically transmitted on its own, since it contained genes II, X and its replication origins which allow it to undergo replication in a plasmid-like state. Filamentous phage have been shown to evolve loss of infectiousness under strict vertical transmission (Bull et al. 1991; Bull and Molineux 1992), though without genomic data, it is unclear if these cases were due to streamlining or drift.

iii) Co-transmission of symbiotic partners

Co-transmission evolved between f1 and IKe in the form of co-packaged genomes. Partner fidelity feedback (Bull and Rice 1991; Sachs et al. 2004) existed during the vertical transmission portion of our protocol and represents selection for cooperation. Whereas during infectious transmission f1 and IKe could spread separately, and without partner fidelity feedback selfish interests were promoted. However, evolutionary enhancement of partner fidelity (Bull and Rice 1991; Sachs et al. 2004) was a result of the evolution of co-packaging, and this finds parallels in many cooperative symbioses.

The extreme case of partner fidelity is vertical transmission, for example where symbionts are locked within a host lineage, and cooperation is favored (Fine 1975; Axelrod and Hamilton 1981). However symbiotic partners are not always locked together as such and diverse mechanisms have evolved whereby individuals enhance their chance of remaining with a current partner. A parallel example from an experimental system is the evolution of host-genome integration in vertically transmitted phage (Bull and Molineux 1992). Other examples include the evolution of adhesive polymers (Rainey and Rainey 2003) and socially-dependent swarming (Velicer and Yu 2003) in social bacteria. In these two cases phenotypes evolved which enhanced adhesiveness between individuals, thus coupling their fates. Enhancement of partner fidelity feedback has also evolved between species. Examples include vegetative and sexual co-propagation in lichens (Sanders and

Lücking 2002) and semi-closed transmission of algal symbionts in symbiotic jellyfish (Montgomery and Kremer 1995).

Similarly f1 and IKe, forced to undergo phases of mixing (horizontal transmission), evolved co-packaging and enhanced co-transmission (partner fidelity feedback) of cooperating partners. Theory predicts that such adaptations enhancing partner fidelity feedback are more likely to evolve in hosts, while symbionts are favored to disperse out of their host lineage, and thus diminish partner fidelity (Frank 1996). However, under Frank's model symbiont dispersal becomes disfavored if the likelihood of successfully colonizing a new host is relatively small. Under the early conditions of our experiment this may have been the case. Initially IKe had extremely low fitness: most cells (>97%) were only infected with f1 when both antibiotics were added and thus were evolutionary dead ends. The mutations in f1 gene V and IKe gene III apparently enhanced co-packaging rates and thus increased partner fidelity between the phages. However this enhancement of partner fidelity (and thus cooperation) is completely explained via the selfish benefit of assured coinfection.

Thus, mechanisms that enhance partner fidelity have evolved under diverse settings in nature, and emerged relatively quickly under our design. It has been suggested that conflict mediation is a major hurdle to be overcome in the major transitions of evolution (Maynard Smith and Szathmary 1995) however the generality of mechanisms that mediate conflict between selfish partners may render this hurdle minimal. Perhaps such evolutionary transitions, where individuals become integrated into a larger whole (Maynard Smith and Szathmary 1995) are more limited by physiological, genetic or mechanistic constraints than conflict between component parts. It would be illuminating to attempt another experiment like the present one in which co-packaging of genomes was impossible, and see if other mechanisms emerged which promoted partner fidelity feedback.

Partner fidelity feedback is a general force which by which conflict is mediated between those individuals (Bull and Rice 1991; Sachs et al. 2004). While extrinsic forces (i.e. viscous environments) can augment repeated interactions between partners, partner fidelity is limited by the strength of those forces and other mechanisms (discussed above) may evolve by which to enhance partner fidelity. The evolutionary origin of cooperation may work in such a fashion, where *extrinsic* forces initially allow fitness interests between partners to be aligned, and further cooperation is favored only when *intrinsic* mechanisms that enhance partner fidelity evolve. However, we do not suggest that selection necessarily favors the evolutionary enhancement of partner fidelity, only that further evolution of cooperation may be limited without such enhancement.

Conclusion

This experiment provides the first empirical analysis of the genotypic and phenotypic details inherent to the evolution origin of cooperation. The origin of cooperation between f1 and IKe, defined by overall increased fitness in both players, was characterized by the evolution of co-transmission of partners and a reduced genome driving obligate coexistence (in IKe). While further study would be necessary to establish the generality of these features, convergent evolution of enhanced partner fidelity in diverse natural systems of cooperation is suggestive its significance. These data also provide empirical data to test theory on the evolutionary origins of cooperation. Our experiment matches many characteristics of the stochastic corrector model for the evolutionary origin of replicator cooperation (Szathmary and Demeter 1987). The similarity of our results to the model's predictions lend credence to the assumptions of this model, and provide the first empirical demonstration of *de novo* evolution of cooperation between genomes.

CHAPTER III:

Cheating in the Cnidarian symbiont *Symbiodinium microadriaticum*

CHAPTER ABSTRACT

Symbioses with horizontal transmission are ubiquitous, however their evolutionary maintenance remains obscure. Theory predicts exploitation of the host by horizontally transmitted symbionts, yet they are commonly beneficial. We experimentally altered algal symbiont transmission between jellyfish hosts to examine the evolution of cheating in a horizontally transmitted mutualism. Our experiments uncovered cheater algal symbionts. Cheaters emerged under repeated horizontal transmission, significantly lowering host fitness. However, transmission of cheaters appears to be limited by the severe harm they cause their hosts. Our results demonstrate the dynamic nature of this infectious symbiosis, and illuminate predictions about the evolution of bleaching in algal hosts.

INTRODUCTION

Understanding the stability of cooperation against cheating remains a critical problem in evolutionary biology (Hardin 1968; Axelrod and Hamilton 1981; Bull and Rice 1991; Frank 1994, 1996a,b; Sachs et al. 2004). Symbioses, intimate interactions between species, provide some paradoxical examples of mutual aid (Savage 1977; Sprent et al. 1987; Trench 1993; Ruby 1996). For symbionts which offer costly benefits to hosts, we must explain what prevents them from cheating and thus gaining reproductive advantage over cooperating (beneficial) symbionts (Hardin 1968; Axelrod and Hamilton 1981; Bull and Rice 1991; Frank 1994, 1996a,b, Sachs et al. 2004). Cheating occurs when a symbiont

receives benefits from its host with little or no reciprocation (Soberon and Martinez 1985; Bronstein 2003): the symbiont enjoys fitness benefits at a cost to the host's fitness (Sachs et al. 2004). Optimal virulence theory, developed to study pathogen evolution, predicts cooperation in symbionts that are transmitted from host parent to offspring (vertical transmission) because symbionts are locked within a host lineage, leaving little opportunity for conflict (Fine 1975; Axelrod and Hamilton 1981; Bull and Rice 1991; Bull 1994; Frank 1994). However, horizontal transmission, in which symbionts are acquired by hosts from the environment, decouples symbiont fitness interests from the host, allowing cheaters to spread (Fine 1975; Axelrod and Hamilton 1981; Ewald 1983; Bull and Rice 1991; Bull 1994; Frank 1994, 1996a,b; Sachs et al. 2004). Horizontal transmission also promotes co-infection by unrelated symbionts that compete for host resources, potentially to the detriment of the host (Frank 1994, 1996a,b). Paradoxically, horizontally transmitted mutualists are common and diverse in nature. They include algal symbionts in a wide array of marine invertebrates (Trench 1993), mammalian gut-symbionts (Savage 1977), nitrogen-fixing rhizobia in legumes (Sprent 1987), and bioluminescent bacteria in fish and squids (Ruby 1996). While some work suggests that cheating may be common in cooperative systems (Soberon and Martinez 1985; Bronstein 2003), the data in support of symbiotic cheating has been meager. Demonstrating cheating requires showing that symbionts enjoy fitness benefits at a fitness cost to the host, as opposed to the presence of ineffective symbionts or a poorly matched host-symbiont combinations in which both symbiont and host potentially suffer lowered fitness.

THE EXPERIMENT

Here, we determine the effect of altering transmission mode on symbiont cheating in a horizontally transmitted algal symbiont of the upside-down jellyfish (*Cassiopea xamachana*). The dinoflagellate algae provide their marine hosts

including corals hydras, and jellyfish with photosynthates (Balderston and Claus 1969), in exchange for nitrogen and inorganic nutrients (Muscatine 1990). Upside-down jellyfish are born symbiont-free, disperse from their mother as planula larvae, and acquire algae from the environment once they have reached the sessile polyp stage (Fig. 12). The asexual polyps can reproduce via clonal budding and infected polyps transmit algae to polyp offspring via vertical transmission. Once infected, polyps undergo metamorphosis to the medusa stage. Most Cnidarian hosts acquire symbiotic algae infectiously (Fadlallah 1983; Babcock and Heyward 1986; Harrison and Wallace 1990) and their symbionts may thus be selected for host exploitation (Fine 1975; Axelrod and Hamilton 1981; Ewald 1983; Bull and Rice 1991; Bull 1994; Frank 1994, 1996a,b). While such exploitation is predicted on theoretical grounds, symbiont cheating has been little investigated in marine symbioses.

We gathered algae from wild jellyfish medusae, infected them into a clone of symbiont-free polyps, and experimentally manipulated transmission mode. In one treatment, buds produced by infected, isoclonal polyps were used as offspring for new host generations, thus enforcing vertical transmission. In the other treatment, horizontal transmission was enforced by infecting a new generation of uninfected host polyps (from the same isoclonal line) with algae expelled from the previous generation of hosts. Treatments were each replicated threefold. Two rounds of experimental transmission followed initial infection, with seven weeks between transmission rounds (Fig. 13). To determine the effects of the treatments we estimated the fitness of hosts and symbionts after experimental selection. During selection algal density within a polyp may have diverged between treatments. To control for this during our fitness assays, we created new populations of infected polyps by: 1) extracting the selected algae from experimental polyps, 2) separately exposing uninfected isoclonal polyps to equal densities of the evolved algae from each treatment, and 3) waiting 90 days to allow

symbionts to fully populate hosts. Host fitness was estimated by counting buds released from polyps (asexual reproduction) and measuring growth rate of polyps (a predictor of time to maturation) over two week periods. Symbiont fitness was estimated by measuring the rate that algae were expelled from hosts (a measure of infectiousness), the density of algae within the host (a measure of symbiont effectiveness), as well as mitotic index (proportion of algal cells undergoing cytokinesis within the host). To assess whether fitness effects of experimental selection generalized across host genotypes, we also infected the evolved algae into three novel host genotypes.

Cheaters could potentially exist in the initial pool of algae or emerge over the course of the experiment. We assume a genetically diverse initial pool of algae as they were sampled over a wide range. Opportunity for generation of novel mutants during the experiment was minimal: based on the fastest doubling-time reports of 1.4 - 6 days for related algae within-host, there were maximally 60 - 257 doublings during the experiment (Hansen and Nielsen 1997; Fitt 2000).

MATERIALS AND METHODS

Host and symbiont collection

Hosts for experimental evolution originated from a planula larva of a female jellyfish collected at Keys Marine Lab, Long Key (N 24^{o} 49', W 80^{o} 49') that was grown up to a clone of symbiont-free polyps in the lab. Alternate hosts for re-infection experiments were half-sibling groups of polyps from a single mother, and unknown father(s), gathered at Grassy Key (N 24^{o} 45', W 80^{o} 59'), Upper-Matecumbe Key (N 24^{o} 54', W 80^{o} 38') and Key Largo (N 25^{o} 05', W 80^{o} 27'). Host planulae were rinsed of maternal tissue and raised into algae-free polyps. Algae were collected from 2 medusae (one large, one small) at each of ten sites along a 120 mile transect ranging from Key Largo in the northeast (N 25^{o} 05', W

80° 27') to Geiger key in the southwest (N 24° 35', W 81° 39'). Previous work confirmed that multiple strains of one species, *S. microadriaticum* infects *C. xamachana* in the sampled range (Wilcox, personal communication).

Infection and experimental selection

An equal density mix of algal isolates was added to 180 isoclonal polyps in artificial sea water (ASW), at a final concentration of 10^3 algae/ml. Infection lasted 48 hours before the polyps were divided into 6 flasks: 2 treatment lines – horizontal and vertical transmission, 3 replicates per line, 30 polyps per replicate. All infected polyps were fed tri-weekly to repletion on *Artemia salina* nauplii, and incubated at 21°C on a 12:12 hour light-dark cycle. *Cassiopea* polyps will not undergo metamorphosis to the medusa stage at 21°C, but will continue to produce asexual buds. ASW was changed each feeding, and flasks replaced weekly to minimize free algae. In the vertical treatment, polyp buds were collected in separate flasks. In the horizontal treatment, buds were discarded. Polyp lines were maintained until algal expulsion was detected (free swimming *Symbiodinium* could be seen in the culture water – seven weeks in all cases). Transmission of symbionts to the next generation of hosts was then initiated. In the vertical treatment, new lines were established using 30 randomly chosen polyps arising from buds previously saved. These polyps inherited their algal symbionts from the parent polyp, so symbiont transmission was strictly vertical. New polyps were then placed in a clean flask with fresh ASW and maintained as previously described. In the horizontal treatment, the next generation of hosts were to acquire their symbionts from environmental sources. Therefore, for each line the previous generation of polyps were placed in clean flasks containing filtered ASW. These flasks were placed in the incubator and left undisturbed for 48 hours. Infected polyps were then removed from the flasks, while the ASW that contained expelled algae was retained. Into this water were placed 30 uninfected polyps (from the original isoclonal polyp line). The new polyps were

allowed to acquire algae from the water for 48 hours before a normal feeding and water change. The polyp lines were then maintained as previously described. After two rounds of transmission, within-host algal densities between treatments may have diverged. Therefore, prior to estimating fitness we created another host generation in which all lines were exposed to equal densities of algae. For each line, evolved algae were extracted from hosts by grinding. The density of algae within the resulting slurry was estimated and new host lines created by adding equal densities of algae to flasks containing 30 uninfected polyps from the original isoclonal line. In parallel, the three alternate hosts were also infected. After 90 days of infection, the fitness of hosts and symbionts were estimated.

Fitness estimation.

Two types of fitness assays were performed on the host and three on the symbiont. Host fitness was estimated with budding rate and growth rate. From each replicate flask 12 polyps were randomly chosen and separated into 6-well culture dishes with 5 ml of ASW. The diameter of each polyp was measured using a drawing tube mounted to a dissecting microscope and tracing the polyp with a computer linked drawing board (Wacom TM). Polyps were monitored for two weeks during which released buds were counted and growth assessed. Growth was measured as change in polyp diameter, and units were transformed to mg protein biomass using a curve standardized to uninfected polyps: mg protein = 0.78Log10((Diameter/103)+1)). Algal fitness was estimated using expulsion rate of algae from the host, density within the host and mitotic index. After host fitness assays polyps were separated into microfuge tubes with 1 ml of ASW, returned to the incubator for 48 hours, then removed. The tube was then centrifuged to pellet expelled algae, and the pellet was re-suspended in 50 μl ASW for manual counting of algae on a hemacytometer. All polyps were then frozen and stored at –20^{o}C for the remaining analyses. Density within the host was estimated by counting algae from whole ground hosts

(re-suspended in 100 μl of ASW) and dividing by host biomass. Finally, a squash preparation from each polyp was used to estimate mitotic rate of the algae by estimating the proportion of algal cells with division plates (Wilkerson et al. 1983). All polyps were frozen in late morning (approximately 10-11am), to minimize diurnal variation in mitotic index measures.

RESULTS

As predicted by theory (Fine 1975; Axelrod and Hamilton 1981; Bull and Rice 1991; Frank 1994, 1996a,b; Sachs et al. 2004), experimental enforcement of horizontal transmission selected for cheating symbionts. Algae from the horizontal treatment had significantly higher division rates within their hosts (ANOVA, $P = 0.003$, $N = 51$), attained higher density within hosts (ANOVA, $P = 0.03$, $N = 51$), and had significantly higher expulsion rates (with host biomass as a covariate) from their hosts (ANCOVA, $P < 0.05$, $N = 52$), while causing reductions in host growth (ANOVA, $P < 0.001$, $N = 52$) and budding (ANOVA, $P < 0.001$, $N = 52$: Fig. 14) compared to the vertical treatment. Under the horizontal treatment 30% of hosts shrunk by 10% or more in size and only 35% grew more than 10%. In contrast, in the vertical treatment only 4% shrunk more than 10% while 88% grew greater than 10%. Treatment effects on host growth were consistent even in different host genotypes (Fig. 15): evolved algae that were infected into hosts from three other locales had similar treatment effects on host growth (full factorial ANOVA, $P < 0.001$, $N = 18$).

DISCUSSION

Although cheater algae are present (or evolved quickly) in natural isolates, the fitness costs that they impose upon hosts may ultimately hinder their spread. Two pieces of evidence suggest a trade-off exists between a symbiont's virulence (harm caused to host: Bull 1994) and its own reproduction. First, there is a strong

negative correlation between within-host algal division rate (mitotic index) and host growth (R^2= 0.834, P = 0.011); evidence that fast growing symbionts stunt their host's growth. Second, while algal expulsion rate per unit of host biomass was significantly higher in the horizontal treatment, total expulsion did not differ between treatments (ANOVA, P = 0.39, N = 52). This results from a positive correlation between host size and algal expulsion rate (R^2 = 0.34, P < 0.001) and the smaller size (lower growth rate) of hosts infected with horizontally propagated algae. Thus, fast symbiont division within hosts caused growth deficits in their hosts, and small hosts expelled fewer algae overall. Just as optimal virulence is predicted to be driven by such trade-offs in pathogens (Fine 1974; Ewald 1983; Bull 1994) evolution of symbiont cheating may be limited by a trade-off against the symbiont's spread to new hosts.

Symbiont cheating may be prevalent in symbioses with horizontal transmission (Savage 1977; Sprent et al. 1987; Trench 1993; Ruby 1996). However, sufficient evidence for cheating has been scarce even in well studied systems. This stems in part from the difficulty in estimating symbiont fitness. For example, many plants benefit from infection with bacterial or fungal root symbionts that exchange N (Rhizobia) or P (mycorrhiza) with plants respectively. Cheating may be rampant in these symbionts: 'ineffective' strains delivering little or none of the above nutrients to plants are well characterized (Singleton and Stockinger 1983; Smith 1996). However, data demonstrating that these symbionts enjoy clear fitness benefits while reducing host fitness has been lacking. These data would distinguish exploitative symbionts from ones that are poorly matched with a specific host, offer other benefits to hosts, or offer condition-dependent benefits.

Our results may also have implications for understanding the evolution of coral bleaching. Bleaching, best known in corals, has received widespread interest in biology, and is an important factor damaging fragile ecosystems (Buddemeier and Fautin 1993; Baker 2001; Douglas 2003). Bleaching is a partial to total loss of

algae as a generalized stress response to environmental insult (Cook 1990), and affects corals, jellyfish, anemones, clams and sponges (Douglas 2003). An adaptive hypothesis has been proposed for bleaching: host and/or symbiont benefit from separation and forming novel combinations of partners under conditions of rapid environmental change (Buddemeier and Fautin 1993). Experiments on corals have provided evidence consistent with host benefit to re-sampling symbionts (Baker 2001). Our results question this benefit. Repeated shuffling of algae in our horizontal treatment allowed hosts to sample the symbiont population, however these hosts suffered reduced growth and budding. A tacit assumption to some adaptive bleaching models is that hosts can control symbiont infection or expulsion (Baker 2001). However, in our experiment exploitative algae grew to higher densities within their hosts and this argues against host control, or simply that host control was overridden in our experiment. We conducted our experiments on polyps and not mature hosts, and it is at the polyp stage in which infection initiates. Thus, if hosts have mechanisms of partner choice (Bull and Rice 1991; Sachs et al. 2004) of symbionts we would expect these mechanisms to be active in the polyp stage. The degree of host or symbiont control is poorly understood in this system. Domination of control by either partner can alter the evolution of the symbiosis (Frank 1996b), so this problem is critical to understanding the maintenance as well as the potential breakdown of marine-algal symbioses.

Our results suggest that cheater algal symbionts exist in wild upside-down jellyfish populations. With repeated horizontal transmission, we uncovered symbionts that, relative to vertically transmitted algae, grew faster within their hosts, attained higher densities within hosts and were expelled at higher rates (per-host mass). This gain in within-host fitness came at a cost. Hosts infected with horizontally transmitted algae grew significantly less. Because algal expulsion and host size are positively correlated, the smaller size of these hosts resulted in reduced overall symbiont transmission. Thus, the spread of cheaters in natural

populations may be limited by their detriment to host fitness. We suggest that such trade-offs can stabilize cooperation in many horizontally transmitted symbioses.

Figure 1. Partner fidelity feedback

Benefits transferred from X to Y feed back through an extended series of exchanges. (B_x, B_y = Benefits to X, Y respectively; C_x, C_y = Costs to X, Y respectively. $B_x = f(B_y)$ means that benefits to X are a function of the benefits to Y. $f'(B_y)>0$ indicates that B_x increases as B_y increase. B, C are always positive.)

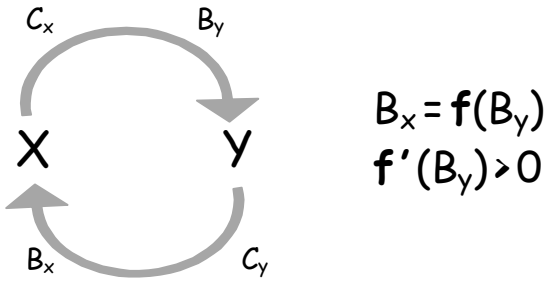


Figure 2. Partner Choice

Either individual X_1 or X_2 receives a benefit from Y, depending on Y's choice. Y chooses to interact with the more cooperative X individual. (B_{x1}, B_{x2} = Benefits to X_1, X_2 respectively, B_{y1}, B_{y2} = Benefits to Y from X_1, X_2 respectively; C_y, is the cost to Y.)

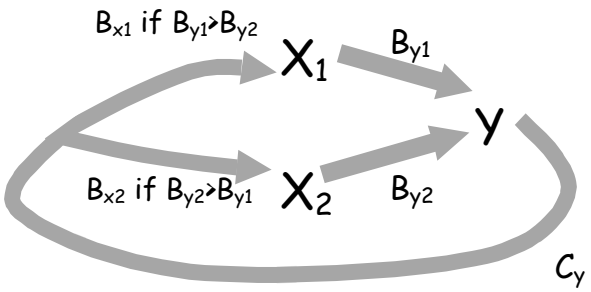
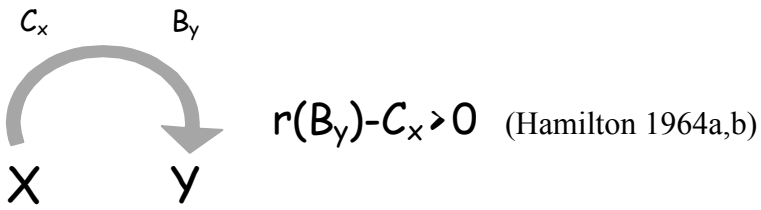


Figure 3. Kin Selection

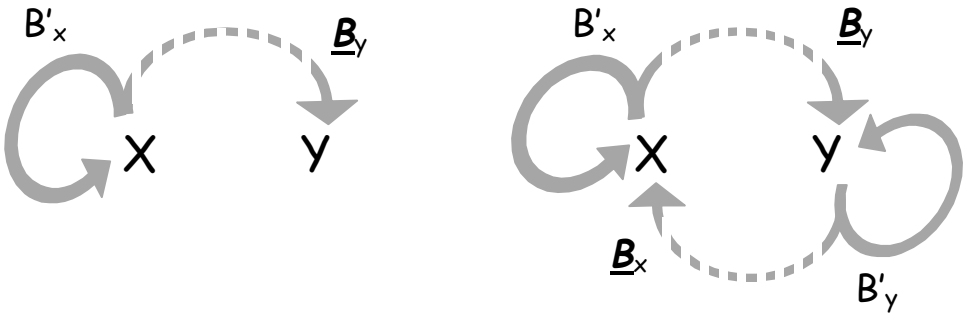
X evolves to benefits Y if $rb_y - c_x > 0$. (r = coefficient of relatedness between X and Y; c_x = the cost of the act to X; b_y = the benefit of the act to Y.)



(Hamilton 1964a,b)

Figure 4. Basic byproduct benefits

Left: An act of X benefits Y as an automatic consequence (byproduct) X's self interested action (one sided). *Right*: Likewise, individual Y may, when performing an act that benefits itself, also benefit X (two sided). (B'_x, B'_y are benefits of self interest to X, Y respectively. $\underline{B}_x, \underline{B}_y$ Are byproduct benefits to X, Y respectively. Dashed lines refer to byproduct benefits).



One Sided Two Sided

Figure 5. Byproduct reciprocity

Y evolves to enhance its benefit to X, which in turn increases the by-products it receives from X. (B'_x is a benefit of self-interest to X, $\underline{B}_y$ is a by-product benefit to Y. B_x is a benefit to B. Dashed lines refer to byproduct benefits.)

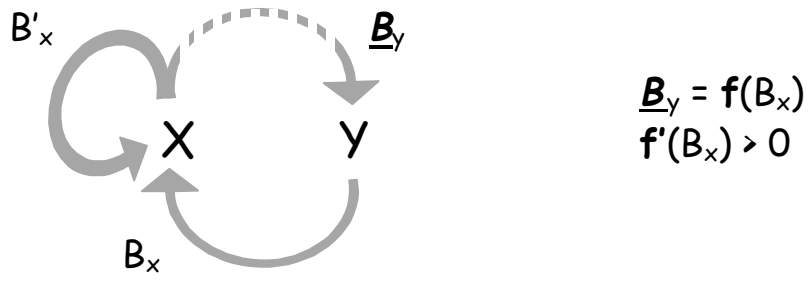


Figure 6. Passaging protocol

The iterated three step protocol: I) Encounter: coinfection of f1 and IKe in *Escherichia coli*, II) Growth: enforcement of paired vertical transmission, III) Reproduction: production of infectious bacteriophage progeny to complete the cycle. Fifty passages of the above cycle were performed.

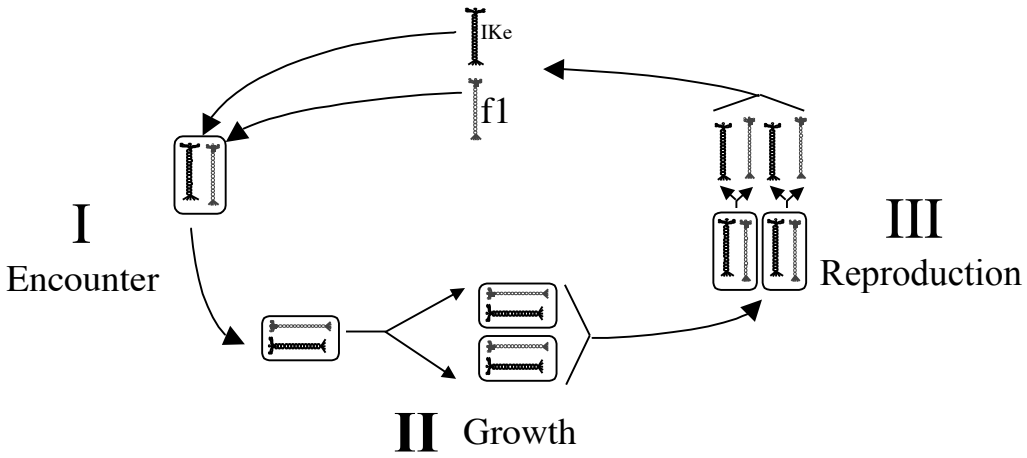


Figure 7. Cross-packaging and co-packaging assays

Phage supernatants from time points in the evolution were infected into each of the cell lines at low multiplicities. A527 cells (N3 pili) could only be infected by phages with IKe coats, K19 cells (F pili) could only be infected with phages with f1 coats, and K1037 cells (both pili), could be infected with either. Resultant colonies growing on Kanamycin (Kn^R) contain an IKe genome, growing on Chloramphenicol (Cm^R) contain an f1 genome, and colonies growing on both (Kn^R/Cm^R) contain both genomes. Cross packaging rates were calculated as B/A+B (f1 genome in IKe coat) and D/D+E (IKe genome in f1 coat). Rates of co-packaging for each coat type were calculated as C/A+B+C (both genomes in IKe coat), F/D+E+F (both genomes in f1 coat). Total co-packaging rates were calculated in cells with both pili I/G+H+I (both genomes in either coat).

Pilus:	Kn^R	Cm^R	Kn^R/Cm^R
N3 (Ike coats)	A	B	C
F (f1 coats)	D	E	F
Both (either coat)	G	H	I

Figure 8. IKe genome minimization

IKe minimal phage visibly emerges in passage 41 and spreads to apparent fixation by passage 43 (Extinction of IKe-full). Agarose electrophoresis of f1 and IKe DNA genomic preparations of co-infected cells from passage numbers 38-45. Passages are indicated above each lane, and two standards are on the right: IKe-min is a DNA preparation of the minimal IKe phage from passage 50, whereas IKe-full is the IKe used to initiate the experiment. The asterisk indicates the passage that the minimal IKe genome is first detectable by electrophoresis.

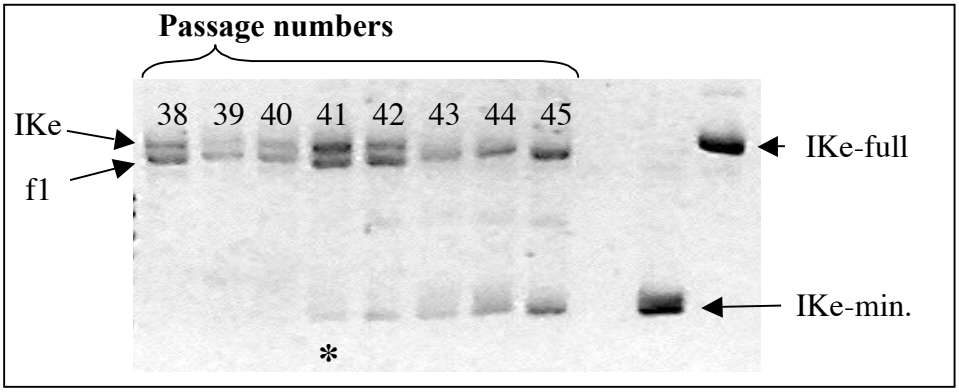


Figure 9. IKe and f1 cross-packaging and co-packaging rates

Cross packaging rates evolved in opposite directions and co-packaging rates increase 150 fold. Percentage and standard error of cross-packaging and co-packaging rates are shown for f1 and IKe at passages 1, 20 and 40. At passage 50, only co-packaging rates are shown, as all phages were packaged in the f1 coat.

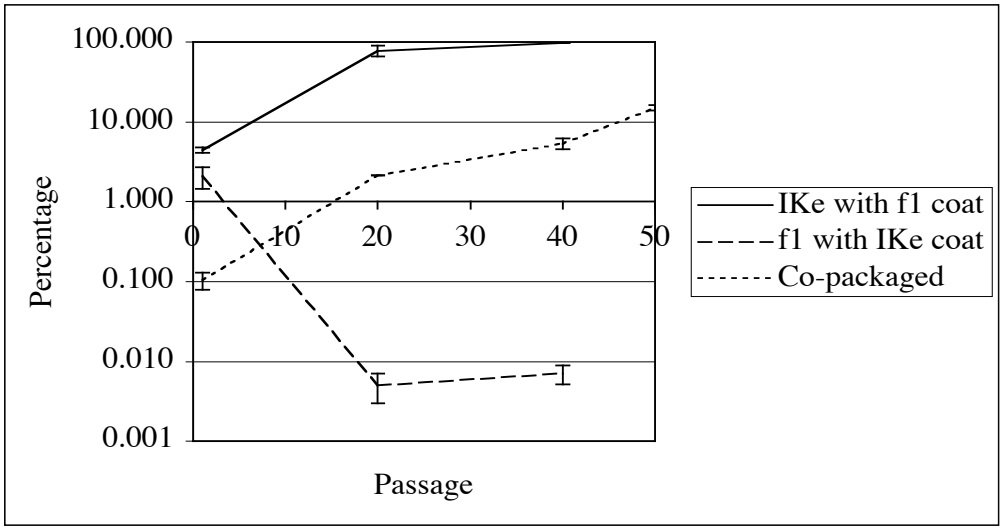


Figure 10. Vertical transmission fitness component

Vertical transmission fitness increased significantly over the course of the experiment. Mean and standard errors of relative fitness are shown for passages 38 and 50 relative to passage 1. Replicated competition experiments were conducted with passage 1 versus 38, and passage 1 versus 50.

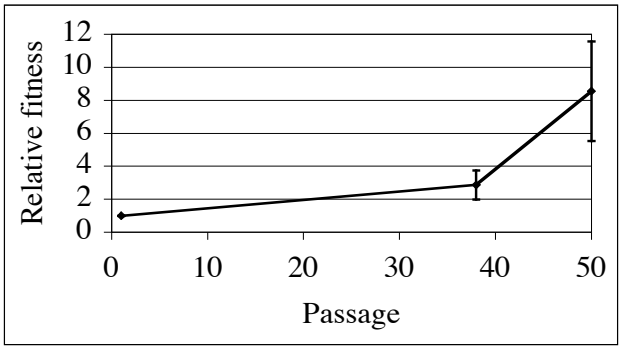


Figure 11. Infectious transmission fitness component

Infectious transmission fitness increased significantly for both phages over the course of the experiment. Mean and standard errors of phage produced per hour are indicated for passages 1, 20, 40 and 50. Ike-0, f1-0 lines indicate the individual phage production rates of the two ancestral phages.

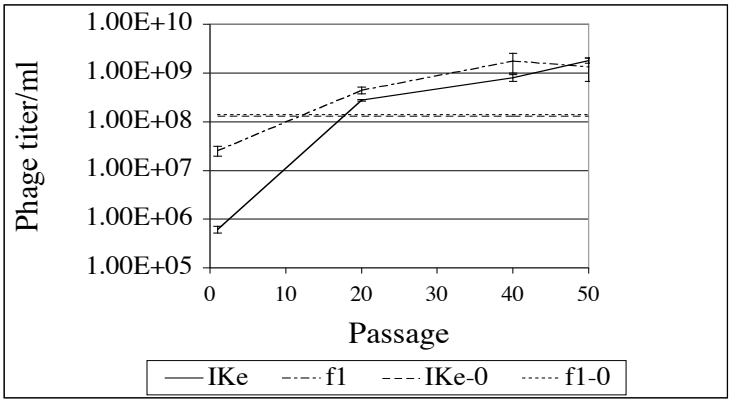


Figure 12. *Cassiopea-Symbiodinium* symbiosis life cycle.

Algal symbionts are transmitted horizontally between generations. Female medusae (sexual adults) release planula larvae (a) that disperse and settle as uninfected polyps. Uninfected polyps bud to produce clonal offspring (b) ultimately becoming infected by environmental algal symbionts (c). Infected polyps bud producing clonal offspring that inherit algae via vertical transmission (d). Once infected, polyps undergo metamorphosis (e). Both medusa and infected polyps release algae into the environment (f) and may be the source of new infections (c).

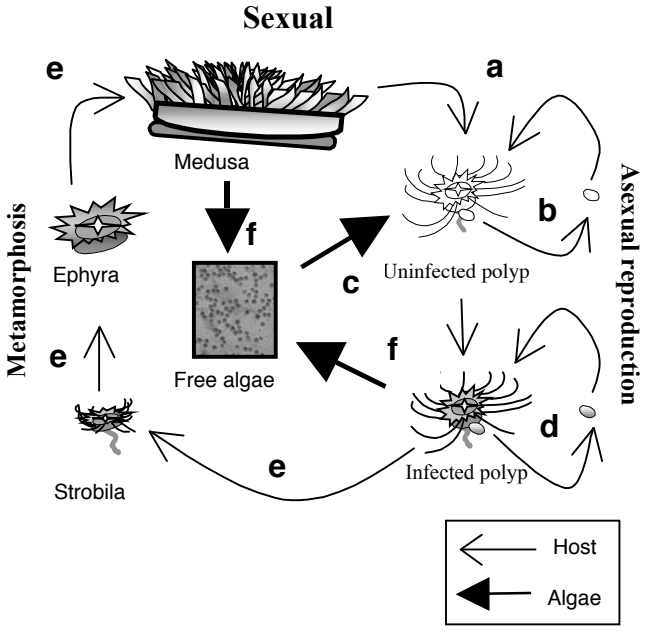


Figure 13. Experimental design

Vertical treatment is above (V): buds released from polyps are saved in a separate flask, where they settle into new polyps. After seven weeks of infection 30 polyps are randomly selected from the newly settled pool. These settled polyps represent the next generation. Horizontal treatment, in which buds are discarded, is below (H). After 7 weeks, polyps are put into a new flask with ASW for 48 hours of algal expulsion. Thirty isoclonal uninfected polyps are infected with the expelled algae. The newly infected polyps represent the next generation. In both treatments there are two rounds of transmission after initial infection.

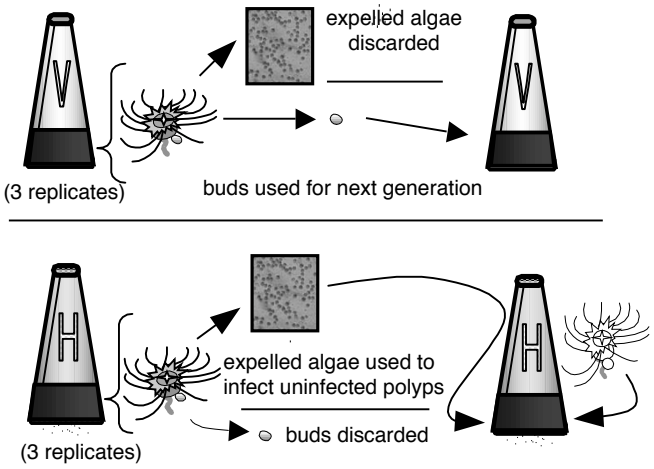


Figure 14. Host and symbiont fitness measures

Hosts experienced higher fitness in the vertical treatment, while symbiont fitness was higher in the horizontal treatment. Replicate means of horizontal (H) and vertical (V) treatments are shown. Polyps re-infected with vertical treatment algae grew at significantly higher rates (a), and budded at higher peak rates (b). The algae from the horizontal treatment divided at significantly higher rates in their hosts (c), and had significantly higher expulsion rates from their hosts - using host biomass as a covariate (d). All treatment effects were significant: in nested ANOVA a, $P < 0.001$, $N = 52$; b, $P < 0.05$, $N = 52$; c, $P = 0.003$, $N = 51$; nested ANCOVA with host biomass as a covariate d, $P < 0.05$, $N = 52$.

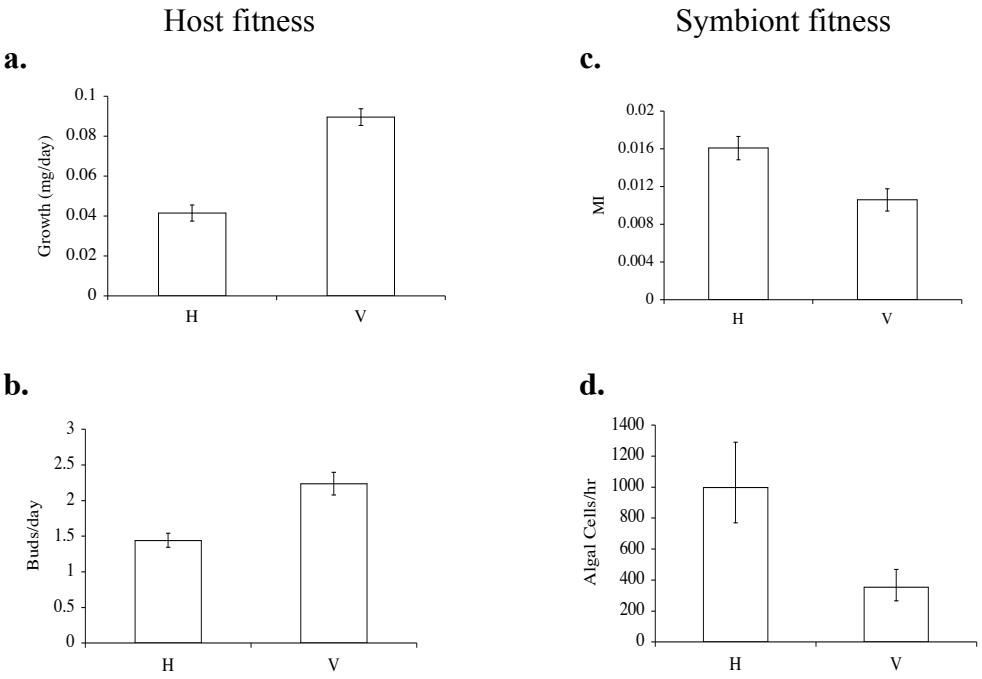


Figure 15. Alternate host fitness

Horizontal treatment algae reduced host growth irrespective of host genotype. Hosts were gathered from three disparate sites and infected with the experimentally evolved algae of each treatment: Coco Plum (CCP), Grassy Key Quarry (GKQ), and Matecumbe Key (MTK). *Significant treatment effect in full factorial ANOVA, $P < 0.001$, $N = 17$.

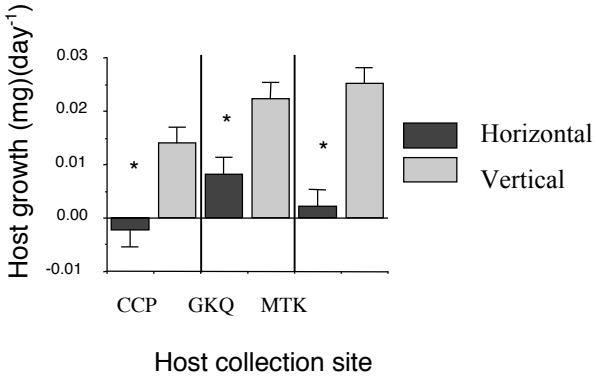


TABLE 1. Framework for the evolution of cooperation

General Models:	Specific Models:	Examples in text
Directed Reciprocation X undertakes a significant cost to benefit Y, and Y in turn reciprocates that benefit back to X. Operates within or between species. (Trivers 1971)	**Partner Fidelity Feedback:** X and Y are associated for an extended series of exchanges that last long enough that a feedback operates: the effect of benefits transferred from X to Y returns and enhances the fitness of X. Thus, by failing to cooperate, individual X ultimately curtails its own fitness. (Bull and Rice 1990, Nowak and May 1992, Frank 1994, Doebeli and Knowlton 1998,Simms and Taylor 2002)	-Vertically transmitted symbionts, optimal virulence evolution, ant-acacia mutualism.
	Partner Choice: Either individual X_1 or X_2 receives a benefit from Y, depending on Y's choice. Y chooses to interact with the X individual that offers greater fitness returns. (Darwin 1859, Eshel and Cavali-Sforza 1982, Noë 1990, Bull and Rice 1991, Peck 1992, Noë and Hammerstein 1994, Batali and Kitcher 1995, Frank 1995, 2003, Wilson and Dugatkin 1997, West et al 2002b, Kiers et al. 2003)	-Squid-light organ symbiosis, legume-rhizobium symbiosis, yucca-yucca moth symbiosis
Shared Genes X benefits Y that tends to carry the same genes as X. Operates within species only. (Hamilton 1964a,b)	**Kin Choice:** X *recognizes* and directs benefits to more closely related X_1 as opposed to more distantly related X_2 based on phenotype(s) of X_1, X_2. (Hamilton 1964a, Queller 2000)	-Admission rules in sweat bees, GP9 locus in fire ants, M-factors in beetles, csA genes in social Amoebae.
	Kin Fidelity: X directs benefits to X_1 base upon X_1's proximity to X. This proximity denotes shared genes with X. (Hamilton 1964a, West 2002a)	-Supression of conflict in siblingss and clonal microbes.
Byproducts X benefits Y as a byproduct of an otherwise selfish act of X. Operates within or between species. (West-Eberhard 1975, Brown 1983)	**One Way:** An act of X benefits Y as an automatic consequence (byproduct) of X's self interested action. (West-Eberhard 1975, Brown 1983, Connor 1995a)	-Vultures and lions, carrion feeders.
	Two Way: Both X and Y each benefit the other as automatic consequences (byproducts) of their own selfish actions. Includes synergism: actions or coordinated behavior that are automatically more fitness-enhancing when performed in groups. (Hamilton 1971,Queller 1985, Connor 1995a)	-Predator dilution in bugs, selfish herds, Mullerian mimicry.
	Byproducts Reciprocity: Y evolves to enhance its benefit to X, which in turn increases the byproducts it receives from X. The byproduct from X does not evolve, but the effect of Y on X does. (Connor 1986)	-Honeyguide-man mutualism

Table 2. f1 point mutations. We identify loci by Genbank nucleotide number (nt), 'Cm' indicates a locus within the chloramphenicol gene insert. Gene numbers are indicated where appropriate and functions of genes/intergenic DNA are predicted as well as putative function of the mutations in those regions. The passages in which mutations arose in and fixed were estimated via sequencing mutant stretches over multiple passages. 'Arose' indicates the earliest passage for which there is evidence of a mutant allele and 'fixed' indicates the first passage in which no peak was detected from the ancestral allele. Missense mutations are indicated with the gen-bank number of the mutant amino acid and the type of transversion is indicated. Non-coding mutations that affected secondary structure are indicated where the change affected predicted stem and loop structures.

nt	**gene**	**Function**	**Putative evolution:**	**Arose**	**Fixed**	**Amino-acid/ 2^0**
*Cm*186	na	Non-coding insert DNA	Unknown	2	10	Not applicable.
5674	IG	(-) Strand Origin	Anti-interference	11	20	Loop → Stem
957	V	ssDNA binding	cross-packaging	15	20	#39 Asn. → Asp.
5529	IG	Morphogenetic signal	cross-packaging	30	35	Stem → Loop
2988	VI	Minor coat protein	adaptation to *IKe-minimal* 44	50	45	Ala. → Ser.
4072	I	Assembly initiation	cross/co-packaging	44	50	#293 Asp. → Tyr.
5521	IG	Morphogenetic signal	cross-packaging	44	50	Stem → Loop
5529	IG	Morphogenetic signal	cross-packaging	42	44	Loop → Stem

Table 3. IKe point mutations and deletions: We identify loci by Genbank nucleotide number (nt). Deletion sizes are indicated. Gene numbers are indicated where appropriate and functions of genes/intergenic DNA are predicted as well as putative importance of the mutations. The passages in which mutations arose in and fixed were estimated via sequencing mutant stretches over multiple passages. 'Arose' indicates the earliest passage for which there is evidence of a mutant allele and 'fixed' indicates the first passage in which no peak was detected from the ancestral allele. Missense mutations are indicated with the Genbank number of the mutant amino acid and type of transversion is indicated. Non-coding mutations that affected secondary structure are indicated where the change affected predicted stem and loop structures. The asterisks indicate genes which never fixed.

nt	gene	Function	Putative evolution:	Arose	Fixed	Amino-acid/ 2^0
deletion 206nt	Non-coding	insert	Fast reproduction	12	15	Not applicable
2173	III	Minor coat protein	Multiple particles	20	30	#31 Tyr. → Met.
6377	IG	(-) Strand Origin	Anti-interference, Packaging	30	38	Stem → Stem
6379	IG	(-) Strand Origin	Anti-interference, Packaging	30	38	Loop → Stem
6382	IG	(-) Strand Origin	Anti-interference, Packaging	30	38	Loop → Stem
6379	IG	(-) Strand Origin	Anti-interference, Packaging	40	42	Stem → Loop
6377	IG	(-) Strand Origin	Anti-interference, Packaging	40	42	Stem → Stem
deletion	4541nt	All but II, X, and insert	Fast reproduction, anti-binding	40	43	Not applicable
6681	IG	Non-coding intergenic	Unknown	40	42	Not applicable
1116	II,X	DNA replication	Copy number, anti-interference	*	*	#70 Leu.→ Phe
1156	II,X	DNA replication	Copy number, anti-interference	*	*	#84 Val.→ Ala.

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