A MOLECULAR AND EVOLUTIONARY STUDY OF THE OBLIGATE ENDOSYMBIONT *WOLBACHIA* IN *TRIBOLIUM CONFUSUM*

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ABSTRACT

Various aspects of the presence of *Wolbachia* in *Tribolium confusum* were investigated. In particular, the goal of the molecular biology component of the experiment was to develop a way, using GFP (Green Fluorescent Protein), to assess population size of *Wolbachia* within its *Triolium* host. The purpose of the evolutionary part of the experiment was, subsequently, to design multi-level selection experiments, with *Wolbachia* population size as a quantitative character, to determine whether host genes, *Wolbachia* genes, or an interaction between the two, determine the population size of *Wolbachia* in host eggs. Only the molecular biology component was done. A plasmid with GFP, two antibiotic resistance genes (spectinomycin and tetracycline), and a ftsZ *Wolbachia* promoter was constructed. Microinjection of this plasmid into *Tribolium* eggs of strains infected by *Wolbachia*, in order to attempt intrahost genetic transformation of the *Wolbachia*, was attempted unsuccessfully. A variety of troubleshooting recommendations are presented in the conclusion. Furthermore, a philosophical discussion of two distinct models of the evolution of cytoplasmic incompatibility in *Wolbachia*, each stemming from one of the two main theoretical perspectives of evolutionary genetics (Fisherian or Wrightian), is offered. In this case, different conclusions appear from different sets of ontological assumptions applied to the same problem.

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

I) Taxonomic Information

Wolbachia is an extremely common bacterial endosymbiont. It is found in over 16% of insect species, across all major orders, found in the New World (Werren et al. 1995a; Wenseleers et al. 1998). Other estimates of its presence in insect species include ones as high as 77% (Jeyaprakash & Hoy 2000; for a useful table summarizing the percentages of *Wolbachia* prevalence sampled thus far across arthropod and nematode taxa, see Stevens et al. 2001, p. 520). This is a wide range of percentage estimates (16-77%; 20-75%, Wade 2001) and, thus, there are a wide range of potentially infected species worldwide. Using an estimate of as many as 10 million insect species worldwide, there are anywhere from 1.6 to 7.7 million infected insect species in the world. And this estimate of infected species does not even include the other arthropod as well as nematode taxa, let alone potential taxa in other potential phyla, thus far documented. *Wolbachia* is thus the most widely spread bacterial endosymbiont documented to date.

Regarding *Wolbachia*'s host distribution, it has been found in the following insect taxa (Werren and O'Neill 1997; for background host taxonomic information, see Brusca and Brusca 1990; http://tolweb.org/tree/): i) Coleoptera (beetles, such as *Tribolium*, the flour beetle), ii) Diptera (flies, such as *Drosophila*, the fruit fly and *Culex pipiens*, the common mosquito), iii) Hemiptera ("true" bugs, including "assassin and kissing bugs"; cf. Brusca and Brusca 1990, p. 550), iv) Hymenoptera (haplo-diploid social insects, including *Nasonia*, a parasitic wasp, Breeuwer and Werren 1993; and many ant species, Wenseleers

et al. 1998), and v) Orthoptera (grasshoppers, locusts, crickets and katydids). In addition to insects, *Wolbachia* has been found in mites (Breeuwer & Jacobs, 1996), crustaceans (Rousset et al. 1992) and nematodes (Sironi et al. 1995). *Wolbachia*'s presence in this last non-arthropod host is of great scientific and public/world health interest. *Wolbachia* infects the nematode worm *Onchocerca volvulus*, etiological agent of "river blindness" or Onchocerciasis, a disease that affects some 17 million people in the Tropics. It seems likely that nematodes acquired, evolutionarily, their *Wolbachia* from their host, *Simulium* black flies. Furthermore, since the worm has an obligate reciprocal relationship with *Wolbachia*, curing the worms of *Wolbachia* seriously interrupts the worm's life cycle within its human hosts, thereby showing great promise as a remedy for this serious illness (Stevens et al 2001; see http://www.cdc.gov/travel/diseases/oncho.htm). *Wolbachia* is thus extremely common in a number of arthropod and nematode taxa.

II) Basics of *Wolbachia* Evolutionary Strategies

Wolbachia, as an obligate intracellular parasite, is found primarily in the reproductive tissues of its hosts, but recently attention has been drawn to its presence in host *somatic* tissues of various kinds, including muscle and nervous tissues (Dobson et al. 1999; Min and Benzer 1997 discuss a virulent *Wolbachia* variant in *Drosophila melanogaster* that "begins massive proliferation in the adult, causing widespread degeneration of tissues, including brain, retina, and muscle, culminating in early death." p. 10792). Perhaps an evolutionary levels of selection story (gains at somatic cell lineage level vs. losses at organism cross-generational transmission level), along the lines of cancer, as presented in the classic work of Buss 1987 (e.g., p. 51) and developed further by

Maynard-Smith and Szathmáry 1995 and Michod 1999 (see also Winther in press), could be developed for this scenario.

Speaking from an evolutionary point of view it is, in fact, the presence of *Wolbachia* in *reproductive* tissues that is of key importance. This is because, as an obligate endosymbiotic parasite, with rare horizontal transfer (either direct or vector-mediated host-to-host, non-offspring-reproduction mediated, transfer of parasite) (e.g., O'Neill et al. 1997), the only way for *Wolbachia* to be transferred from one host to another is through reproduction. It has been shown that horizontal transfer is rare. It almost certainly must have occurred in the evolutionary past, given the widespread taxonomic distribution of *Wolbachia*, especially in cases of similar strains infecting, for example parasitic wasps such as *Nasonia* as well as their arthropod hosts, the blowfly of the taxon Protocalliphora (for phylogenies, see Werren et al. 1995b, p. 57; Werren and O'Neill 1997, p. 27). Furthermore, horizontal transfer has been observed in a few laboratory investigations involving male-killing *Wolbachia* (Hurst and Majerus 1993). In addition, paternal transmission (i.e., "infected" sperm transmitting *Wolbachia* to unfertilized and uninfected eggs) is *also* exceedingly rare. Although full biochemical details are still lacking, the reason for this seems to be that the tight packing of sperm, which includes primarily chromosomes, leaves little physical space for *Wolbachia* (Hurst and Majerus 1993). However, as we shall see, this does not mean that *Wolbachia* cannot leave its "trace" in the sperm. Thus, transmission through the maternally-produced *egg*, which gives rise to a new adult, is the main means of parasite transmission. The central fact is that in order to be evolutionarily successful, *Wolbachia* must ensure that it is present *in the egg cytoplasm*, a place that will allow it to be transmitted to new hosts, the offspring of the original host. A

corollary to this fact is that males and their reproductive product, sperm cells, are an evolutionary "dead end" for *Wolbachia*.

The central fact, and its corollary, leads to a very important *modeling* consequence, as explained by Dunn et al. 1995, p. S92: "…Furthermore, since infection is *inherited*, the mathematical approach bears more similarity to that of population genetics than to the population dynamics of contagious infection: we can represent the parasite as a uniparentally inherited gene." Sociologically speaking, then, it is no accident that population geneticists, rather than evolutionary ecologists—both of which are highly mathematically sophisticated—have been the force behind the development of mathematical models of the evolution of *Wolbachia*.

Given that *Wolbachia* can only be transmitted through the maternal egg, an interesting levels of selection conflict arises. As is well-known, at least since R. A. Fisher's classic 1930 argument, stable and equilibrium investment in the two sexes is 50-50. Put differently, since nuclear genes are transmitted in equal quantities through males and females, any nuclear gene that has some effect in *skewing* the sex-ratio of the offspring of that organism, will, under standard Fisherian "large population size theory" assumptions (Wade and Goodnight 1998), be selected against (i.e., will have a relative fitness for that particular fitness component lower than a subset of the other alleles also found at that locus). However, genes within *Wolbachia* itself are selected to increase the *ratio of females* in the population precisely because *Wolbachia*, and its associated genes, are only transmitted through females. In fact, in an "ideal world" from the point of view of *Wolbachia*, only infected female hosts would exist; male hosts would vanish (perhaps there are some background modeling assumptions under which this would not be true).

Metaphorically put, this would be a world consisting of a sea of purely hospitable and transmissible cytoplasm – an endless and bountiful ecology colonized by *Wolbachia*. Needless to say, this scenario is in fact impossible since selection at the host level (i.e., Fisherian sex-ratio equilibrium) acts strongly against too large a skewing of host sex-ratio.

III) Details Regarding *Wolbachia* Evolutionary Strategies

Although the extreme form of the *Wolbachia*n Elysian Fields (ubiquitously colonized cytoplasm) just described is not possible due to the inherent levels of selection conflict, many attenuated versions of the story are possible and, in fact, exist. *Wolbachia* exhibits a diverse repertoire of strategies to increase the proportion of infected cytoplasm in the total "cytoplasmic population" of its host, which also involves sperm cytoplasm and uninfected egg cytoplasm. These strategies include: 1. cytoplasmic incompatibility, 2. parthenogenesis, and 3. femininizing genetic males.

1. Cytoplasmic incompatibility (CI) occurs when an uninfected egg is fertilized by the sperm of an infected male. Such an egg does not develop. All other fertilization combinations lead to developing eggs. Note that cytoplasmic incompatibility can also occur in populations with multiple kinds of *Wolbachia* infections ("superinfections" as described in Sinkins et al. 1995). In all such cases, "infection" is a relative term. That is, a sperm is considered infected (relative to an uninfected egg) as long as it contains at least one *Wolbachia* strain *not* present in the egg. This is the general and proper way to talk about infected and uninfected gametes.

2. Parthenogenesis occurs when *Wolbachia*, for example, induces eggs in females to start mitosis and, upon duplication of the haploid chromosome set (found in all

eggs), hinders the two identical chromosome sets from segregating during the first mitotic anaphase. This inhibition leads to a single cell with a completely homozygous diploid set of chromosomes (Stouthamer et al. 1990; Stouthamer et al. 1993). This reproductive cell can then develop into an adult female (Stouthamer and Kazmer 1994; Dunn et al. 1995).

3. Feminizing genetic males occurs when *Wolbachia* affects the development of genetic males, probably by influencing the androgenic gland of the males, making them into phenotypic females. This phenomenon has been studied extensively in the isopod *Armadillidium nasatum* (Dunn et al. 1995; Rigaud et al. 1997).

In general, we can consider all of these mechanisms strategies for increasing the number of infected females in the population *relative to* either uninfected females or males, or both. Sometimes the direction of infected female skewing can *also* be in the interest of the host, for example in cases of local mate competition (an evolutionary phenomenon perhaps first clearly explained by William Hamilton in 1967), particularly common in highly structured breeding populations, such as that which exists in highly inbreed mite populations. However, in most cases female skewing is *not* in the interest of the host.

In the cases of parthenogenesis and feminization, the direct selective advantage to *Wolbachia* is readily apparent. That is, relatively more infected cytoplasm in the overall population is produced. There is no production of wasted (sub-optimal) infected males in the case of parthenogenesis, and there is no eventual loss of fertilizations (by the male that was feminized) through CI in the case of feminization. In the case of cytoplasmic incompatibility, which will be the focus of the experiment presented in this thesis,

however, the direct selective advantage is more opaque: sperm, as a "warrior caste" (MJ Wade's term), kill potential offspring that would have become uninfected males and females. The infected sperm is *already* an evolutionary dead end: it will not transmit *Wolbachia*. So why, from *Wolbachia*'s point of view, should its fertilization of an uninfected egg lead to no development? Is this not just an act of spite? (e.g., Hurst 1991) Indeed, in cases with *no* population structure, cytoplasmic incompatibility is a "neutral trait" or "is favoured [only] because of pleiotropic correlations among [with other] parasite traits." (Frank 1997, p. 327, cf. Frank 1998) Such cases were formally explored by Prout 1994 and Turelli 1994. Without population structure, then, the spread of CI in a population will only occur if that character is correlated with other *Wolbachia* traits with a direct selective advantage, such as, hypothetically speaking, a direct fitness gain of the host through, for example, more efficient host metabolism, or increase in maternal fecundity, aided by *Wolbachia*.

With population structure, however, there is a clear kin selection advantage for the *Wolbachia*, and thus its behavior is not simply an act of spite (Frank 1997, 1998; see also Wade and Stevens 1994). That is, by destroying uninfected cytoplasm, *Wolbachia* directly increases the relative proportion of infected (with the same *Wolbachia* strain) cytoplasm in that *very* (sub)population. Unlike the famous case of kin selection in social insects, however, this is not a case in which there is *direct* kin interaction that both reduces the fitness of one kin (sterile female worker) and increases the fitness of the other kin (gynes or males). Nevertheless, there is a clear kin-mediated genetic benefit to *Wolbachia* that have left their "trace" on sperm [through chromosomal imprinting (Hoffmann and Turelli 1997; Reed and Werren 1995) that, in order to be restored to a functional state, must be

rescued by some (set of) substance(s) in the eggs] in that this allows highly related *Wolbachia* in eggs to increase their relative proportion in the population cytoplasm pool. Although there is no direct kin interaction, there is, nevertheless, a kin-mediated effect.

Another way of classifying the strategies available to *Wolbachia* for increasing their relative representation in the host population is a *functionalist* one, focusing on the fitness structure of the situation rather than on the processes—that is, mechanisms—involved in the situation, as the one I have just presented (i.e, cytoplasmic incompatibility, parthenogenesis, feminizing genetic males). Let us now turn to the functional classification presented by Werren and O'Neill 1997. They state that there are four ways for the maintenance and increase of vertically-transmitted symbionts (Werren and O'Neill 1997, p. 3): 1. increase fitness of the infected hosts, 2. increase sex ratio (proportion females) of infected hosts, 3. decrease fitness of uninfected hosts, and 4. decrease sex ratio (proportion females) of uninfected hosts.

1. Increasing the fitness of the infected hosts is, or at least *was*, the "received view" on parasite effects on host. That is, parasites and hosts evolve a mutually beneficial relationship. (An interesting recent example of this is the relative advantage that sperm from infected *Tribolium confusum* seem to have over sperm from uninfected males, in fertilizing *both* uninfected and infected females. This seems to be a case of sperm competition, Wade and Chang 1995.) Needless to say, fitness increase of the host is not always, or even primarily, the case. In the case of *Wolbachia*, for example, there can be a survival or fecundity cost (i.e., a lowering of female reproductive output), which can sometimes be severe (on survival cost, see Min and

Benzer 1997). Despite this cost, and contra the received view, parasites can still increase in frequency through other strategies, such as the next two.

2. Increasing the sex ratio (proportion females) of infected hosts is what occurs in the cases of parthenogenesis and feminizing males discussed above. As discussed, this increases the relative amount of infected cytoplasm present in the population.

3. Decreasing the fitness of uninfected hosts is what happens in the case of CI where eggs of uninfected females, when fertilized by sperm of infected males, do not give rise to offspring. However, in this context, an interesting question arises: does this act also decrease the fitness of the *infected* hosts by wasting one of its potential offspring? If we consider a heritable element in the cytoplasm—e.g., mitochondrial or *Wolbachia* gene—the answer would be "no" (in fact, host cytoplasm, as already extensively discussed, increases in proportion through CI). *But*, if we consider a nuclear genetic element, the answer seems to be "yes." That is, CI also has a cost to the infected host in lost offspring (though the cost is not in lost sperm per se – sperm on a per-unit-basis, is much cheaper than egg). However, kin selected benefits outweigh this cost.

4. Decreasing the sex ratio (proportion females) of uninfected hosts is what occurs in CI of haplo-diploid Hymenoptera. In these cases, rescue of the genomically imprinted paternal chromosomes does not occur, and a haploid ("fertilized") egg develops into a male. This is another way of increasing the proportion of infected cytoplasm in the population.

IV) Some Observations Regarding Theoretical Models Pertinent to Cytoplasmic Incompatibility

Of these strategies, the one that has received the most theoretical attention is cytoplasmic incompatibility, in which uninfected hosts have their fitness decreased. Although they vary from model to model, depending on a variety of assumptions, three crucial factors (i.e., variables) involved in these models include: 1. amount of fitness reduction in terms of egg production (fecundity) in females due to presence of infection, 2. transmission efficiency of infection from mother to offspring, 3. level and severity of cytoplasmic incompatibility (which will affect the "hatch rate," sensu Turelli 1994).

Note that (1) can be due to a variety of reasons, females can become ill more often or reproductive production might be compromised as females have to fight the *Wolbachia* infection. This reduction, however, might actually be negative; in other words, there might be a net fitness increase – this is the classic case of symbionts as beneficial passengers. Note that (1) should be clearly distinguished from (3). The former concerns the net number of eggs a female can produce *independently of*, and *prior to*, whatever sperm should fertilize these eggs; this value is determined by physiological mechanisms, with their origins in the infection, affecting egg production. The latter is related to the relative number of eggs that *develop post-fertilization*. In the case of infected eggs, this variable (e.g., "hatch rate") should have a value of 1 (i.e., the level of cytoplasmic compatibility is perfect), whereas that is not the case with eggs with lower, or no, levels of infection (i.e., the variable value is < 1).

Other variables can also be introduced in the models, including: 4. relative survival rate of offspring from embryos to adulthood (Fine 1978), 5. the amount of relatedness in a

population (Frank 1997), and 6. mean population fitness as measured by population productivity (Stevens and Wade 1990). However, a basic result can be learned by just exploring the first three variables.

This result, shown by Caspari and Watson 1959, is that provided one assumes perfect maternal transmission of the parasite by infected mothers *and* complete CI (the second assumptions is specifically made on p. 568 when they talk about the sterility of a certain cross), the infection will only increase in frequency in the population if the [frequency of the infection] is higher than [the relative fecundity cost on females with the infection] (summarized on p. 570; cf. Hoffmann and Turelli 1997, pp. 62-63). If the infection frequency is lower than the relative fecundity cost (note that both can be expressed in, and subsequently compared as, percentages), then the frequency will go to 0. If it is higher than (equal to) the relative fecundity, the frequency will go to 1 (be at an unstable equilibrium). Now, if there is population subdivision, then it might seem that in small populations, due to, say, random mortality, the infection would go to 0 or 1 faster. However, Wade and Stevens 1994 have shown that with population subdivision, the spread of *Wolbachia* (or any other parasite mediating CI) through a population is actually slowed down: "…by slowing the rate of movement away from the unstable equilibrium point in some sense 'stabilizes' these interior [unstable] equilibria." (p. 86)

A corollary to this result is obviously that there *are* conditions under which infection can increase in frequency *even when it has a fecundity cost on its host*. This result, first shown in 1959, must almost certainly have been surprising during its time, given the context of a received view that had been stressing the mutualistic symbiotic interaction between parasite and host.

A number of other results have been shown with the more complex models, some of which have already been discussed. For example, Frank 1997 distinguished between the Prout 1994 and Turelli 1994 models assuming panmictic reproduction and his own, assuming kin selection and population structure. Both of these kinds of models have led to more subtle results and have shown how, and under what conditions, the parameter values of, for example, levels of cytoplasmic incompatibility can change. It is, admittedly, odd that, given his focus on structured population models concerned with cytoplasmic incompatibility, Frank did not even mention Wade and Stevens 1994, a paper also concerned with population structure (though not explicitly with kin selection; however, the paper also *implicitly* dealt with kin selection if we follow Wade 1980 and 1985's logic of population structure as being, in an important sense, *equivalent to* kin selection). Further work attempting to unify, or at least show the theoretical relations among, Frank's and Wade's (among others) work on population structure and kin selection in the evolution of parasite-mediated cytoplasmic incompatibility would be highly welcomed.

V) Density Effects of *Wolbachia*

There are a number of other issues that need to be discussed in order to present a comprehensive understanding of the evolution of *Wolbachia*. What is the long-run equilibrium condition, if any, of *Wolbachia* in the host population? Are infected strains of *Wolbachia* evolutionary replaced by "insensitive"/"rescuing" types (Bourtzis et al. 1998) that then, in turn, are replaced by uninfected hosts [which now have a higher fitness since there is very little (no) CI in the population consisting mostly (only) of hosts with rescuing *Wolbachia* and uninfected hosts; furthermore, the uninfected hosts pay *no* fecundity cost],

as proposed by Hurst and McVean 1996? With what frequency is a mutualistic relationship between *Wolbachia* and their hosts *eventually* reached (i.e., is there any truth to the received view that mutualistic symbiosis will eventually be reached)? What is the equilibrium density, if any, of the *Wolbachia* in the cells of their hosts? How much plasticity can the *same* strain of *Wolbachia* present in different hosts, and how much of such plasticity is due to additive genetic variance in the same strain of *Wolbachia* vs. parasite-by-host genetic interaction variance? Regarding this last question, consider the following quote regarding *Wolbachia* strategy plasticity in different host species:

> …the *Wolbachia* MK[male-killing]-inducing strain of *T*[*ribolium*] *madens* is indistinguishable from the cytoplasmic incompatibility (CI)-inducing *Wolbachia* in the closely related *T*[*ribolium*] *confusum*. Studies with non-MK *Wolbachia* demonstrated that the particular host-*Wolbachia* interaction plays an important role in the induction of reproductive phenotypes. Because the DNA sequences in the *Wolbachia* infecting these two closely related *Tribolium* species are identical, the MK effect appears to follow the pattern of being specific to the particular host-symbiont interaction. (Stevens et al. 2001, 528).

In short, there is no doubt that many (types of!) questions, empirical and theoretical, remain unanswered, and even mostly unexplored.

Be this as it may, my experiment, which I will briefly describe in the section immediately below, concerns particularly the role of bacterial *density* in host cells.

In a vein similar to Buss's argument regarding cancers in multicellular organisms, it is very plausible that—qualitatively speaking, at least—there is intra-cellular level selection for higher intra-cellular density levels of *Wolbachia*. As Werren and O'Neill 1997 write:

> A second feature in common with mitochondria is the hierarchical structure of symbiont populations. There is the individual symbiont, population of symbionts within individual host cells (nutritive symbionts are often

> localized in specialized cells, mycetocytes or bacteriocytes, until the time of host reproduction and transmission), population of infected host cells within an individual host organism, and populations of infected hosts. The population dynamics of symbionts will be dependent upon stochastic processes of transmission and selection at the different levels. (p. 33)

However, a conflicting-levels-of-selection argument for increased *Wolbachia* density is unlikely to be simple. Complexity arises, in part, because it is unclear how *Wolbachia* density correlates with higher-level effects. For example, Bourtzis et al. 1998, write: "…there was no simple correlation between the absolute densities of *Wolbachia* in testes and the ability of these strains to act as either a mod+ [cause genomic imprinting of paternal chromosomes] or a mod- strain [do *not* cause such genomic imprinting]." (p. 852) This might be similar to the case of cancer, in which we have some idea that there is some kind of qualitative relationship (even if non-linear) of cancer cell density with cancer cell lineage *as well as* organism fitness (the former, being higher density, higher fitness; the latter, being higher density, lower fitness). However, more studies of the qualitative, let alone precise quantitative, relationship of *Wolbachia* density with phenotypic and fitness effects, at various compositional levels, is required.

Breeuwer and Werren 1993 presented what they called a "bacterial dosage" verbal model in which:

> …cytoplasmic incompatibility apparently involves an action of the bacteria in the male (*e.g.*, "imprinting" of sperm chromosomes) and a counteraction in the egg (*e.g.*, production of a "rescue" substance). It is an interaction between these two effects that determines whether the sperm chromosomes are fragmented and lost. We propose that unidirectional compatibility is strongly influenced by the "dose" of bacteria in the male (*e.g.*, in spermatocytes) relative to the dose in the egg. Sperm will be incompatible with an egg when the number of bacteria in the male strain is greater than in the female strain. Conversely, a cross is compatible when the paternal strain harbors equal (*e.g.*, intrastrain crosses) or lower numbers of cytoplasmic bacteria. (pp. 571-572)

Although they presented some supporting evidence for the relationship between bacterial density and physiological effects, including the fact that females that have been cured of *Wolbachia* only produce eggs that have lost the infection a few days *after* being cured (thereby suggesting that bacteria must decrease in density before their presence and effect in the egg disappears), their verbal model (particularly the claim regarding comparative densities in sperm and egg) would require significant fleshing out and further investigation. For example, it should be combined with variables regarding superinfections (Sinkins et al. 1995).

VI) Motivation and Design of Experiment

In the experiment I planned to do, the main idea was to use artificial multi-level selection experiments on parasite population size as a quantitative character to determine whether host genes, *Wolbachia* genes, or an interaction between the two, determine the population size and density of *Wolbachia* in host eggs.

In order to facilitate censusing the size of *Wolbachia* populations inside host eggs, the plan was to transform *Wolbachia* with GFP (green fluorescent protein) and two antibiotic resistance genes. With the guidance of Carl Bauer, David Rollo, and James Smart (the latter two were members of the Bauer laboratory), a construction of a plasmid, containing the following genes, was planned (and done successfully!): 1. the GFP gene, 2. spectinomycin and tetracycline resistance genes (MJ Wade and I determined that it would be useful to have two antibiotic genes available with which to do the selection experiments), and 3. a *Wolbachia* ftsZ promoter. After this, the intention was to insert the

plasmid into *Wolbachia*, which is necessarily within its *Tribolium* host. By culturing the host on a medium containing antibiotic(s), non-transformed *Wolbachia* would be killed (O'Neill 1989), and we would be left with host lines containing only transformed *Wolbachia*. Once successfully transformed and microinjected into the host, we would be able to census *Wolbachia* population size and density in individual host eggs as variation in the intensity levels of fluorescence.

The idea then was to perform two kinds of selection experiments on this quantitative character (population size and density of *Wolbachia* within *Tribolium* cells and eggs). In one experiment, we would select on the host genome and randomize the inter-generational transmission of the parasite genome. In the other experiment, we would select on the parasite genome and randomize inter-generational transmission of the host genome. Performing these experiments concurrently would also allow us to select simultaneously on the host and parasite genomes, both in the *same* and in *opposing* directions.

In the first experiment, we would select females with large values of the quantitative character. We would look for natural variation in this trait. But if we were to mate these females with random males we would confound selection on host and parasite genome since the selected females (focal females) would transmit not only their genes, but also their *Wolbachia* (this would essentially amount to a form of correlated selection). Therefore, instead, we would mate the *brothers* of the females with (a) random females and (b) arrays of sisters stemming from unrelated families. Doing both would allow us to compare the difference between (a) completely random host and parasite genome background *versus* (b) semi-controlled host genome and controlled parasite genome

background (on randomizing versus controlling as two ways of attempting to eliminate background confounding factors, see Cartwright 1989). Either way, the *Wolbachia* present in the females that we would mate to the brothers of the focal females would *not* be subject to selection. In our design and analysis we would also account for the fact that brothers only share, on average, half of their genes identical by descent with their sisters (the focal females). Thus, this selection regime would only be half as strong compared to one on focal females. But, this design would allow us to select on host genome without also selecting the parasite genome.

In the second experiment we would also select focal females with large values of the quantitative character, but now we would want to select on the parasite genome. Here we would also look for natural variation in this trait. In the best of all possible worlds, this could be done by removing the parasite in the selected females and distributing these parasites among random females. But the biology of the situation does not allow this. In order to randomize host genome as much as possible we would, instead, mate the selected focal females with the brothers of the *unselected* females (i.e. the females with the lowest values of the quantitative character). Although a dilution effect again exists (expectation of sib-to-sib $r = .5$), this protocol would significantly cancel the effects of selection on host genome. Put differently, any (combination of) female host alleles causing (in some sense or other) high numbers of *Wolbachia* would be offset by male host alleles causing (in some sense or other) low numbers of *Wolbachia*.

This protocol would allow us to separate selective effects on host genome from those on parasite genome. This design complemented with further experiments on simultaneous selection on both genomes, both in the same and in opposing directions,

would allow us to determine whether there is significant host or parasite, or both, additive genetic variance, as well as epistatic variance, for the quantitative character under study. Causal mechanisms through which such genetic variance could act include variance in the immune system of the host, and variance in parasite competitive efficiency within the cytoplasmic ecology in which they find themselves. Since *Wolbachia* can be interpreted as an incipient organelle, our experiment is also pertinent to the processes of endosymbiosis, evolutionary transitions, and levels of selection, as I have discussed, to an extent, above (e.g., Margulis 1993, Maynard Smith and Szathmáry 1998). Whereas there may be selection at the higher host level to control the population size and density level of *Wolbachia,* there may be selection at the lower parasite level for selfish or defector *Wolbachia* that grow uncontrollably. This is a hypothetical scenario, but our experiment would allow us to both assess hierarchical genetic variance (underlying genetic variance available for selection), and suggest selective causal reasons for the regulation of *Wolbachia* population size and density in *Tribolium.*

The experiment, as conceived and described here, only reached the plasmid construction stage. A significant number of microinjection attempts, aimed to transform *Wolbachia* with the plasmid, failed. Needless to say the selection experiments were therefore, unfortunately, not even attempted.

Chapter 2. Methodology

First, the plasmid construction procedures will be detailed (molecular biology section). Second, the microinjection and population biology procedures will be indicated.

I) Molecular Biology Methods

One goal of the project was to produce the right plasmid for the microinjection for the selection experiments. The two goals for this molecular biology part of the project were: 1. construct the relevant plasmid (pRW4) and 2. prepare the plasmid for microinjection (as pure plasmid and as a plasmid within a "promiscuous" strain of *Escherichia coli,* SM10 lambda-pir).

With respect to the first (major) step, a plasmid (see Figure 7 in Chapter 3) was constructed that contained the following: 1. GFP (for the purpose of measuring quantity of *Wolbachia* per *Tribolium* cell), 2. tetracycline and spectinomycin resistance sites (for the transformed *Wolbachia* bacteria to neutralize the effects of the antibiotics to be put in the *Tribolium* flour), 3. *Wolbachia* promoter sequence, so that the whole plasmid could be transcribed and translated by a *Wolbachia* bacteria. The plasmid was constructed using, first, a commercially-available vector with GFP (pEGFP-N1), and then a broad-host vector (pRK415, designed by James Smart, Bauer Laboratory, Indiana University). *Wolbachia*, as a *Rickettsia* alpha proteobacteria, can almost certainly process this vector. In order to construct this plasmid, standard gene cloning procedures, to be explained below, were employed. In the results section, the modules of procedures used for each of the

intermediary plasmids (pRW1, pRW2, pRW3) will simply be stated and summarized in a table.

The second step is more straightforward. Isolation of the plasmid was done using the Standard Alkaline Lysis Preparation procedure and the plasmid was suspended in microinjection buffer, kept on ice, and microinjected. The plasmid was also chemically transformed into the SM10 lambda-pir strain which was then cultured, concentrated, and microinjected in the second part of the experiment.

CONSTRUCTING THE PLASMID

Basic Frame for the Experimental Process of Constructing a Plasmid:

(To be detailed in what follows.)

A. Isolate insert using digest technique with which a DNA segment is cut out of another plasmid. If pure insert is desired, can employ gel purification procedure. Open up vector at the appropriate places (same restriction enzyme sites as insert) using digest procedure.
B. Ligate insert and vector using ligation procedure.
C. Transform plasmid into host bacterial cell. (Relatively small success rate per bacterial cell.)
D. Grow colonies on relevant antibiotic plates in order to, eventually, select successfully transformed bacterial cells.
E. Select growing overnight colonies and culture them.

F. Isolate plasmid from overnight colonies using miniprep or standard alkaline lysis preparation. Quantity of plasmid can be assessed using spectophorometer.

G. Diagnose plasmid by strategically digesting it. If plasmid has been produced successfully, then can go to step A to make a *new* plasmid.

A. Enzyme Digests

2 µl 10x NEB2 (buffer)

1 µl BSA

.25 µl of each restriction enzyme used (e.g., HindIII, XbaI, etc.)

10 µl DNA

13 – (.25 x number of enzymes used) µl of ddH2O

(The exact amount of restriction enzymes depends on the amount of DNA present in µg, which can be determined using the spectorphorometer. The amount in µg is then used to determine the number of enzyme units necessary. Consult: URL = <http://www-personal.umd.umich.edu/~mparsons/474/setting_up_enzyme_digests.pdf>)

1. Run for 60 minutes at lowest optimum temperature indicated for the set of enzymes used.
2. Run diagnostic gel.

B. Ligation

2 µl 5x ligase buffer

1 µl T4 DNA ligase

2.5 µl ddH20

X µl of insert (on the order of a few µl)

Y µl of vector (on the order of a few µl)

(The exact value of X and Y depends on the relative number of insert and vector per µl in its own solution. This number can be determined using the spectrophorometer, which provides the amount of DNA per ng/µl (step 2 of *spectrophorometer* protocol below). This number together with the relative sizes (in kb) can be used to calculate the value of X and Y. For example, consider the case where the vector is Q times the size (in kb) of insert. Let us then say that we know, from the spectrophorometer, that we have A ng/µl of insert and B ng/µl of vector. Under those conditions, X = A/Q and Y = B. This gives the relative amount of each. The absolute amount is not too important as long as there is an excess of ligase.)

Incubate at 16°C for an hour.

C. Transformation

Electroporation

1. Desalt DNA (plasmid) before electroporation. Place 20 minutes on nitrocellulose filter paper in a bath of ddH20.
2. Pipette 2 µl of ligation product into prepared eppendorf tubes with relevant electroporation competent bacterial cells. Mix with tip of plastic.

3. Take 40 µl of this and place in electroporation kuvette between electrodes.
4. Activate electrodes at 1.5 volts until beep is heard. Time constant should have been around 4, or else discard.
5. Immediately pipette 1 ml of recovery solution (LB or SOC medium) into kuvette.
6. Remove cells and place them into 13 ml tubes.
7. Put in 37°C bath for 1 hour.
8. Place 50, 100, and 500 µl of this solution on plates with relevant antibiotics and allow colonies to grow overnight.

Chemical Transformation

1. Pipette 3µl of ligation product into prepared eppendorf tubes with relevant chemically-competent cells. Mix with tip of plastic.
2. Put on ice for 30 minutes.
3. Heat shock at 42°C for maximum 90 seconds.
4. Put on ice for 5 minutes.
5. Transfer to 1 ml of LB (or SOC) and place in 37°C bath for 1 hour.
6. Place 50, 100, and 500 µl of this solution on plates with relevant antibiotics and allow colonies to grow overnight.

D. Antibiotic Plates

1. Grab appropriate plate from Bauer cold room.
2. Add any new antibiotics to the plate according to *adding antibiotics* procedure below. Do this next to a burning Bunsen flame (to create sterile conditions). After

pipetting relevant antibiotic(s), use sterile glass triangle (alcohol & flame) to spread it (them) around on plate (use spinning wheel).

3. Let plate sit to dry and warm under the hood for approximately 20 minutes.
4. Take relevant bacterial sample (either from freezer or from recovery solution after transformation) and a. (for freezer stock) use sterile wooden stick to scrape out bacteria and gently scrape the stick over the surface of the agar plate in zig-zags, making sure lines are not crossed, or b. (for recovery solution) pour solution on plate and use glass triangle and spinning wheel to spread bacteria across plate.
5. Cover plates and label them. Place them upside down (agar side up) in the 37º room overnight (anywhere from 12-24 hours).

E. Overnight Colonies

1. Either take colonies at the end of the zig-zag path (from freezer stock), or from successfully transformed colonies (from recovery solution after transformation). Basic rule: one colony for each test tube of LB broth. Do all of this next to an open flame.
2. Air-Pipette 5 mL of LB broth into a sterile test tube with plastic top. Make sure that glass pipette is sterile, using Bunsen Burner, before and after transferring the broth.
3. Pipette appropriate amounts of relevant antibiotics. Swirl gently.
4. Sterilize an inoculating needle using an open flame. After it cools down (approximately 20 seconds) use it to transfer a *single* colony from the plate to the test tube with LB and antibiotic in it (the overnight).
5. Place tube in 37º room overnight (anywhere from 12-24 hours).

F. DNA Isolation from Cell Cultures

Standard Alkaline Lysis Preparation (Protocol from James Smart; also adapted from URL = <http://preuss.bsd.uchicago.edu/protocols/Alkaline.html>)

1. Centrifuge 1-1.5 ml of bacterial culture for 5 minutes at 15 k. Remove all supernatant. (First round of tubes.)
2. Dissolve pellet in 100 µl of Solution I. Cell resuspension and weakening of cell walls step. Vortex vigorously. Get rid of all clumps. Can leave for 10 minutes.
3. Add 200 µl of Solution II. Lysis step. Do not vortex. Mix gently by inversion. Leave on ice for absolutely no longer than 5 minutes. If left longer, the plasmid DNA will be irreversibly denatured.
4. Add 150 µl of Solution III. Renaturing of DNA and cleaning step (gets rid of linear DNA and SDS). Mix by flicking. Store on ice for 3-5 minutes.
5. Centrifuge for 5 minutes at 15 k, at 4°C. Supernatant has the DNA; transfer it to a fresh tube. (Second round of tubes.) Throw out pellet of "old" tube.
6. Phenol:$CHCl_3$ extraction. Removing protein and activating DNA for restriction enzyme step. Add 450 µl of equal amounts of Phenol and $CHCl_3$ to each tube. Mix by inverting 5-10 times. Spin for 5 minutes at 15 k, at 4°C. Supernatant has the DNA; carefully transfer it to a fresh tube. (Third round of tubes.) Throw out pellet of "old" tube.
7. Alcohol (ethanol) precipitation stage. Add 1 ml of –20°C 100% ethanol. Mix gently by inversion. Let stand for 2 minutes at room temperature.

8. Centrifuge for 15 minutes at 15 k. Small pellet, sometimes invisible, will form. This is the desired DNA. CAREFULLY remove the supernatant by gentle aspiration using a micropipette. Remove any drops of fluid adhering to the walls of the tube. If care is not taken during this step, the pellet of DNA could be lost even from breathing into the tube.
9. Rinse the pellet of DNA with 1 ml of –20°C 70% ethanol at 4°C. Spin briefly (2 minutes at 15 k). Remove the supernatant as described in step 8.
10. Speed vac dry 15 minutes at 4 k.
11. Resuspend in 10 µl of TE8/RNAseA. Redissolve the nucleic acids in 50 µl of TE (pH 8.0) containing DNAse-free pancreatic RNAse (20 µg/ml). Or, for microinjection, resuspend in 10 µl of microinjection buffer (100 mM KCl, 10 mM KPO_4, pH 7.0). Vortex briefly. Store the DNA at -20°C. Purity of isolated DNA can be assessed using spectrophorometer. DNA can be used for immediate further experiments or can last for a few weeks. (Long term plasmid storage must be done *inside* transformed bacteria that, upon culturing, are suspended in 2 ml 10% glycerol at -80°C.)

Necessary Solutions for Standard Alkaline Lysis Preparation:

Solution I

50 mM glucose

25 mM TrisoCl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved, and stored at 4 °C.

Solution II (make fresh for each Lysis)

0.2 N NaOH (freshly diluted from a 10 N stock)

1% SDS

Solution III

60 ml 5 M potassium acetate

11.5 ml glacial acetic acid

28.5 ml H_2O

TE8/RNAseA

40 µl of 5 mg/ml of DNAse-free pancreatic RNAse

2 ml of TE pH 8 (from equal amounts of 10mM Tris pH 8 and 1 mM EDTA)

Minipreps

Refer to URL =

<http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_QP_Miniprep/1027678_HB_QP_0504_WW_LR.pdf>

Necessary "Background" Protocols for Plasmid Construction:

Spectrophorometer

(To assess purity of DNA sample isolated by Standard Alkaline Lysis Preparation or by micropreparations.)

1. Take 99 µl of ddH2O and 1 µl of DNA sample. Place in spectrophorometer.
2. The 260 nm reading provides the quantity of DNA. Take the reading and multiply it by 5000 [multiply the number by the dilution factor (100) and then by 50]. This will provide the quantity of DNA in ng/µl.
3. The 260 nm/280 nm reading provides the purity of the DNA. For sequencing, the purity has to be between 1.8 – 1.9. Lower than 1.8 means there are impurities such as protein. Higher than 2.0 means that there is RNA contamination.

   Between 1.9 – 2.0 is acceptable for cloning purposes, but preferably the value should be between 1.8 – 1.9

Tips:

• Do not touch the glass side of the kuvette

• After every use of the kuvette, clean it with ddH2O and then dry it carefully with kimwipes.

• Calibrate it first with 99µl of ddH2O.

• Hit "read sample" after every time a new sample is placed in the kuvette.

• Make sure the visible and UV lamp is turned off at the end of the day – bulbs are very expensive, on the order of thousands of dollars.

Gel Electrophoresis

1. Use approximately 40 ml TAE for minigel, 100 ml TAE for normal gel.
2. Add between .7 and 1.4 %, by weight, of agarose to the TAE. Heat for approximately 20 seconds in a microwave oven, until agarose is melted. Solution should not be too hot.
3. Add 2 µl of Ethidium Bromide (for DNA visualization under UV light) for minigel, 5 µl for normal gel.
4. Place in tray with comb and with blue tape used to cover ends of the tray. Allow to cool to room temperature (approximately 1 hour).
5. Remove comb and load with 10x loading buffer and 2 µl from each PCR reaction. Load one lane with 1 kb ladder.
6. Place in appropriate direction in TAE bath in electrophoresis machine.
7. Turn on electricity: 120 volts, approximately 30 minutes; 30 volts, approximately 2 hours.

Adding Antibiotics to Plates and Overnight Solutions

On agar need twice the concentration as in overnights, since antibiotic in agar moves solely by diffusion.

1. For Tetracycline (Tet):

Use 5 mg/ml (5 µg/µl) of stock Tet solution.

On agar (with 25 ml Luria Broth (LB)):

need 500 µg total of Tet, so need 100 µl of Tet solution

In overnights (with 5 ml LB):

need 10µg/mL, or 50 µg of Tet, so need 10 µl of Tet solution.

2. For Spectinomycin (Spec):

Use 100 mg/ml (100 µg/µl) of stock Spec solution.

On agar: already made

In overnights (with 5 ml LB):

need 50 µg/ml, or 250µg of Spec, so need 2.5 µl of Spec solution.

3. For Kanamycin (Kam):

Use 10 mg/ml (10 µg/µl) of stock Kam solution.

On agar: already made

In overnights (with 5 ml LB):

need 50 µg/ml, or 250 µg of Kam, so need 25 µl of Kam solution.

Gel Purification

(Extracting DNA of a particular kb size from an electrophoretic agarose gel.)

Refer to URL =

<http://www1.qiagen.com/literature/handbooks/PDF/DNACleanupAndConcentration/MinElute/1027886_HB_QQ_MinElute_0604.pdf>

PREPARING THE PLASMID FOR MICROINJECTION

Making Plasmid (pRW4) for Microinjection

1. Plate and grow overnight colonies from freezer stock according to IV and V above.
2. Isolate plasmid according to VI (Standard Alkaline Lysis Preparation) above (resuspend in microinjection buffer).
3. Clean DNA thus isolated using Zymo Research Zymoclean kit. This is necessary to make the plasmid solution very clean (i.e., get rid of any phenol residue, which is highly toxic). URL = <http://www.zymoresearch.com/products/dna/zymoclean_gel_dna_recovery_kit.asp>

Washing and Diluting SM101pir (promiscuous bacteria) for microinjection (Bauer Lab)

1. Put overnights of transformed SM101pir, made from freezer stock, in appropriate plastic test tubes for Sorvall centrifuge.
2. Spin very slowly in Sorvall centrifuge (approximately 2k) for 10 minutes; make sure that temperature is not near freezing. Ensure that there is *some* bacterial pellet at the bottom of the tube after spin. If not, spin for longer (5-10 minute increments).
3. Remove supernatant.
4. (Wash step) Add 5 mL of fresh LB, shake the test tube gently to resuspend pellet.
5. (Wash step) Repeat steps 2 and 3.

6. Add approximately 250 µl of LB to plastic test tubes. (CAVEAT: this is another part of the experiment that can be varied. It is unclear how concentrated the bacterial broth should be. It should be as concentrated as possible, but if it is too concentrated, it becomes too viscous and sticks to the side of the capillary tube used for microinjection; furthermore, the eggs may only be able to survive injection with a limited number of bacteria. Attempt at different concentrations.)
7. Bacterial broth is now ready for microinjection. Place on ice until ready to use.

II) Microinjection and Population Biology Methods

(Done after pRW4 plasmid was constructed and prepared.)

In this part of the experiment, both naked plasmid (pRW4) and SM101pir (promiscuous bacteria) were microinjected into eggs. Eggs that survived microinjection were then sexed according to larval characteristics. Eventually, isolated P females were mated with P males, and the F1 eggs and larvae were tested for successful transformation.

Isolating and Mounting *Tribolium* Eggs (Wade Lab)

1. Make an *egg factory* by putting sterile fine-sifted flour in a sterile glass jar (should be a 2-3 centimeter thick layer at the bottom of the jar). Add desired adults to this (approx 30-50; depending on how many eggs the factory produces). Use Flagstaff, Arizona and Vejle, Denmark populations. The former certainly has *Wolbachia* in it; there is some uncertainty vis-à-vis the infection status of the latter (see results

chapter). Other populations could be used, but these first have to be tested for *Wolbachia* in them (see PCR section below). Label the jar.

2. How to isolate eggs in three steps. Use the coarse-meshed sieve to remove the adults. Place the contents of the jar onto a coarse-meshed sieve on top of a piece of black construction paper. From the side, tap the sieve and let the flour fall on a piece of black construction paper. Gently tap adults from sieve onto a sterile glass plate and place them back into the jar.
3. By forming the construction paper with flour + eggs into a funnel, pour the flour + eggs onto a fine-meshed sieve on top of another piece of black construction paper. From the side, tap the sieve and let the flour fall on the construction paper. Large white oblong pieces that stay on the fine-meshed sieve will be observed. These are the eggs. Get as much flour through the sieve as possible by continuing to tap it. Return that flour to the jar with the adults.
4. Turn the sieve with eggs upside down on a piece of black construction paper. Tap the sieve to allow all eggs to fall on the paper. Now gently angle the paper and watch the eggs roll down the side onto another piece of construction paper. Do this a few times. This is an egg *selective sieve*.
5. First, discard eggs isolated with steps 2-4.
6. After eggs have been discarded and egg factory has sat undisturbed for 2 hours, repeat steps 2-4, but this time keep the eggs. This provides eggs that are equal to or less than 2 hours old.
7. Once all the eggs are separated, place the eggs in a test tube with a few ml of 2.5% bleach solution. Shake a few times; let sit for 1 minute. This action washes off the

flour and removes the outer egg coat. Pour this mix through a small plastic sieve. Rinse with ddH2O to get rid of bleach (exact amounts and concentrations in this step are not crucial).

8. Take a glass slide and, using a fine camel's hair brush, move the eggs from the sieve and place them, in a row or two, on the glass slide. Use a simple microscope to do this carefully. They are gelatine-like, transparent, and egg-shaped. Label the slide.
9. Take the glass slide to the microinjection apparatus in the Kaufman lab. (A NOTE ON TIME: Step 6 is done after 2 hours because this gives enough time to perform steps 6-9 and the microinjection protocol before the eggs start cellularizing after 4 hours. It is important that time regimen be strictly followed. *Eggs need to have received treatment (plasmid or bacteria) before they are 4 hours old*.)

Microinjecting Tribolium Eggs (Kaufman lab has superb machines; also did some in Preer lab)

1. Use plasmid solution and diluted bacterial broth, on ice, respectively. Add a small amount of purple dye (highly diluted tetramethylrhodamine-dextran, 10,000 molecular weight, from Molecular Probes) to give the plasmid a purple color. The bacteria already have a different yellow color from the egg. These colors provide a way to observe successful injection of material into the eggs.
2. Take slides with eggs up to the Kaufman lab. (Previous training for how to use the microinjection machine is necessary; this is a rule of the Kaufman lab. Training was received from Paul Liu.)

3. Microinject eggs at different specs of the microinjection machine, a Narashige IM 300. Use the inverted microscope, a Nikon Eclipse TE300 to see what you are doing. Again, this is one of the variables of the experiment. Specs for the microinjection machine:

   For plasmid: .1 sec; 10 psi for Injection; 1 psi Balance; .4 psi Hold.

   For bacteria: .1 sec; 15 psi for Injection; 1.5 psi Balance; .5 psi Hold

   These specs can be varied, but the eggs are very delicate and especially sensitive to injections with the bacterial broth (relative to mortality rates for injection with naked plasmid, mortality rates for injection with bacterial broth were up to 5 times greater). So, possibly, a new experiment should be done in which the amount injected is diminished –the specification values would be decreased.
4. After microinjecting all the eggs, place them in large plastic petri dishes, place a piece of kimwipe soaked with distilled H2O in the petri dish to provide humidity, cover the petri dish and place the petri dish in the Wade Lab incubators, which should be at approximately 28º C. Make sure the kimwipe is moist at all times. This usually requires checking it twice a day.

Rearing and Mating Ps, Rearing F1s (Wade Lab)

1. Make a mix of, by weight, 95% fine-sifted flour and 5% yeast. Bake this overnight. Take, by weight, 99.75 % of this and add .25% of 95% Tetracycline powder. May have to increase the amount of tetracycline, or use spectomycin instead. (This is another variable of the experiment that has not yet been worked out.) Keep this at

room temperature in a sterile and closed container: a 50 ml Falcon tube would work.

2. Sterilize an appropriate number of foam tops for the eppendorf tubes you will use. The sterile eppendorf tubes and racks can be purchased from the biology supply room.
3. With the fine-meshed sieve, check the eggs daily to see whether they have hatched into larvae. Usually they start hatching after 3 or 4 days. They can continue hatching until about day 6 since microinjection. Check until day 8 to be sure.
4. When an egg has hatched into a larva, carefully move the larva, with a camel's hair brush, into one of the tubes with a few centimeters of the flour prepared in step 1. Label the tube with date of egg injection, egg hatching, home population, and treatment type. These larvae are the P generation.
5. After about a week** after hatching, the larvae should metamorphose into pupae. Once they have been in the pupal phase for a few days (3-5), it is easier to identify their sex ("sexing them"). Sex them by looking for the presence of "horns" in their genital area. If they have horns, they are females. (Jacob Moorad or Jeff Demuth, from Wade lab, helped.) Adults can also be sexed. Adult males have a small pit in the first third of the first section of their first pair of legs. Label which sex the larvae are.
6. The pupae metamorphose into adults after approximately 1 week**. The adults can be left, by themselves, for up to a few months.
7. Now that the pupae have been sexed, and adults eclose after approximately 2.5 weeks upon hatching, adults can be mated. Separate males from flour by gently

tapping the contents of a tube onto a coarse-meshed sieve. Place the single male in a tube with a single female. Place the flour back in the eppendorf tube in which the male was.

8. The male will copulate with the female within the first day. Wait 2 days after introducing the male, and separate the eggs using the isolate *Tribolium* egg protocol above. Place these eggs in their own labeled vial. These eggs are the F1 generation. It is these eggs, larvae and adults that must be tested for the presence of Wolbachia and GFP.

Identification of *Wolbachia* and pRW4 DNA from *Tribolium* treatments using Polymerase Chain Reaction (Bauer Lab)

(50 µl)

5 µl 10x Tricine Buffer

4 µl 10m dNTP

.5 µl forward primer*

.5 µl reverse primer*

.15 µl taq polymerase + pfu

1 µl 100mM MgCl2

1 µl purified DNA (e.g., plasmid) or 2.5 µl *Tribolium* grindate**; template DNA

37 µl or 35.5 µl ddH2O

1. Add primers and template at 4°C.
2. Hot start (96°C) for 2 minutes.

3. Add cocktail of remaining ingredients (to make the mix fully reactive) at end of hot start.
4. 4.1. Denature at 96°C for 30 seconds, 4.2. anneal at 62°C for 45 seconds, and 4.3. extend at 72°C for 3 minutes. Repeat 30 times.
5. Extend at 72°C for 6 minutes.
6. Lower to 4°C. Can be kept (practically) indefinitely before diagnostic gel is run.

*Forward and reverse primers used are of two sorts: 1. one pair (*Wolbachia* promoter segments) to identify the presence of *Wolbachia* in the experimental treatments (woFor CCA AGG TTA AAA GTT GCA AGA ACT ATT GCA; woRev GGA CCG GTA GTG CTT GAG CAT CGG TAT; see Chapter 4), and 2. another pair (GFP segments) to identify the presence of pRW4 in the experimental treatments (GFPFor ACG TAA ACG GCC ACA AGT TCA G; GFPRev GCT CGT CCA TGC CGA GAG TG).

***Tribolium* grindate is prepared in the following way:

1. Make glass mortars by melting thin 2 ml capillary tubes over a Bunsen burner. As they melt, rotate them to get a smooth surface at the tip. These are now sterile.
2. Use 50 uL of squishing buffer (SB) (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl) with Proteinase K. SB and Proteinase K (added to a concentration of 200 ug/ml) can be gotten from Paul Liu, or made fresh.
3. Pipette 50 uL of SB/Proteinase K mix in eppendorf tubes with 3-5 eggs, larvae, or adults (of same treatment!). (Larvae give the best PCR amplification signal.) Use oblong glass mortar to crush the eggs, larvae, or adults in the buffer. Let sit for 45

minutes (Proteinase K denatures significantly already after approximately 15-20 minutes).

4. Bring this to a near-boil (95º C) for 5 minutes on the PCR block.
5. Crush again with the second glass mortar.
6. Repeat step 4 and crush again.
7. Dilute 1:100 in ddH20.
8. Place *Tribolium* grindate on ice. (NOTE: Use this beetle soup as soon as possible. It may be able to keep for a week in the –20º C freezer, but any DNA in it will denature relatively quickly due to DNAses that may be present.)

Chapter 3. Results

Here, I will first describe the results of the molecular biology part by detailing each plasmid construction step. I will then describe the ultimately negative results of the microinjection and population biology part of the experiment.

I) Molecular Biology Results

Basic Plasmid Constructs

I followed the molecular biology methods outlined in the previous methodology chapter.

pRW1: This step was done primarily by another person in the Bauer lab. David Rollo amplified the *Wolbachia* sequence, possibly containing the ftsZ promoter by PCRing a segment of the *Wolbachia* ftsz from *Tribolium* grindate. Dr. Carl Bauer and David Rollo designed primers using a BLAST search for this gene segment of *Wolbachia*. The initial procedures involving pCRscript SK(+)Amp (Stratagene) are unclear to me. Significant more detail regarding this sequence is to be found below even if the exact nature of the sequence remains problematic. (Lesson: *an experiment should be started and finished by the same person*.) It is this 1.087 kb sequence that was cut and ligated into the various plasmids. The segment was cut using HindIII ad AgeI and ligated into pEGFP-N1 (Clontech), which has a Kam resistance gene. This plasmid was subsequently electroporated into the bacterial cloning strain DH5.

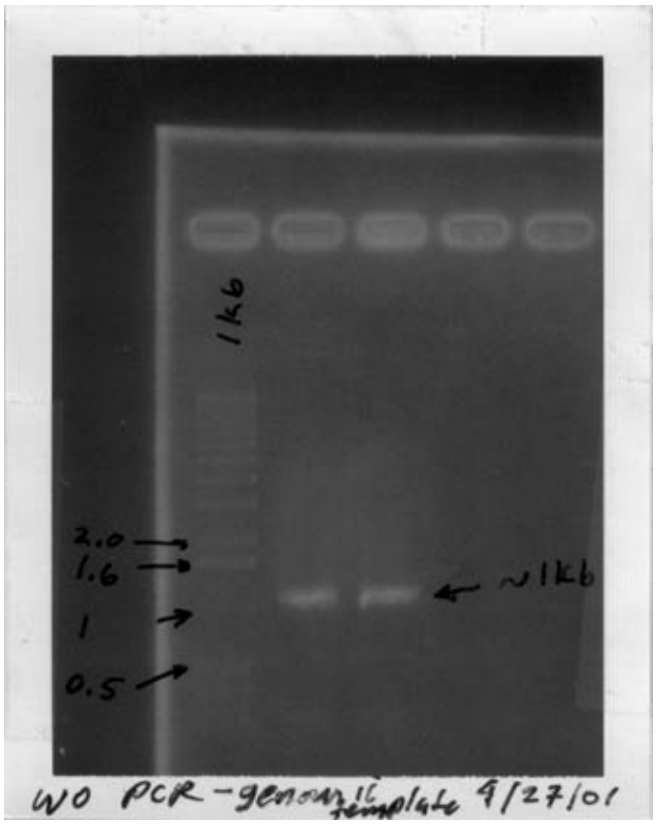


Figure 1. Diagnostic cuts of pRW1. Writing by David Rollo. All writing on subsequent figures is by author.

pRW2: It was a mistake to put pRW1 into DH5, since that is a dam+ strain, and the pEFGP-N1 vector has a methylated XbaI cutting site. Under this condition, the site is not active. This cutting site, which is convenient to use since it marks one end of the GFP segment (see Figure 5), needs to be unmethylated by transforming it into a dam- strain. This was done using a Bauer lab strain, NS2626. The plasmid was chemically transformed into that bacteria. After growth in NS2626, and subsequent isolation of the plasmid, diagnostic cuts showed that the appropriately-sized segments were there. Furthermore, since the Xba1 site did cut now, it was clear that this step was successful.

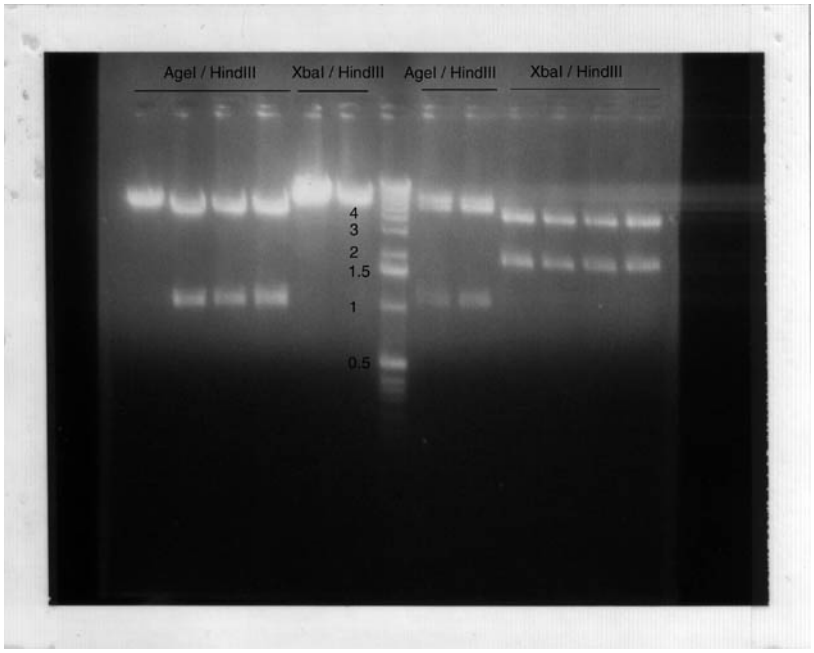


Figure 2. Diagnostic cuts of pRW2 with pair-wise Hind III, Age 1, Xba1 to see if there were 1.1 and 1.8 kb fragments. All lanes match predictions. Lanes 1-4 AgeI and HindIII on pRW1: there should only be a 1.1 kb fragment (*Wolbachia* sequence). Lanes 5-6 XbaI and HindIII on pRW1: since XbaI does not cut in pRW1, there should be no fragments here. Lanes 7-8 AgeI and HindIII on pRW2:

there should only be a 1.1 kb fragment (*Wolbachia* sequence). Lanes 9-12 XbaI and HindIII on pRW2: XbaI should cut, so there should be a 1.8 kb fragment (*Wolbachia* sequence and .741 kb GFP).

pRW3: The purpose here was to insert the *Wolbachia* sequence and GFP segment into a broad host-range plasmid. After all, I want the plasmid to be recognizable by *Wolbachia*, and there is more of a chance of this occurring if the plasmid is a broad host-range one. Here HindIII and XbaI were used to cut both pRW2 (at each end of the *Wolbachia* sequence-GFP segment) and pRK415. The desired segment of pRW2 was gel isolated before it was put into the ligation mix. In the pRK415 vector, the HindIII and XbaI cutting sites are part of the MCS (multiple cloning site) (see Figure 6). Upon electroporation and growth in strain DH5, diagnostic cuts showed that all the appropriate bands were there.

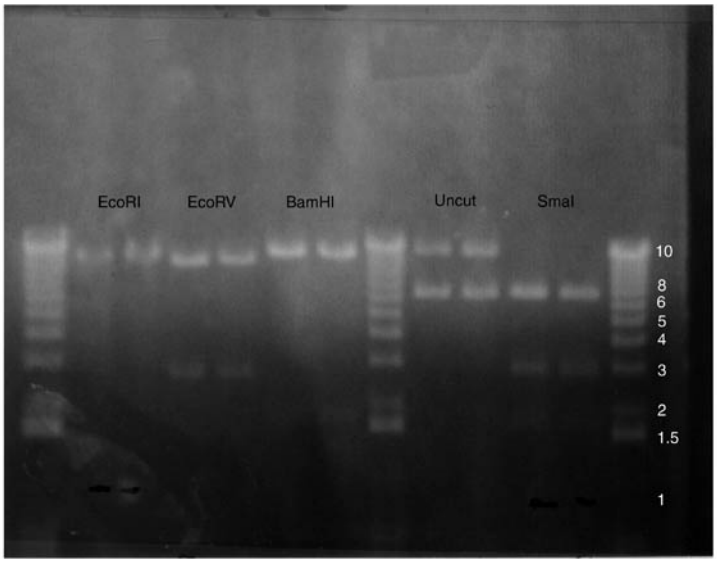


Figure 3. Diagnostic cuts of pRW3 with single enzymes to see if appropriate fragments were there. All lanes match predictions. Appropriate fragments: EcoR1, .9 and 11.4 EcoRV, 2.8 and 9.5 kb; BamHI, 1.8 and 10.5 kb; SmaI, .9, 1.8, 2.8, and 6.8 kb. Weak bands are highlighted. 7 kb fragment in uncut lane almost certainly indicates supercoiled plasmid.

pRW4: The purpose here was to insert a spec cassette into pRW3. The HindIII cutting site was used for both the spec cassette and pRW3. Spec cassette was gel isolated and ligated into the cut pRW3. The plasmid construct was successful as can be seen from the diagnostic cuts and from the fact that DH10b with this plasmid survives in overnights with both tet and spec.

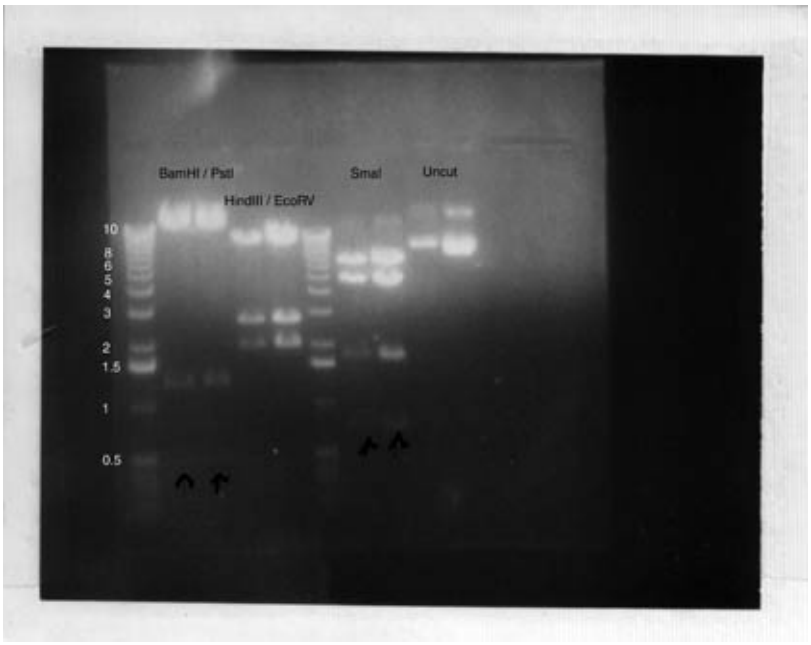


Figure 4. Diagnostic cuts of pRW4 with single and pair-wise enzymes to see if appropriate fragments were there. All lanes match predictions. Appropriate fragments: Bam H1 and PstI, .5, 1.2, and 12.5 kb; HindIII and EcoRV 2, 2.5, and 9.7 kb; SmaI, .9, 1.8, 4.7, and 6.8 kb. Weak bands are shown with an arrow.

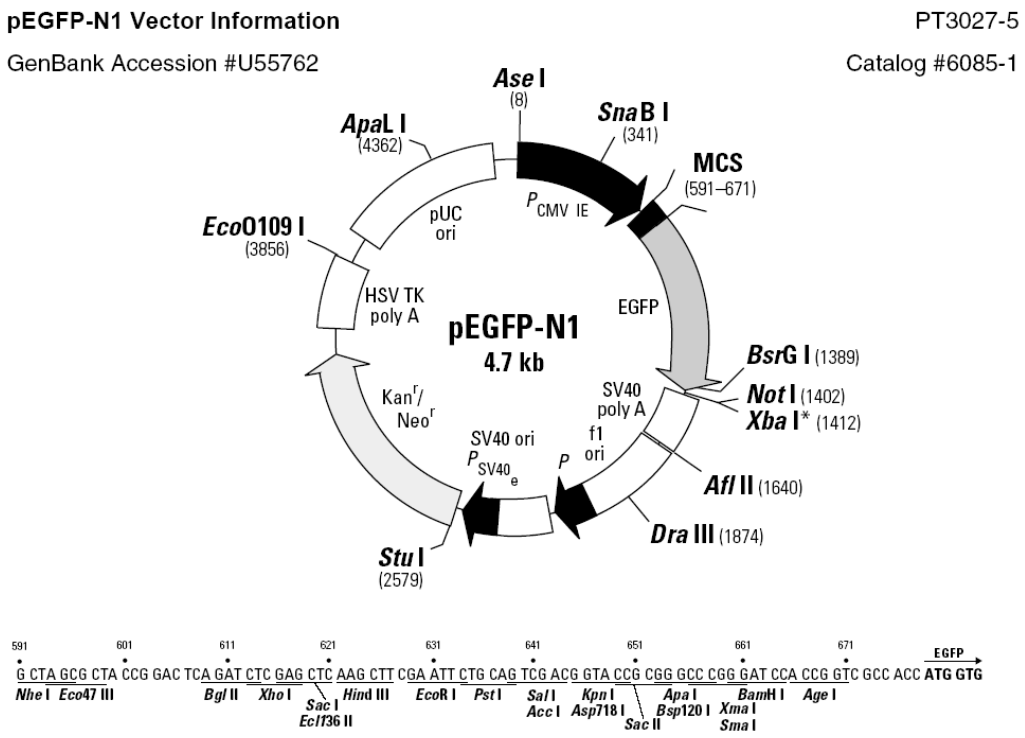
	Contents of the Plasmid	**Purpose of the Plasmid**	**Antibiotic resístance genes**
pRW1	*Wolbachia* sequence in pEGFP-N1 vector in strain DH5. High-copy plasmid	To place *Wolbachia* sequence in a vector with GFP	Kam
pRW2	*Wolbachia* sequence in pEGFP-N1 vector in NS2626, a dam- strain. High-copy plasmid	To activate XbaI cutting site in the vector	Kam
pRW3	*Wolbachia* sequence + GFP in pRK415 vector in strain DH10b Low-copy, broad host-range plasmid	To insert the *Wolbachia* sequence and GFP segment in a broad host range vector	Tet
pRW4	*Wolbachia* sequence + GFP in pRK415 with spec cassette in strain DH10b. Low-copy, broad host-range plasmid	To add a spec cassette to the vector, so that final plasmid has two antibiotic sites	Tet Spec

	DNA Isolation/ Amplification	**Ligation-Transformation**	**DNA Diagnosis**
pRW1	From *Tribolium confusum* larvae. PCR using forward/ reverse primers designed from *Wolbachia* ftsZ	- Cut pCR script and pEFGP-N1 with HindIII and Age I - Ligate with PCR amplified *Wolbachia* sequence into first pCRscript SK(+)Amp* and then pEFGP-N1. - Electroporation	Cut with Hind III and Age 1 and see if there is a 1.1 kb segment
pRW2	Isolated pRW1 using miniprep	- Simply move plasmid into new bacteria - Chemical transformation	Cut with pair-wise Hind III, Age 1, Xba1 and see if there is a 1.1 and 1.8 kb segment
pRW3	Alkaline Lysis prep (Need to use because the plasmid is low-copy.)	- Cut both pRK415 and pRW2 with HindIII and Xba1 - Gel isolate *Wolbachia* sequence -GFP from pRW2 - Ligate - Electroporate	Cut with single enzymes: Bam HI, EcoRV, SmaI and see whether appropriately sized bands are there**
pRW4	Alkaline Lysis prep (Need to use because the plasmid is low-copy.)	- Cut pRW3 and spec cassette with HindIII - Gel isolate spec from spec cassette - Electroporate	Cut with single and pair-wise enzymes: Bam HI, EcoRV, HindIII, PstI, SmaI***

*The initial procedures involving pCRscript SK(+)Amp are unclear to me.
**For pRW3: EcoR1, .9 and 11.4 EcoRV, 2.8 and 9.5 kb; BamHI, 1.8 and 10.5 kb; SmaI, .9, 1.8, 2.8, and 6.8 kb
***For pRW4: Bam H1 and PstI, .5, 1.2, and 12.5 kb; HindIII and EcoRV 2, 2.5, and 9.7 kb; SmaI, .9, 1.8, 4.7, and 6.8 kb

Table 1. Table, in two parts, summarizing various aspects of each of the plasmids constructed.

Vectors Employed



Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N1 Vector. All restriction sites shown are unique. The *Not* I site follows the EGFP stop codon. The *Xba* I site (*) is methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with this enzyme, you will need to transform the vector into a *dam*⁻ and make fresh DNA.

Figure 5. Commercially available pEGFP-N1 vector in which the *Wolbachia* sequence was inserted at/between the HindIII (622-627) and AgeI (666-671) sites.

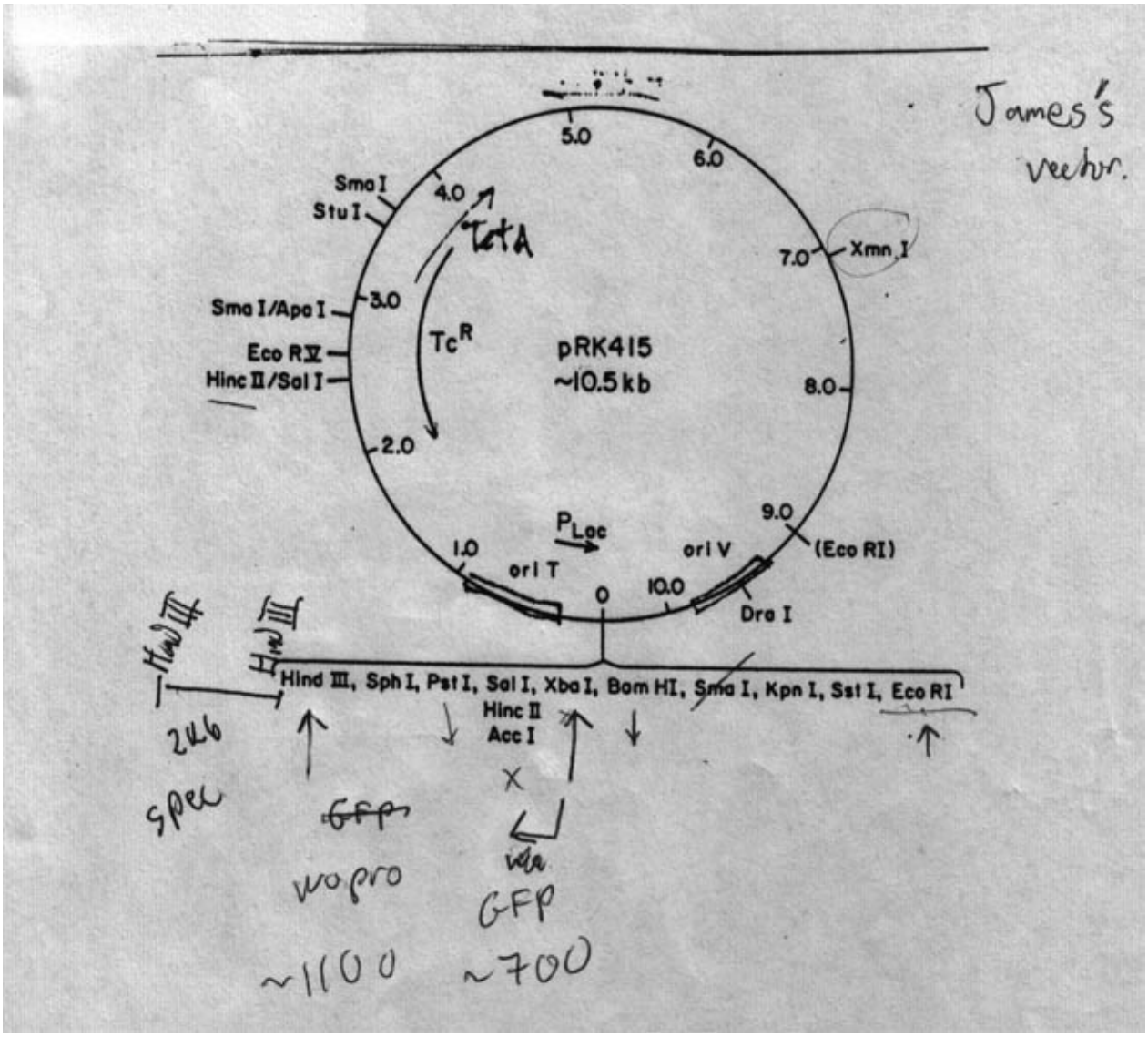


Figure 6. pRK415 vector provided by Dr. James Smart from Dr. Bauer's lab. GFP and the *Wolbachia* sequence were inserted at/between the HindIII and XbaI sites.

Final Plasmid Construct (pRW4). Figure 7.

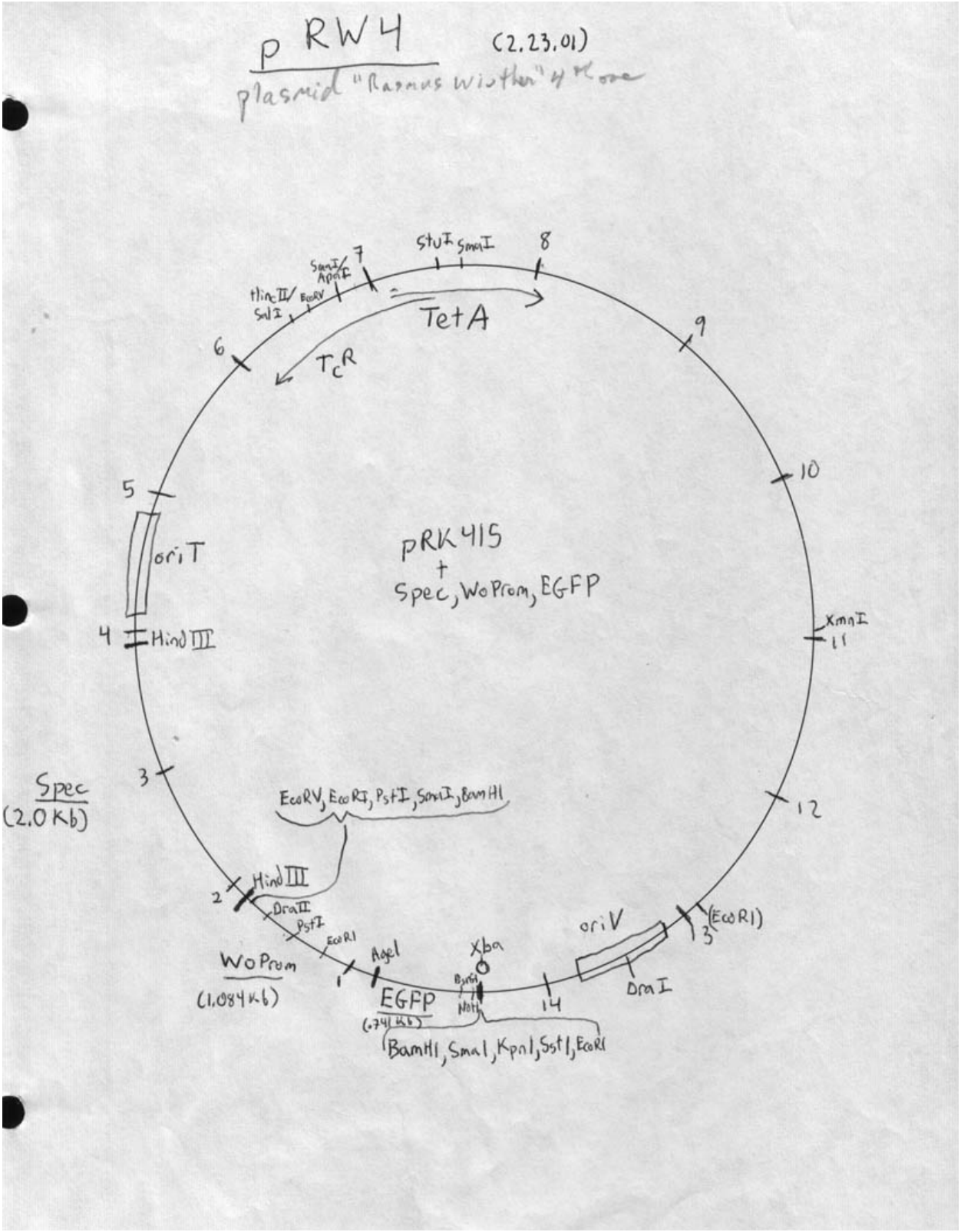


Figure 7. Map of pRW4 plasmid. All known restriction enzyme sites are indicated.

Figure 8. Genomic Analysis of *Wolbachia* sequence, possibly containing an ftsZ promoter

Wolbachia sequence isolated, amplified and put into all plasmids

5′AAAGCTGGAGCTCCCCGCGGTGGCGGCCGCTCTAGCCCGGACCGGTAGTGCTTGAGCA
TCGGTATTTGCTACAACAAAATTTACTCCTTGCAAATTGGATTGAATCATGTTATTCACA
GCATTTCCACCAGCACCACCCACTCCCACAACGGTAATCCTTGGGTGYAATACAGGTAGC
TCTGGTAAACTAAGGTCAATTGACATTTAAACTTTACTCAAATATTAGTGAATTCACNTT
GATAACAAGCNTACCTGCTTTTTTAACAATATGCAAACATTTTTCATTTTGACATATGCA
AATAGTAAATAAGATTTACTTATTCTTAACATTAATAGATGTGATAATTATAGATTTTCA
CATAACTCTACTAAAAATTTAAAAAGGGACAGAAACCTATCCCTTTTGTTGAAAACTAAC
TTACAGAACGAACTGGCAAGCAAGGATGTACTTTAAGTTCATTAATCTTAGTGTCTGGTT
CTTTATCCCCATCCTTTACTATTGTTCGTATTGTATCAATATTGCCATTGAGGGCTTTAA
TTTCCTCTGTATAACCACTGTTTTTATATGCAAGCACAGGTGCAGGTATTAAAGCTGCTA
CTGCAGCAAGGACTACCAAACTAGCAATAAGTGGATGAGCAACTGCAAATGCAGACATTG
CAGTAATAGCAGGACTAACAAGTGTTGCAGCTTTCGTTCCAACTGTACCAACAAAAGTTG
CATAAGCTGGACTTAAAAAATAAGCAGCCGTAAGCGAAGCTACTGCTGCAATTGCTACAA
AAGTACCAGCTGTTTTACGCGGATTTTCTTTTACAAGATCCCAACTTTGCACAGGAACTT
CTTTAGCTGCTGACCAAAGGCCCTTCACTTTTGTATTTATTTTTTATCATTTCAATTCTT
GTCATAAATCTGCTCCTAAAATATTAAAATTTCACTAACGGCAAGTATATAGCAAAAAAA
AGCTGCCCGTCAGGTAAATTAATTTATTTATACATCTTTATTCTGTAATTCAGCGCTTCT
GCAATGTGTGCTTTTTATTTCCTCACTTTTCACAAGAGCTGCAATAGTTCTTGCAACTTT
TAACCTTGGGGGCGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGT
CGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCT
CACTGGCC 3'

(1087 bases***)

Key to Figure 8

- … primer worev (primer; forward direction; underlined 6-base sequence is AgeI site)
- … primer wofor (primer; reverse complement direction)
- … pCRscript SK(+)Amp (plasmid piece, in the MCS, from 728 to 609, of plasmid; NCBI sequence BLAST and comparison with URL = <http://www.stratagene.com/>, reverse complement direction; underlined 6-base sequence is HindIII site.)
- … wolbachia hit 1 (ends on blue TTT) (sequence piece; NCBI sequence BLAST "Wolbachia sp. wTai DNA, insertion sequence ISWI in orfB", forward direction)
- … wolbachia hit 2 (begins in light gray TAG embedded in worev primer) (sequence piece; NCBI sequence BLAST "Wolbachia endosymbiont of Drosophila melanogaster"; forward direction)
- … wolbachia hit (included in wolbachia hit 1) (sequence piece; NCBI BLAST; forward direction)
- … Contig1230 74_1contig.ace.340 (sequence piece; NCBI BLAST; forward direction)

… Cell division GTPase, ftsZ (NCBI BLAST for ORF, reverse complement direction). Blast reference is Holden et al. 1993.

… ftsZ of *Wolbachia* endosymbiont of *Drosophila melanogaster*; obtained as a *promoter* sequence from Regulatory Sequence Analysis tools (http://embnet.cifn.unam.mx/rsa-tools/); also referred to in Holden et al. 1993 (reverse complement direction); no overlap with sequence in Fialho and Stevens 1997. [Total bases in both, partially overlapping, functionally interpreted sequences of ftsZ: 423]

ACT Indicates part of the sequence that is known to be homologous with some known sequence or other. Otherwise, letters are in black.

***Please note that sequence above, provided by IU Biology sequencing service, is itself in reverse complement order, which is why, for example the reverse primer is in the forward direction (and vice-versa). The order indicated for each colored entry (e.g., Contig1230 74_1contig.ace.340 as "forward") is the order *with respect to* the order given by the sequence as shown above. Thus, a "forward" sequence is, *in reality*, a reverse complement sequence. As DNA can be functionally interpreted in either direction, this issue is not crucial.

According to Holden et al. 1993 and BLAST:

Ftsz: "[Function] This protein is essential to the cell-division proves. It seems to assemble into a dynamic ring on the inner surface of the cytoplasmic membrane at the place where division will occur, and the formation of the ring is the signal for septation to begin. Binds to and hydrolyzes GTP.

[Subunit] Aggregate to form a ring-like structure (by similarity).

[Subcellular location] Cytoplasmic. Assemble at the inner surface of the cytoplasmic membrane (by similarity).

Note that there is no overlap between my sequence and Fiahlo and Stevens' 1997 sequence (as determined by BLAST searches). However, there is significant overlap between my sequence and Holden et al.'s 1993 sequence, as indicated by the (light and dark) grey segments. These are the only segments of my sequence for which there is *functional information*. The dark grey region is, with very high likelihood, the ftsZ *promoter*, whereas the light grey region is probably a fragment of the coding region of ftsZ. For purposes of this experiment, presence of the promoter region is crucial.

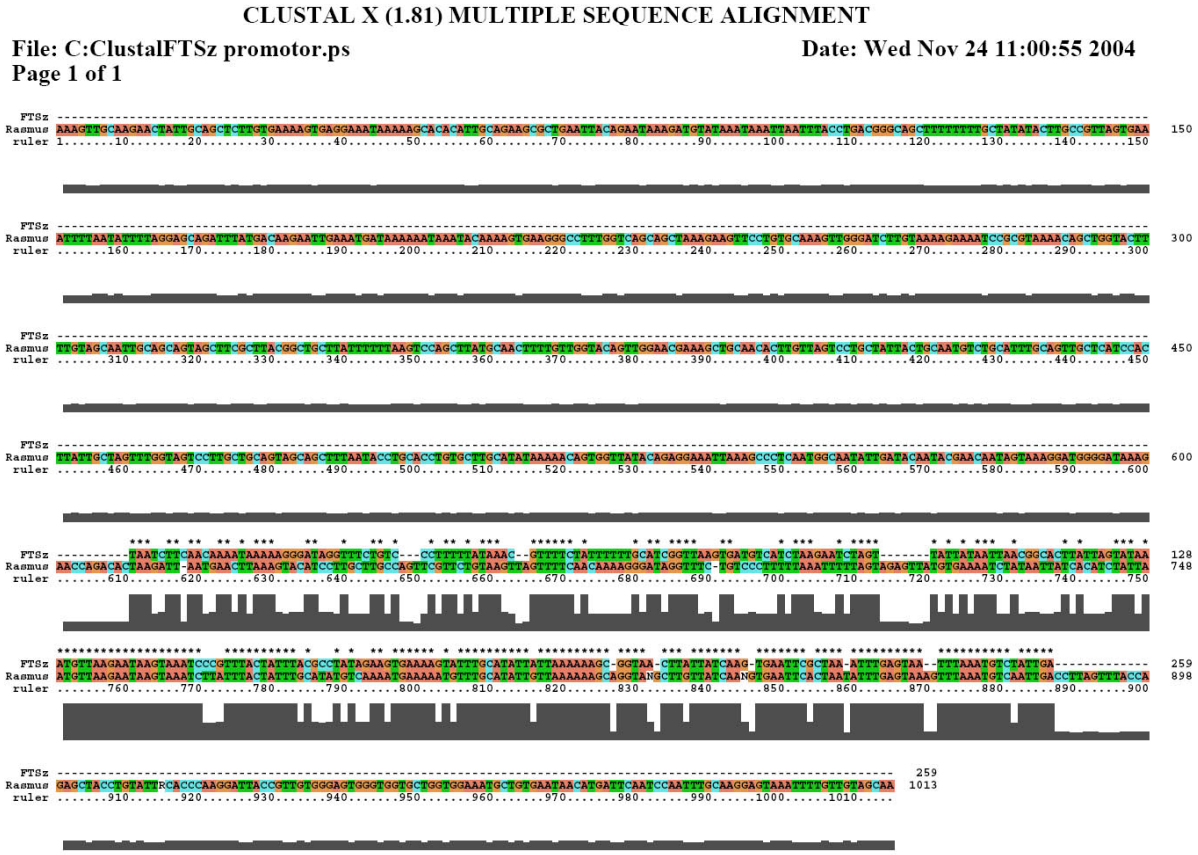


Figure 9. Comparison of *Drosophila melanogaster Wolbachia* ftsZ promoter sequence (from <http://embnet.cifn.unam.mx/rsa-tools/>) with sequence isolated from *Tribolium*, using the ClustalX program URL = <http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html>; see also Thompson et al. 1997. Note that there is significant overlap between the two sequences, as also indicated in dark grey in Figure 8 above. The large overlap of 275 bases suggests that there is a significant chance that the entire promoter has been captured.

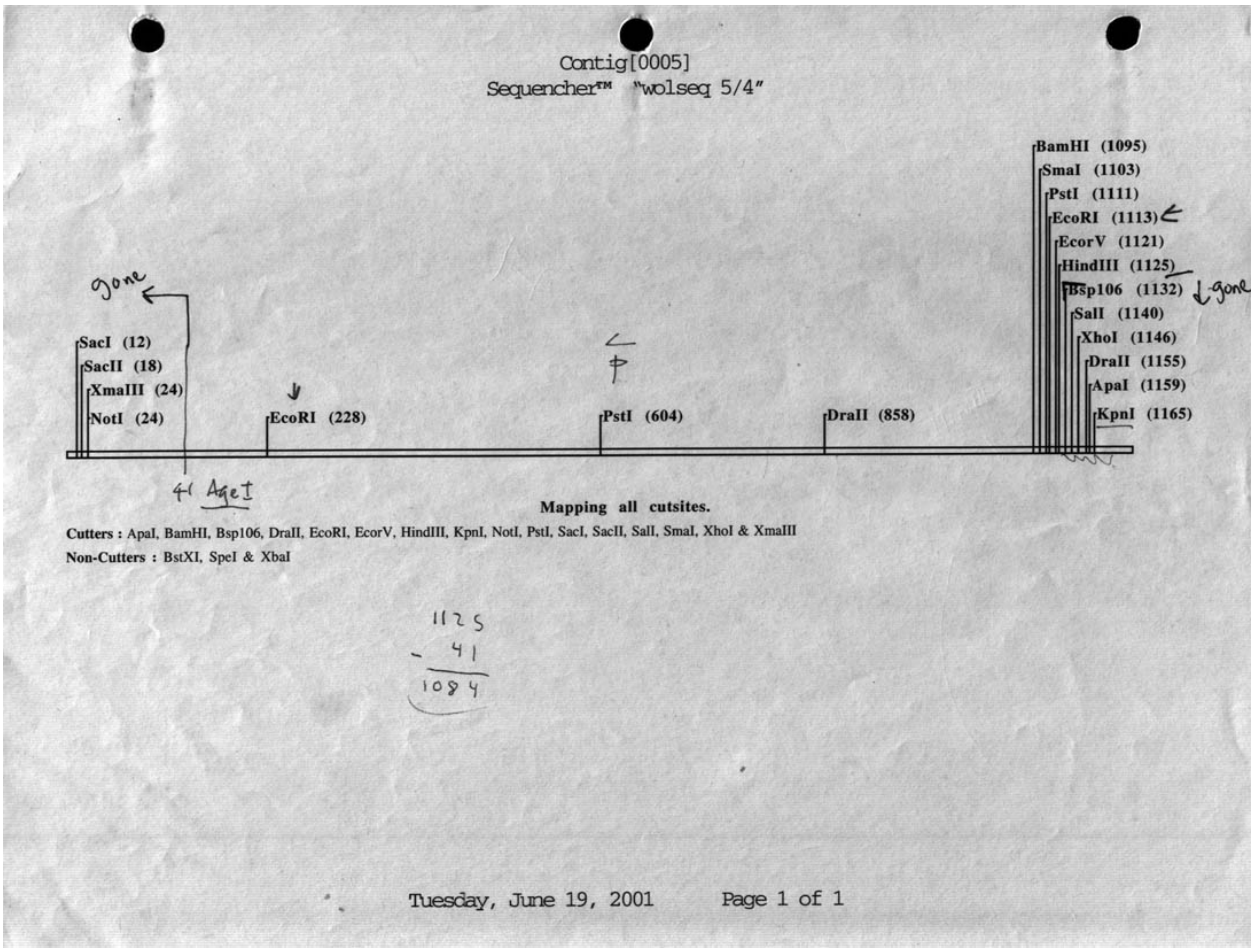


Figure 10. All cutting sites for sequence isolated from *Tribolium*. Again note that the sequence is in reverse complement order. All restriction sites outside of AgeI (worev) and HindIII were eliminated.

Microinjection Preparation

pRW4 plasmid was prepared in the manner indicated in the methodology chapter. It is in *E. coli* strain DH10b.

pRW4 was also transformed chemically into SM10Lpir, a promiscuous bacteria, that is, a bacteria that will readily mate with a broad range of other bacteria. The transformed SM10Lpir, after recovery, were grown on tet/spec plates to ensure that they had the plasmid. They grew as lawns on these plates, indicating that the transformation was successful. After overnights were made, the SM10Lpir was concentrated as described in the methodology chapter.

Location of materials

The two strains of bacteria, DH10b and SM10Lpir, both with pRW4, are found in rack G7 of the new Bauer freezer. They are in the box labeled "Chemically-Competent Cells 12-18-01."

II) Microinjection and Population Biology Results

Microinjection was done both in the Preer and Kaufman labs. The Preer lab equipment relied on hand pressure for microinjection, whereas the Kaufman lab has a fully automated and precise machine. For future work on this project, use of the Kaufman lab machine is highly recommended. This is also a machine with which different pressure settings should be tried, in order to increase the survivorship of microinjected eggs (see, e.g., Chang and Wade 1994, 1995).

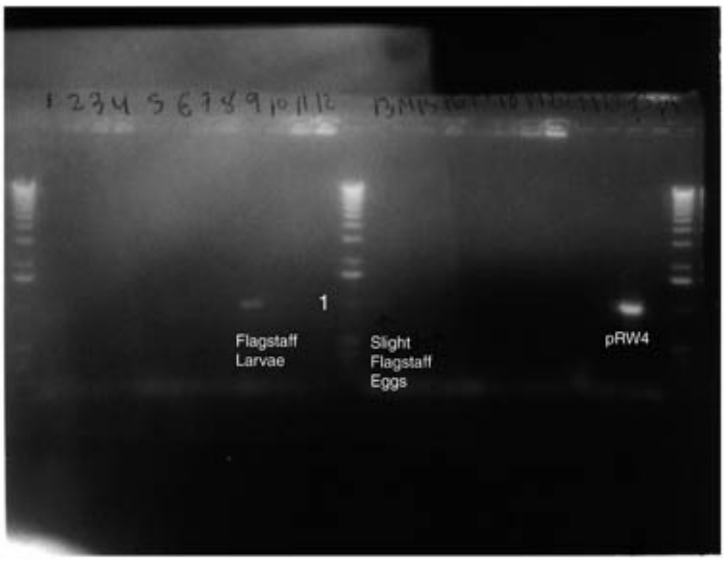


Figure 11. According to this diagnostic PCR, only Flagstaff, Arizona populations of *Tribolium confusum* (lanes 9-16) have *Wolbachia*. Neither Vejle, Denmark populations of *T. confusum* (lanes 1-8), nor North Carolina *T. castaneum* (lanes 17-20) showed any signal of *Wolbachia*. Note that positive control, pRW4, with the *Wolbachia* promoter region, provides signal. Later experiments by Matt Roberts,

however, indicated that the Vejle, Denmark populations had *Wolbachia* as well. These results are therefore equivocal and further work is required (see Chapter 5).

In the two tables that follow (Table 2), I indicate the numbers of eggs microinjected and the numbers of larvae/adults that survived. I also present the percent survivorship of larvae and adults. The adults, as can be seen in the table on the next page, were also sexed.

	Eggs Injected	Larvae Hatched	% Larval Survivorship	Adults Surviving	Dead Adults	% Adult Survivorship
Vejle Plasmid Preer	46	N/A	N/A	5	3	10.9
Vejle Plasmid Kaufman	99	11	11.1	9	2	9.1
Vejle Bacteria Preer	42	N/A	N/A	19	1	45.2
Vejle Bacteria Kaufman	56	4	7.1	4	1	7.1
Vejle Total/ Average	243			37	7	18.1
Flagstaff Plasmid Preer	50	N/A	N/A	11	4	22
Flagstaff Plasmid Kaufman	63	10	15.9	6	3	9.5
Flagstaff Bacteria Preer	63	N/A	N/A	17	6	27
Flagstaff Bacteria Kaufman	88	3	3.4	1	3	1.1
Flagstaff Total/ Average	264			35	16	15
TOTAL/ AVERAGE	507			72	23	16.5

	Female Adults	Male Adults	% Female Surviving
Vejle Plasmid Preer	2	3	40
Vejle Plasmid Kaufman	7	2	77.8
Vejle Bacteria Preer	11	8	57.9
Vejle Bacteria Kaufman	4	0	100
Vejle Total/ Average	24	13	68.9
Flagstaff Plasmid Preer	6	5	54.5
Flagstaff Plasmid Kaufman	4	2	66.7
Flagstaff Bacteria Preer	11	6	64.7
Flagstaff Bacteria Kaufman	1	0	100
Flagstaff Total/ Average	22	13	71.5
TOTAL/ AVERAGE	46	26	70.2

As can be seen from these tables (Table 2), 507 eggs were microinjected, and 72 of those eggs reached adulthood. Of those 72, 46 were females. This is not an insignificant amount of females. Did any of those females contain *Wolbachia* transformed with the pRW4 plasmid? Unfortunately, the answer is "no," even though justifying this answer is a rather complex affair. It is to this that I now turn.

First of all, it should be noted that it is unclear whether the Vejle *Tribolium* strain contains *Wolbachia*. A diagnostic test, run with both positive (pRW4) and negative (ddH20) controls, indicated that *only* the Flagstaff strain are infected (Figure 11). I therefore chose Vejle as a negative control for the microinjection experiments. The Flagstaff strain *consistently* provided a strong signal for *Wolbachia* in a number of different PCRs. The Vejle, Denmark population provided no signal. However, a later PCR run by Matt Roberts of the Wade lab indicated that there was *Wolbachia* in three Vejle adults *after* microinjection. Given this contradictory result, further diagnostic tests for the presence of *Wolbachia* in Vejle are needed. These include running more PCRs with Vejle *Tribolium* grindate and, even, doing matings with other *Tribolium* populations that are

known *not* to harbor *Wolbachia*, in order to see whether CI, at any level, is expressed. This latter method is more time-consuming since it involves a large number of controlled matings and assessment of fertility measures, but it would provide independent data for the presence or absence of *Wolbachia* in the Vejle population.

In the microinjection experiment, it remains unclear whether a *negative* control (which was the original intention with using Vejle) is needed. That is, if a positive result is actually obtained (i.e., a *Tribolium* strain is observed to have both *Wolbachia and* GFP), it seems highly likely that a transformed *Wolbachia* did actually survive into the next generation of *Tribolium*. A negative control would therefore seem unnecessary. Furthermore, if negative controls are omitted, then *all* the microinjections would be potential candidates for success. Given the cumbersome nature of both microinjections and *Tribolium* rearing, this is a tremendous advantage. Furthermore, there are other *independent* means of testing whether a positive result actually is a successful result. For example, such a beetle could be placed directly in the appropriate microscope to see whether the *Wolbachia* it putatively contains actually turns out to emit fluorescent light as a consequence of its GFP. Having said all of this, however, the importance of negative controls is clearly crucial in science and the trade-offs between including these vs. omitting them (and thereby having more potentially successful candidates) need to be considered carefully.

Regardless of whether the Vejle strain actually has *Wolbachia*, a diagnostic PCR test for GFP of larvae from 35 females (some of the 42 females died or did not produce eggs and larvae even after mating) was done.

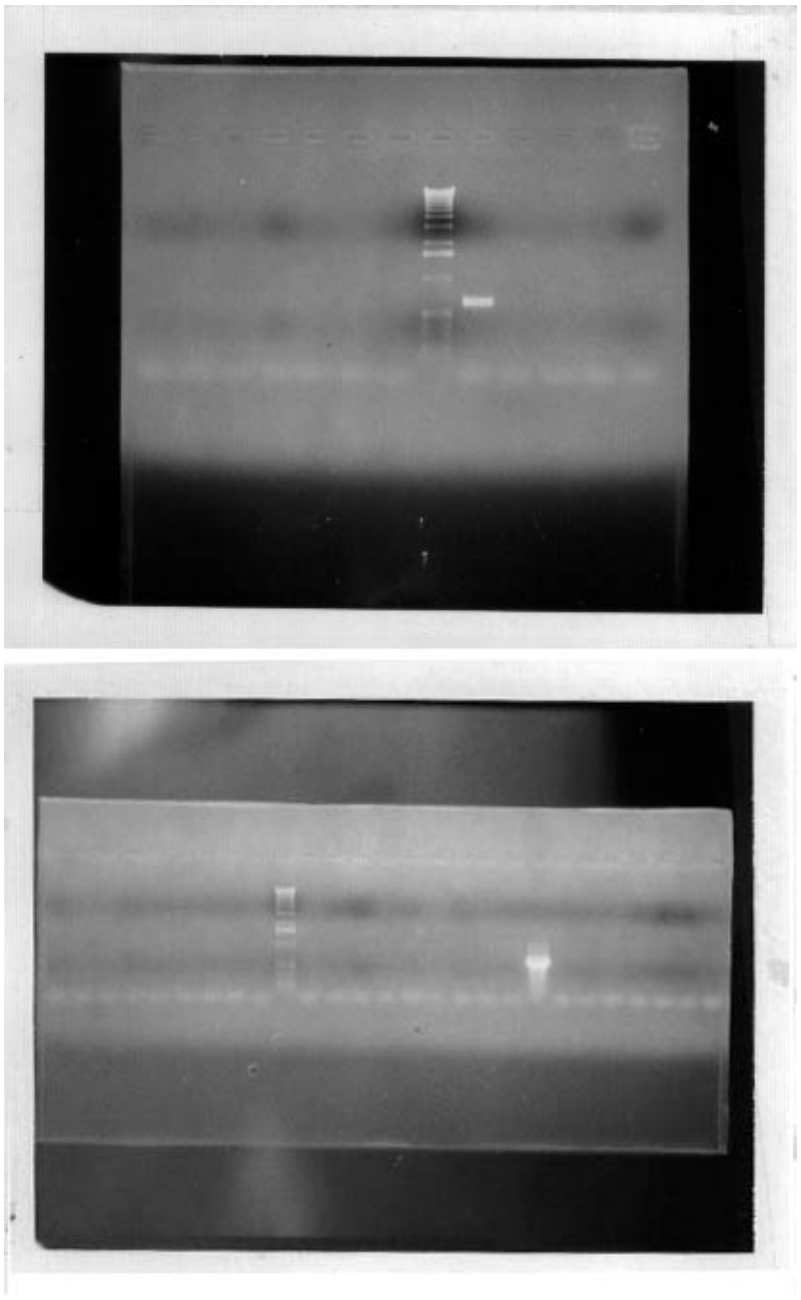


Figure 12. Negative results for the presence of GFP in larvae from 35 microinjected females. Only the pRW4 plasmid lights up in each gel.

As can be seen, only the positive control (pRW4) lit up. All the larvae failed to show a signal for GFP (experiment done July 18, 2002). A few months after this

experiment, Matt Roberts ran a diagnostic PCR test for *Wolbachia* (*not* GFP!) in 20 surviving females and found *Wolbachia* in 5 of them (including 3 Vejle beetles) (Michael Wade, pers. comm., October 28, 2002). This is an interesting result, since the beetles were raised on flour with antibiotics (only tet, at this stage of the experiment). There are two possibilities here: 1. the beetles kept some *Wolbachia without* the plasmid. This might be the case if the amount of antibiotic was not sufficient to clear non-transformed *Wolbachia*. This is an aspect of the experiment that has to be troubleshooted: how much antibiotic is sufficient to clear beetles of *Wolbachia*? (see, e.g., Stevens and Wade 1988) 2. alternatively, the experiment might actually have been successful. That is, a successfully transformed *Wolbachia* might actually have survived within a *Tribolium*. In order to test these two alternatives, a diagnostic PCR test for GFP had to be done. Unfortunately, lack of data presents itself here. I remember doing a GFP PCR test when I returned to Indiana University a few weeks after Matt Roberts had found signals for *Wolbachia*. This test showed no GFP signal, but, unfortunately, I am unable to locate the gel picture. Even if there had been GFP in those adults, they are, unfortunately, dead by now. Again, the microinjection part of the experiment would have to be redone from scratch in order to get transformed *Wolbachia* into the *Tribolium*.

Chapter 4. Biological Reification of the Mathematically Abstract: Two Distinct Models of the Evolution of Cytoplasmic Incompatibility

In an essay I recently wrote, "Biological Reification of the Mathematically Abstract," I developed a model for tracking how mathematically-inclined biologists impose their ontological assumptions onto their understanding of the world. This essay is part of a research project aimed at understanding the nature and importance of reification in science. The OED Online defines reification as: "The mental conversion of a person or abstract concept into a thing." I called my model the *SMEO* model ("set-up, manipulate mathematically, explain, objectify") and applied it to a particular case study from population genetics: two models of the evolution of CI by Michael Turelli and Steve Frank, respectively. In what follows, I will briefly describe the structure of my SMEO model and will then apply it to the particular case study, which I analyze in some detail.

In an important sense, the SMEO model originated from a passage found in Oyama's *The Ontogeny of Information*. Her central worry in this text is how the assumptions of the genetic program, a particular kind of metaphorical and potentially mathematizable model, affect our understanding of the *actual* processes of development and evolution. Let us turn to an insightful and important passage from Oyama's classic book:

> There is a subtle, repeated process at work here [in understanding and explaining the regularity of systems]. Order in a process is perceived and formulated as descriptive rules. From these, prescriptive rules are derived and imposed on a mechanical medium to allow simulation of the original process. The prescriptive rules are then projected back into the original process as cognitive agents, programs, accounting for the original order in terms of the simulated order. The working of the original is then said to be

> "like" that of the imitation, and therefore due to the same kind of intentional control that created that imitation. To say it another way, order is abstracted from one system and imposed on a second, then the imposed order-as-program is abstracted from the second and projected into the first. Rather than assuming that ontogenetic processes fit our notion of programs, we should be asking (and in fact people involved in computer simulation *do* ask) whether our notions of programs do justice to ontogenetic processes. We do well to remember that the word "model" is ambiguous, referring at times to the original, as in artist's model, and sometimes to the replica, as in model airplane. (1985, pp. 62-63)

Here Oyama provides a fascinating account of the inferential and theoretical process of the ascription of "programhood" to systems, especially the biological (onto)genetic systems on which her work has focused. Her work has primarily analyzed the pernicious role of the *metaphor* of the genetic program as a commitment to causally potent and relevant *pre-existing* form in development; my interest here, on the other hand, is in tracing the role of explicitly *mathematical* models in the explanatory and ontological practices of science.[1] We both worry about reification.

According to Oyama's critical analysis, human agents perceive orderliness in a material system, and then formulate this *actual* regularity as a set of descriptive rules, from which the *necessary* regularity (at least ideally) can be eventually derived, in the form of *prescriptive* rules. I would emphatically add that *much* of the integrative work necessary for the abstraction stems from the theoretical perspective employed for the particular mathematical modeling case.[2] Thus, the overarching and guiding theoretical perspective is of key importance in the first step of mathematical modeling. (Step 1-S: here the model is

[1] I am grateful to Cor van der Weele for pointing this difference out to me.
[2] I am grateful to Marcel Boumans, Vivette García Deister, Sabina Leonelli, Sergio Martínez, and Cor van der Weele for discussing this with me.

set up.)[3] Empirical assumptions, metaphors and causal images from the theoretical perspective, as well as phenomenological perceptual biases, enter into this putatively descriptive stage because all of these shape the perceived actual regularity of the material system. This is a clear and complex case of the "theory-ladenness of observation" – or what I call "theory-drivenness."[4]

These prescriptive rules are subsequently "imposed on a mechanical medium," such as a computer (or even paper or a blackboard, that is, the brain?!), for formal manipulation and/or simulation. (Step 2-M: here the mathematical model is *mathematically manipulated*.) In this step, many assumptions, techniques, and biases regarding the *structure* and *processes* of mathematical models and programs are instantiated and employed. These techniques often stem from internal or even metaphorical mathematical and computer science concerns. For example, in the "organism as computer" metaphor, the genetic program is interpreted as "software" for the cell's "hardware," and may even be characterized as containing both routines and data, organized in distinct (sub-)programs or modules. There are, of course, also a large variety of mathematical manipulation rules. The important point is that, in the mathematical models, mathematical or logical structures and processes replace mechanistic material structures.

The third step concerns the model-material system *relation*, in particular the way that the model is used to explain and increase understanding of the structure and process of

[3] van Fraassen 1989 critiques the objectivity and realism (and necessity) ascribed to *laws of nature*, which are always abstracted, in some form or another, from empirical regularities. Van Fraassen's critique of laws is analogous to Oyama's critique of the "objective" structure and reality of genetic programs. In stark contrast to these critiques, some have defended the view that the behavior of the universe can be explained by programs (qua laws of nature?) underlying it. See, for example, Wolfram's notion of "the principle of computational equivalence" in which every natural regular process can be considered equivalent to a computation, Wolfram 2002.

[4] I thank Steve Crowley for this suggestion.

the material system. (Step 3-E: here the model *explains*.) To put it strongly, model-based explanation, I hold, requires at least an "as if" ontology (weak reification, see "Biological Reification of the Mathematically Abstract"), but more often, biologists engage in strong reification in their acts of model-based explanation. In my manuscript, I explore van Fraassen's (1980) as well as Cartwright's (1983, 1998, 1999) strong influence on my view of explanation. I differ from them mainly in that I argue that explanation has a strong ontological component. Returning to the quote above, Oyama thinks of the third step as the imposition of the metaphor of the gene program onto the material system.

The fourth step concerns the status of the material system *itself*. The reification has been completed when the ontological commitments are considered objective and independent in the material system itself. (Step 4-O; here the ontological commitments, carried by the model, are *objectified*.) The act of reification is *forgotten* in step 4 and the reified ontology is taken as the fabric of reality and used as mere background against which *future* scientific investigations are done. Here Dewey's "philosophic fallacy"[5] is finally completed and observable: the ontology carried in the theoretical perspective and model is actually understood as really existing in, and as being causally efficacious of, the material system. Perhaps Oyama is alluding to this step when she writes "the working of

[5]Dewey 1929/1958 writes: "Selective emphasis, choice, is inevitable whenever reflection occurs. This is not an evil. Deception comes only when the presence and operation of choice is concealed, disguised, denied. Empirical method finds and points to the operation of choice as it does to any other event. Thus it protects us from conversion of eventual functions into antecedent existence: a conversion that may be said to be *the* philosophic fallacy, whether it be performed in behalf of mathematical subsistences, esthetic essences, the purely physical order of nature, or God." (1929/1958, p. 29) That is, an empirical study of the act of reflection and abstraction will invariably point out that reflection always heavily involves theoretical activity (e.g., selective emphasis and choice), and that this activity generates strong ontological commitments. Consequently, it is a mistake (i.e., a fallacy), to reify outcomes and fruits of *this* reflection, whether they be mathematical, aesthetical, physical, or theological outcomes and achievements, as *prior* existences. I would add that despite being a fallacy, it is practically inevitable – the best we can do is to be *aware* of it and consider *alternative* existences.

the original is then said to be 'like' that of the imitation, and therefore due to the same kind of intentional control that created that imitation."

This is the basic nature and structure of my SMEO model. Note that the theoretical perspective plays a crucial organizing role in my account. The presence and power of such perspectives is clear in the case study to which I will now turn. Perspectives provide different empirical assumptions, mathematical techniques, mathematical abstraction techniques, and causal images. The two models articulated using the resources of the perspectives produce very different ontologies, through their distinct reifications. Michael Turelli's model employs classic population genetic methodology and, because of its assumptions of panmixia and negation of kin selection can be considered a Fisherian kind of model (named after R.A. Fisher, 1890-1962, a key founder of mathematical evolutionary genetic theory, e.g., Fisher 1930). It is committed to an ontology appealing to intracellular bacterial density and genetic pleiotropy as the operative mechanisms for the evolution of CI. Steve Frank's model uses newly developed quantitative genetic techniques and, because of commitments to kin structure and kin selection, can be seen as a Wrightian kind of model (named after S. Wright, 1889-1988, another key founder of mathematical evolutionary genetic theory, e.g., Wright 1968, 1969, 1977, 1978). The ontology of this model fundamentally concerns kin selection as the key mechanism for the evolution of CI.

In the context of these models, then, it is useful to also note that over the last few years, a *contemporary* incarnation of the general Fisher-Wright debate over the nature and relevance to the evolutionary process of (1) population structure and genetic epistasis and, more generally speaking, (2) context and interaction, has graced the pages of *Evolution*, almost certainly the most important journal in evolutionary biology (e.g., Coyne, Barton,

and Turelli 1997, 2000; Wade and Goodnight 1998, Goodnight and Wade 2000). I will show in detail how the two models can be seen as instances, or parts of, this general debate. Here we also return to the issue of relevance and the question of what is at stake. One very striking feature of Fisher's approach is its atomistic treatment of organisms and genes, and its neglect of interactions, including kin interactions. This highly individualistic approach might have an ideological component that could be useful to contrast with the interaction-oriented relational approach of Wright, although that line of investigation is speculative at this point. Certainly, however, given the social and political relevance of such potentially ideologically-generated differences, this is a pressing issue.

I will now turn to an analysis of the two models using my SMEO account.

Turelli's Model

In what follows, I will outline parts of Turelli's model using my SMEO account.

Step 1-S in Turelli's Model

There are two particularly strong empirical assumptions, which guide the mathematical moulding, or integration, of Turelli's model, and which stem from Turelli's theoretical perspective: the absence of direct selection on *Wolbachia* traits in sperm and the lack of population structure. With respect to the first, in imagining actual biological selective forces on the parasite, Turelli considers sperm evolutionary "dead-ends" for the *Wolbachia* – there is no paternal transmission of *Wolbachia* and hence no direct selection on the *Wolbachia* trait of affecting sperm to express CI. Put differently, any variance in the *Wolbachia* genome for the trait of CI (a trait which is necessarily expressed *only* in the

infected sperm x uninfected egg fertilization combination, a combination which leads to *no* offspring) cannot be effectively selected upon since the realized heritability is zero (it cannot be *transmitted*). From an evolutionary point of view, selection without transmission is dynamically and kinematically ineffective – it does not cause any changes of gene frequencies (Arnold and Wade 1984).

The further assumption that Turelli then makes, stemming from the Fisherian theoretical perspective, is that there is no population structure: panmixia, or random mating within a large population, is assumed. Thus, there can be no *indirect* "transmission through the sperm" either, in the sense of kin selection. In cases with population structure, and thus kin selection, destruction of uninfected eggs, by infected sperm, would increase the fitness of (also related) hosts with the *same Wolbachia* strain as the infected sperm through, for example, increased resource allocation (e.g., space and food). But Turelli assumes that this does not occur and, in the set up of his model, has no variables and parameters (henceforth also just "terms") that could measure kin structure.

These, and other, assumptions are present in the mathematical set up of his model – i.e., in the terms that he does define.

Step 2-M in Turelli's Model

Turelli's mathematical manipulation is complex. Here I will only emphasize the aspects of step 2-M pertinent to the reification and which, therefore, can be usefully and directly contrasted with Frank's model.

Turelli considers the fitness function for the evolution of CI *when there is some level of incompatibility* (1994, p. 1504):

$$p_{i,t+1} = [p_{i,t}F_i(1 - \mu_i)]H_{bar,i}/W_{bar} \quad [1]$$

where F_i is the fecundity of strain i, μ_i is the fraction of uninfected ova produced by infected female of strain i (i.e., this is a measure of the lack of fidelity of host maternal transmission), $p_{i,t}$ is the population frequency of strain i in generation t, and W_{bar} is the mean population fitness. This is a well-known population genetic form of fitness function, with multiplicative elements (see Wade et al. 2001), wherein $F_i(1 - \mu_i)H_{bar,i}/W_{bar}$ is the relative fitness of *Wolbachia* strain i, or the "effective fecundity." Note the absence of *any* term tracking kin structure. $H_{bar,i}$ is defined—relative to each *Wolbachia* strain i, where i = 1, 2, 3… n, where n is the total number of partially incompatible strains—as (p. 1504):

$$H_{bar,i} = p_1H_{i1} + p_2H_{i2} + \ldots + p_nH_{in} + q \quad [2]$$

H_{ij} is the relative hatch rate from fertilizations of ova from mothers infected by strain i, by sperm from fathers infected with strain j, and for which $0 <= H_{ij} < 1$ indicates a corresponding inverse level of CI (e.g., $H_{ij} = 0$ indicates complete incompatibility), p_i is the relative frequency of strain i, and q is the relative frequency of the uninfected strain. Total CI in the population, relative to each strain, changes both as the *relative frequency of different strains* changes and as *each pair-wise* H_{ij} changes.

Given the general fitness function (equation 1), Turelli subsequently arrives at a general result for the nature of the parameter relations necessary for a partially incompatible rare variant (of type 1) to *increase* in frequency in the population (of type 2) when genetic variance in parasites, not hosts, is considered. The mathematical condition that he derives is the following (p. 1504; parameters summarized on p. 1502):

$$F_1(1 - \mu_1)(1 - p_2s_{h12}) > F_2(1 - \mu_2) \quad [3]$$

where terms are as stated above, and s_{hij} is $1 - H_{ij}$ (i.e., s_{hij} is a measure of cytoplasmic incompatibility, with 1 representing complete incompatibility and 0 representing complete compatibility). Equation 3 can be described verbally as follows:

> If parasite variants are partially incompatible, a new variant will increase when rare only if it increases the 'effective fecundity' [i.e., $F_1(1 - \mu_1)$] of infected females enough to offset the progeny it loses through incompatibility with the infected males already present [i.e., $(1 - p_2 s_{h12})$]. (p. 1509)

This is the condition for frequency increase in the population when the variant is rare.

Recall that, according to Turelli, there is neither direct selection on sperm nor kin selection. From this condition, it then follows that a *Wolbachia* variant (type 1) increasing levels of CI (H_{21}), will increase in proportion in the population *only* if it increases "effective fecundity." Note that s_{h21} (a function of H_{21}) does not appear on the right side of equation 3. Therefore, changing CI independently of every other variable is causally ineffective in changing the population frequency of type 1.[6] Thus:

> the level of incompatibility between infected males and uninfected females evolves under parasite control *only as a correlated response* to direct selection on the fecundity of infected females, efficiency of maternal transmission of the infection and levels of compatibility between parasite variants (1994, p. 1509)

There is no direct selection on CI. For CI to increase[7], it has to be positively correlated with effective fecundity.

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[6] A proponent of a Wrightian perspective could argue here that the missing and causally ineffective $(1 - p_1 s_{h21})$ would be significant precisely with population structure. Under such structured conditions, type 1 would have a large enough frequency locally and the parenthesis could not be ignored! I thank Ben Kerr for this observation.

[7] There is a lack of clarity in Turelli's article regarding exactly which material property of CI is to be explained: level of CI (i.e., s_{hij}) or gene frequency of a CI strain (i.e., p_i), or both. His population genetic modeling practices suggest gene frequency, but the citation immediately above implies level of CI. Frank, on the other hand, clearly differentiates the two phenomena – he provides two separate conditions for increases of both, for example (Frank 1997, p. 329).

Step 3-E in Turelli's Model

In this step, models are used to explain regularities in the material system. Recall that this step is a *relation* between mathematical model and material system. As we shall see, the ontology implied by Turelli's mathematical model, does not match perfectly the ontology he believes, on mechanistic grounds alone (when considering the mechanistic effect of bacterial density), to exist in the material system. This is one case in which the mathematical model ontology does not consistently impose itself.

For Turelli, the two mechanisms that can mediate the correlations between levels of CI and female effective fecundity are *bacterial density* and *genetic pleiotropy*. He mentions bacterial density significantly more often than pleiotropy as a mechanism, so I will focus on the former. But for reasons pertinent to the nature of ontological reification stemming from the model, I will return to the latter.

Turelli, following earlier empirical work, suggests that an increase in bacterial density has the following *correlated* effects (see especially discussion on p. 1505): (1) it increases the rate of maternal transmission (mother to her eggs) of the bacteria (pp. 1500, 1505), (2) it decreases maternal fecundity (pp. 1500, 1505), and (3) it increases levels of CI (pp. 1505, 1509-1510). Turelli and Frank do agree on the direction of these causal correlations caused by bacterial density. Their strong ontology vis-à-vis bacterial density is congruent. Turelli notes that this phenomenology is observed especially in the laboratory, where the effects are exacerbated. Note that on the surface, Turelli's modeling results can explain these patterns. Direct selection on *bacterial density* among hosts (and the genetics "underlying" it) occurs through direct selection on female fecundity and maternal

transmission (traits that can actually be transmitted), but not on CI (a trait that cannot be transmitted, from the *Wolbachia* point of view).

However, the effect of increased bacterial density, which both increases incompatibility and *decreases* female fecundity (and increases transmission probability), is supposed to be explained by the *opposite* correlation that Turelli derived mathematically (equation 3) to explain the pleiotropic evolution of CI: i.e., a positive correlation, *ceteris paribus*, between increased incompatibility and *increased* maternal effective fecundity [F-$(1 - \mu)$], necessary for a variant, causing both of these, to increase in frequency when rare in the population. Only when increases in bacterial density cause a larger increase in transmission probabilities, $(1 - \mu)$, *relative* to the decrease in female fecundity, F, will the mathematical correlation be in the same direction as the material correlation.** And this cannot be assumed, *a priori*, to be the case. Hence, equation 3 explains some patterns [e.g., when F decreases *more* relative to increases in $(1 - \mu)$] that are *inconsistent* with patterns *independently* inferred through mechanistic (not mathematical) considerations and empirical assumptions – patterns concerning the effects of bacterial density. It is interesting to note that Turelli does not seem to be aware of the tension between the mathematical explanation and mechanistic considerations/ontology[8].

Step 4-O in Turelli's Model

Subsequent to the explanatory act, the model, and its components, are objectified in the system. The material system is understood as inherently containing the ontology. For

[8] Here there are, thus, two different sources of reification. Recall that it is beyond the scope of this chapter to consider the role of abstract material models, as opposed to mathematical models, in the process of reification.

example, concerning the correlations between effective fecundity and levels of CI, in appealing to genetic pleiotropy as a phenomenon inherent to the material system, Turelli can postulate *any* direction for *any* correlation. This leaves the regularities in the material system open to radical reification. Turelli can postulate, and objectify, any correlation required by his model.

Furthermore, Turelli also employs his ontology of correlated selection, through bacterial density and genetic pleiotropy, to account for other phenomena as well as to present *further* tests of that very ontology. The strong ontology is taken as *background* for future work. For example, he discusses how "Variation in… patterns of pleiotropy may underlie the great variation in levels of unidirectional incompatibility associated with *Wolbachia* in different taxa: from complete… to nearly undetectable." (1994, p. 1510) Note that he is appealing to his strong ontology—now objectified and its theoretical source forgotten—to account for a variety of phenomena. In addition, he provides suggestions for further experimentation: "By introducing a novel *Wolbachia* into a large laboratory population, the prediction that $F(1 - \mu)$ should increase might be tested directly." (1994, p. 1510)

Frank's Model

In what follows, I will outline parts of Frank's model using my SMEO account.

Step 1-S in Frank's Model

Frank's paper starts with framing the problem of the increase in frequency of CI by distinguishing between the verbal kin selection models of Hurst (1991) and Rousset and

Raymond (1991) *and* the formal individual-based models of Turelli (1994) and Prout (1994). For example, although Hurst's verbal model lacks clarity, he presents an early formulation of the issue at hand: "All of the costs of the spiteful act inflict the hosts not the spiteful symbiont. Under these conditions, as the simple models demonstrate, spite can evolve and can be stable. Cytoplasmic incompatibility can thus be seen as a special case of kin selective spite (Hamilton 1970, 1971)." (Hurst 1991, p. 276) Spiteful behavior, in general, cannot evolve. But it is precisely because the behavior (CI) *benefits Wolbachia* kin, Hurst argues, that it constitutes a spiteful behavior that can actually evolve. This is an assumption that stands in stark contrast with Turelli's assumptions and which Frank avails himself of: "I show, with a formal model, that weak kin interactions are sufficient to explain the observed patterns of incompatibility." (Frank, 1997, pp. 327-328)

Frank's model is integrated by the Wrightian theoretical perspective, which is committed to the nature, role, and existence of population structure and its associated concept, kin selection (Wade 1980, 1985, 1992).

Step 2-M in Frank's Model

First, Frank presents the overall model: "The first step is to write a fitness function that describes how biological assumptions influence reproduction." (1997, p. 328) He presents an explicit relative fitness function of the parasite, which eventually, after mathematical manipulation, allows him to bring in kin structure as pertinent:

$$w(x, y) = [(1 - a - bx)(1 - \mu)] / [(1 - l)^2 + l(1 - a - by) + l(1 - l)(1 - y)] \quad [4]$$

This fitness function measures the fitness of a parasite as a function of (1) the continuous trait value, x, of that parasite in the host, (2) the average value, y, of that same trait in *other*

neighbors with which the host female interacts, (3) the absolute fitness cost, *a*, the parasite exerts on all infected females, (4) the relative fitness cost the parasite has on its host, *bx*, in which *b* is a kind of cost parameter, (5) as in Turelli's model, the transmission rate of the parasite ($1 - \mu$), and (6) the frequency of infection, *l*, which is Frank's *q*, not to be confused with Turelli's *q*, the frequency of *un*infected types (all on p. 328). Note that the form of this fitness function involves additive elements, as tends to be the case in quantitative genetics (see Wade et al. 2001).

It is beyond the scope of this paper to discuss these terms in detail, but I will briefly elaborate on two: *b* and *y*. The important *b* parameter measures correlated *fecundity cost* as a function of *level of CI* in males. "This parameter [*b*]," notes Frank, "is the reduction in the fecundity of an infected female that arises as a correlated trait of the level of incompatibility expressed in males." (1997, p. 328) It is a genetic correlation (with a value between –1 and + 1) and, as such, does not specify the cost for a focal female or even group of females (*–bx* is the cost). Rather, as a correlation coefficient, it specifies the direction and tightness of the correlation between the two traits (i.e., fecundity cost and level of CI). Put differently, as is well known, the magnitude of the correlation squared (i.e., r^2) is the amount of the variance in one (or the other, depending on which is chosen as the dependent variable) of the parameters explained by the variance in the other. It is important to note that the correlation is independent of the slope/scale or origin of the regression line.

Frank divides his analysis into three main parts: when *b* is, respectively, equal to, less than, or greater than 0. Turelli modeled the *second* case. Frank considers the *last* case the most interesting since that is the biologically most likely one, and is also the one for

which it is challenging to explain how selection could increase levels of CI (since, apparently, CI has a fecundity *cost*).

For Frank, the key to the increase in levels of CI is kin selection. In order to reach this conclusion from his formal model, let me mention, in general and briefly, the mathematical techniques he explicitly uses to *index* kin structure of the host population and, thereby, of the parasite population. Recall that *y* is the average value of the trait in *other* neighbors with which the host female interacts. Frank notes that in differentiating the original fitness function (equation 4) with respect to *x*, under any of the three value ranges for *b*, there will always be some d*y*/d*x* term, which turns out to be "the slope of the group phenotype on individual genotype, which is the kin selection coefficient of relatedness [Hamilton's *r*]." (1997, p. 328). Frank also refers to a methodology providing the meaning of the d*y*/d*x* term, which is not actually presented in his 1997 paper. He developed this methodology with a co-author (PD Taylor; first author) in a 1996 paper entitled "How to make a kin selection model" – this method is further developed in Chapter 4 of Frank 1998. The interested reader of Frank's 1997 paper can *refer* to the 1996 paper and the 1998 book as a kind of generative "mathematical manual" (rather than, say, a "laboratory manual") in which strategies for *how* to build a formal kin selection model can be found:

> [the "direct fitness"] method provides an *orderly set of tools* for studying the multiple pathways by which social interactions influence fitness. More importantly, the *evolutionary processes* stand out clearly during the *analysis*, so that the analysis itself *enhances our understanding* of the problem. (Taylor and Frank, 1996, p. 36; emphasis mine)

Thus, d*y*/d*x* clearly and explicitly provides the grounds for tracking and mathematically manipulating kin structure.

Frank, unlike Turelli, presents clear conditions for both the increase of the *value of the CI trait* (i.e., under the respective parameterizations: Turelli's = s_{hij}, Frank's = x, and, eventually, z) as well as the *frequency of infected types in the population* (i.e., under the respective parameterizations: Turelli's = p_i, Frank's = q, written as l in equation 4 above) (1997, p. 329). It is beyond the scope of this paper to present Frank's mathematical manipulation in more detail. Frank's brief discussion of conditions for increase, and equilibrium conditions, under a range of values of the different parameters and variables is "meant as a rough, qualitative guide to the complex dynamics of the system. *The main point is that relatedness, r, can strongly influence selection of incompatibility*." (pp. 329-330, emphasis mine)

Frank summarizes his theoretically-motivated results in the following fashion:

> Prout (1994) and Turelli (1994) implicitly assumed that $r = 0$. Given that assumption, it is not surprising that they concluded kin selection does not favour incompatibility. I have shown that the simple condition $r > 0$ is sufficient to favour incompatibility when there is no genetic correlation between incompatibility expressed in infected males and reduced fecundity expressed in infected females (the parameter $b = 0$). When there is a correlation, $b > 0$, [i.e., there is a positive fecundity cost] kin selection influences incompatibility, but the net selective effect depends on the relative magnitudes of relatedness, r, negative effects on female fecundity, b, transmission efficiency, μ, and the frequency of infection, q. The direction of selection can shift toward higher or lower incompatibility as these factors change in magnitude. (p. 330)

Kin selection has a significant effect and, for some ranges of parameter and variable values, can increase both the value of the trait and the frequency of infection.

Step 3-E in Frank's Model

Kin selection has significant explanatory power under Frank's model. Guided by his model, Frank appeals to a correlation between "the reduction in the fecundity of an infected female" and "the level of incompatibility expressed in males." (p. 328) The biologically most plausible correlation here is a positive one, contra Turelli. Because of his introduction of another variable, *r*—motivated by a rich theoretical background—Frank suggests (1) that there is indeed *direct* selection (from the point of view of *Wolbachia*) on levels of CI *and* (2) that increased levels of CI could be positively correlated (mathematically and materially) with a *decrease* in (infected) female fecundity. Notice that these two results are the opposite of what Turelli concluded. Turelli found that there was no direct selection on levels of CI and that increased had to be correlated with an increase in (infected) female fecundity.

Frank, in emphasizing kin selection, from a genetic point of view, rather than bacterial density, provides a different, but equally theory-laden, explanation of the material processes. Explanatory resources, contained in his model, include: (1) empirical assumptions regarding the ubiquity of population structure, (2) mathematical techniques (e.g., the "direct fitness" method of Taylor and Frank 1996, Frank 1998) that allow the tracking of kin structure and kin selection, and (3) mathematical structures (e.g., $dy/dx = r$). The ontological commitments associated with 1-3 of the model are imposed onto the material system.

Furthermore, recall that Frank states that "weak kin interactions are *sufficient* to explain the observed patterns of incompatibility." (pp. 327-328, emphasis mine) Although he never makes the claim that kin interactions are *necessary* to (all kinds of) selection, given that he does not consider alternative models that *ignore* kin selection—in fact, in this

work and elsewhere (e.g., 1998) he considers such models suspect—it does seem that he considers kin selection a *necessary component* of any complete explanation of evolutionary change. This, thus, *might* be a case of the particular model-based explanatory relation that I referred to in section 2.4 above: "making the merely explanatorily sufficient explanatorily necessary."

Step 4-O in Frank's Model

Although Frank tends to stay at an abstract theoretical level in his work, he also sees kin selection as an ontologically justified factor in the material system. It is the material cause which must be modeled through the use of theory. His impressive 1998 book continues his research project of modeling kin selection, in order to help explain selection in the process of social evolution.

This comparative case also allows us to introduce step 4-Pl. We can compare the relative merits of the different ontologies (Turelli's and Frank's). To what kinds of further investigations do they point? Can their respective reifications be hybridized (they do seem incompatible)? Which one is more compatible with *other* ontologies? What influences on policy—for example, views on genetic effects and genetic interactions, or pest control—do their different models, and reifications, imply? Pluralizing ontologies allows us to be critical of the reifications and their cascading effects both in further scientific theorizing and experimentation, as well as in social and political contexts. One of the goals in my research project is to argue for the strength of considering a variety of ontologies – of pluralizing.

Further Thoughts

In this case study of formal modeling of CI in *Wolbachia*, I hope to have shown that theoretical perspectives and models reify their ontological assumptions onto their understanding of the material system. Mathematical models, in part because of their precisely defined terms, are a convenient place to track such reification. It is important to analyze reification as it has political and social consequences of many sorts, especially in biology. And it is precisely in a political and social context that theoretical perspectives are embedded. Thus, a full account of reification in this case, will also involve an analysis of the research traditions and theoretical traditions stemming from the work of both Fisher and Wright. The analysis presented here is rather individualistic and formal. A historico-sociological account should also be provided.

Chapter 5. Intracellular Transformation of *Wolbachia*: Where Do We Go from Here?

As can be seen in the explanation of the experiment at the end of Chapter 1, as well as the description of the methods and results in Chapters 2 and 3, my masters experiment combined methods and purposes in a variety of very different biological disciplines: molecular, cellular, and population biology. Not only did the research involved in this thesis give me a much fuller appreciation of the difficulties and subtleties of biological research, but it also provided a much more detailed comprehension of the deep divisions between what I have called formal and compositional biology (Winther 2003). There is no question that the work summarized in this thesis informs my philosophical views and assists further philosophical developments, consequences that might not be immediately recognized by an outside reader surveying the broad mix of methods and objectives in this experiment as well as the rather general Chapter 1 and philosophical Chapter 4.

Having tried to justify the breadth of this thesis by appealing to my studies in philosophy, it remains unclear how all the different chapters (parts, modules) of the thesis fit together, just as the full set of relations between compositional and formal biology remain to be established. Chapter 1 can stand alone as a general introduction to *Wolbachia*. Chapter 4 can also be read independently of the rest of the thesis and is part of a project on reification. MJ Wade and I are collaborating on one of the texts of this project. The remainder of this conclusion concerns Chapters 2 and 3. In particular, I will evaluate weaknesses in my actual experiment and indicate how the experiment can be continued.

This involves both clarifying how current weaknesses can be troubleshooted and solved *and* stating further questions that need to be answered. It is to this that I now turn.

As discussed in Chapter 3, the nature of the 1.087 kb *Wolbachia* segment, isolated as the initial step of the experiment, remains problematic. Although I presented functional genomic evidence supporting the suggestion that it is indeed a ftsZ sequence, further diagnostic tests are necessary. That is, the whole plasmid (pRW4) needs to be sequenced and its different regions need to be robustly characterized using sequencing tools. If the appropriate sequences are *not* found, a new *Wolbachia* segment, isolated from *Tribolium confusum*, will have to be isolated and put into pRW4 to make a new plasmid, potentially called "pRW5." In order to make pRW5, it would be a good idea to first cut out the *Wolbachia* segment already present in pRW4 using Age1 and DraII (this would leave a bit of the segment in the plasmid, but the advantage of *not* using the HindIII cutting site is that, in this manner, the spec cassette would be kept in pRW5). Subsequently, using newly designed primers from BLAST searches of actual *Wolbachia* ftsZ region (with help from Carl Bauer and Michael Wade), the appropriate *Wolbachia* sequence could be PCRed from *Tribolium* grindate, from a population infected with *Wolbachia*, and subsequently ligated into pRW4 (– current *Wolbachia* segment), thereby completing the construction of pRW5. Not being completely aware of the initial steps of the overall experiment is perhaps my main error.

A few more comments regarding the plasmid, pRW4, are in order. (I owe a number of these observations to Victor Anaya.) First, the origin of replication of pRK415 is of *E. coli* and the possibility exists that *Wolbachia* may not be able to interpret it. If this were the case, even after a successful transformation, plasmid reproduction within *Wolbachia* would

not occur. In fact, it is not yet known "whether *Wolbachia* carry plasmids, but indirect evidence suggests an infectious (viral) agent of *Wolbachia* may exist (reference to Williams et al. 1993)." (Werren 1997, p. 595) If it were indeed the case that *Wolbachia* lacks its own plasmids, then uptake of foreign plasmids, even with a *Wolbachia* origin of replication would be difficult. Further information regarding this point needs to be gathered, possibly from experts in the field. Bourtzis' laboratory in Greece (URL = <www.imbb.forth.gr/people/bourtzis>; see especially "Section 4.3 *Wolbachia* genetic transformation system") attempted, unsuccessfully, to transform, using electroporation, *Wolbachia* with a plasmid containing GFP and a *Wolbachia* wsp promoter (!). Subsequently, they constructed two plasmids containing the wsp promoter and transmembrane protein domains – these transformations were also unsuccessful. Undoubtedly contacting members of this laboratory would be extremely useful. Furthermore, to assess functionality of the plasmid in *E. coli*, a useful side-experiment would be to simply grow overnights with the plasmid and *test* those for GFP fluorescence. It is important to test this functionality also because the GFP vector employed (pEFGP-N1) is actually optimized for mammals and this could be a factor affecting its expression in bacteria. Furthermore, *Wolbachia* might have its own restriction system, which would destroy any intracellular plasmid. This possibility also needs to be considered.

Let me now turn to some comments regarding problems pertinent to the microinjection and population biology aspects of my experiment. It is unfortunate that there are no data available regarding the presence of GFP (i.e., pRW4) in the larvae of the microinjected *Tribolium* that tested positive for *Wolbachia* in Matt Roberts' test. However,

given the negative results of my experiment testing for GFP, as well as the passage of so much time since then, this is a moot point. More microinjections need to be made.

Before more microinjections are made, it would also be useful to test the Vejle, Denmark population for *Wolbachia*. At this point, it remains unclear whether this population harbors the endosymbiont. Vejle *Tribolium* grindate could be PCRed for *Wolbachia*, but, as we will see below, there are issues about whether there is enough *Wolbachia* in those beetles to provide enough DNA signal for a PCR. After all, earlier diagnoses using PCR were equivocal – in some cases negative (when RGW did them), and in others positive (when Matt Roberts did them). Another way of testing for the presence of *Wolbachia* would be to perform breeding experiments that tested for the expression of CI. With *Wolbachia* present, CI should be expressed, possibly at different levels.

Now, once the plasmid and the appropriate populations for microinjection have been worked out, there are a number of factors that need to be varied in order to provide as large a breadth of options as possible: 1. pressures of the Narashige IM 300 microinjection machine, 2. concentrations of microinjected solutions of SM10Lpir and plasmid, and 3. the concentration of antibiotics in the flour for *Tribolium* rearing. These are the main independent variables whose values significantly affect the outcome of the experiment.

Microinjection pressure has three variables in the Kaufman lab machine. These should be tried at different settings. Presence of dye in plasmid, as well as the unique yellow color of bacteria, allow the experimenter to observe whether plasmid/bacteria were injected into the eggs. However, quantity of microinjected material matters here. With too little, the *Wolbachia* in the egg may simply never transform – there is not enough plasmid

(naked or in bacteria). With too much, the egg can burst or subsequently, become infected. This is a difficult balance to find.

The concentration of microinjected solutions should also be varied. With a higher concentration, less liquid needs to be injected for the same number of plasmid (naked or in bacteria). As high a concentration as possible seems ideal because cells do get damaged from the quantity of volume microinjected, so introducing as little new volume as possible is important. However, there is a limit to the concentration of bacterial fluid that can be used – the microinjection needles get clogged. Furthermore, it is also crucial to keep in mind that with very high concentrations, relatively little liquid should be microinjected. Otherwise, the eggs will likely get infected.

The concentration of antibiotic(s) in the flour employed to raise potentially transformed *Tribolium* should also be varied. Of course, if it is too low it will not clear non-transformed *Wolbachia* from the *Tribolium* (this is, in fact, what may have occurred with the *Wolbachia*-positive microinjected beetles, as found by Matt Roberts). However, it can also be too high. Transformed *Wolbachia* might require some time, both during development and even during reproduction into the next generation, to process the plasmid and produce its protein products (GFP and antibiotic resistance). Put differently, the selective regime should not be too harsh, even for *transformed Wolbachia*. Admittedly, this is also a difficult balance to find.

Few *a priori* suggestions can be given regarding how to vary these factors. The only absolute recommendation that can be given is that *many* microinjections must be attempted. Even with low probability of success (a low probability, p, that can be improved slightly by varying appropriately the three factors mentioned above), a large

number of events, *n*, will almost certainly lead, eventually, to a successful transformation. And such a success would already in itself be a significant result for molecular biology. However, for the population biology experiment, it would be ideal to have multiple successes. After all, using just one successful female as the founder of a population would radically decrease the amount of genetic variation present for the selection experiments planned.

There is also an unresolved issue regarding the diagnostic method of PCR employed. In particular, it remains unclear whether negative results really means that there is no pertinent DNA in the reaction. For a variety of reasons, to be detailed below, there could in fact be very little plasmid DNA present in the *Tribolium* grindate, even if the plasmid is *actually* present. The amount of grindate placed in each reaction could be increased, but there is also a limit to this because many chemicals in the grindate are bound to interfere with the PCR – hence no amplified DNA in a particular PCR reaction (i.e., in one test tube) might simply indicate that the PCR reaction did not even occur, not that there was an absence of plasmid.

Let me explore some of the reasons for why there could be very little plasmid DNA present even *with* a transformed *Tribolium* larvae. (1) The number of germ cells per larvae, (2) the relative number of germ cells with *Wolbachia*, and (3) the amount of *Wolbachia* per germ cell, each need to be considered. Low values for any (or any combination) of these would lead to the presence of little plasmid. Furthermore, intra-*Tribolium*-individual selection among *Wolbachia* strains, on analogy with cell lineage selection within organisms, should also be considered. Perhaps *Wolbachia* with the plasmid are less fit and are outcompeted by non-transformed *Wolbachia*, provided that there is not sufficient

antibiotic in the flour. Transformed *Wolbachia* may disappear completely, they may be in the process of disappearing, or they may reach some sort of equilibrium (with non-transformed *Wolbachia*). For these reasons, among others, there is a significant chance that hidden positives (i.e., larvae with transformed *Wolbachia* that do not show up in the PCR reaction) could exist.

There are a number of problems that need to be worked out before the long-range selection experiment can even begin to be considered. These problems can be resolved.

I have written this conclusion primarily for someone who plans to continue this experiment. Let me provide a few final remarks, of a motivational sort, regarding this experiment. Good students are almost invariably *creative* and *self-motivated*. It seems unlikely that a good student would be initially interested in doing an experiment already started by somebody else. In this context, I would like to say that *Wolbachia* is an intrinsically interesting system that combines knowledge and challenges from practically *every* discipline of contemporary biology. Many molecular, cellular, developmental, and evolutionary biologists are interested in *Wolbachia* (for example, see the breadth of contributions to O'Neill et al. 1997). Furthermore, my experiment hardly counts as done. Although I have made significant progress in constructing the plasmid and in working out some of the microinjection techniques, there remains much to be done. Furthermore, the fascinating multi-level selection experiment has not even been started.

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URL = <http://www.ncbi.nlm.nih.gov/>

Bourtzis' Laboratory:

URL = <www.imbb.forth.gr/people/bourtzis>

Center for Disease Control article on Onchocerciasis:

URL = <http://www.cdc.gov/travel/diseases/oncho.htm>

ClustalX sequence comparison tool:

http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html

Enzyme digests:

URL =

<http://www-personal.umd.umich.edu/~mparsons/474/setting_up_enzyme_digests.pdf>)

Qiagen Minipreps for DNA isolation:

URL =

<http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_QP_

Miniprep/1027678_HB_QP_0504_WW_LR.pdf>

Regulatory Sequence Analysis Tools:

URL = <http://embnet.cifn.unam.mx/rsa-tools/>

Standard Alkaline Lysis Preparation

URL = <http://preuss.bsd.uchicago.edu/protocols/Alkaline.html>

Stratagene Products:

URL = <http://www.stratagene.com/>

Tree of Life Phylogenetic Database:

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Wolbachia genome project:

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Zymoclean kit:

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<http://www.zymoresearch.com/products/dna/zymoclean_gel_dna_recovery_kit.asp>

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AREAS OF SPECIALIZATION

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PUBLICATIONS

Refereed

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Michael J. Wade, Rasmus G. Winther, Aneil F. Agrawal, and Charles J. Goodnight. 2001. "Alternative Definitions of Epistasis: Dependence and Interaction," Trends in Ecology and Evolution, 16: 498-504.

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Robert J. van Syoc and Rasmus Winther. 1999. "Sponge-Inhabiting Barnacles of the Americas: A New Species of *Acasta* (Cirripedia, Archaeobalanidae), First Record from the Eastern Pacific, Including Discussion of the Evolution of Cirral Morphology," Crustaceana, 72: 467-486.

Invited

In press, 2005. "Evolutionary Developmental Biology Meets Levels of Selection: Modular Integration or Competition, or Both?", to appear in Modularity: Understanding the Development and Evolution of Complex Natural Systems (W. Callebaut and D. Rasskin-Gutman, eds.), MIT press.

2001. Review of Ants at Work: The Organization of a Social Insect Colony by Deborah Gordon, Philosophy of Science, 68: 268-270.

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In Preparation

"Biological Reification of the Mathematically Abstract."

"Part-Based Explanation in Biology."

"'Abstractivist' and 'Concretivist' Philosophies of Science: Michael Friedman and Nancy Cartwright."

"An Analysis of Modeling in Compositional Biology: Textbooks as Loci of Practices and as Ladders to be Thrown Away."

Review of The Cambridge Companion to Darwin edited by Hodge, J and G Radick, for Journal of the History of the Behavioral Sciences.

PRESENTATIONS

Invited Presentations

"Biological Reification of the Mathematically Abstract," Vrije Universiteit, Amsterdam, September 2004, and Instituto de Investigaciones Filosóficas, Universidad Nacional Autónoma de México, México, October 2004.

"An Analysis of Modeling in Compositional Biology: Textbooks as Loci of Practices and as Ladders to be Thrown Away," "Filosofía de las prácticas científicas" symposium at the Instituto de Investigaciones Filosóficas, Universidad Nacional Autónoma de México, México, June 2004.

"El realismo y sus dos enemigos," Department of Philosophy, Universidad Autónoma Metropolitana – Iztapalapa, México, June 2004 (in Spanish).

"Causal Explanation in Compositional Biology," Instituto de Investigaciones Filosóficas, Universidad Nacional Autónoma de México, México, January 2004.

"Parts and Kinds in Evolutionary Developmental Biology," Graduate Student Workshop, Philosophy Department, Stanford University, CA, December 2002.

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"On Integrated Wholes and Competing Parts," History and Philosophy of Science Department, Indiana University, Bloomington, IN, November 2001. Hanson Prize Lecture.

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Presentations

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TEACHING AND RESEARCH EXPERIENCE

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American Philosophical Association
Philosophy of Science Association
International Society for the History, Philosophy and Social Studies of Biology

Referee

Philosophy of Science
Journal of Experimental Zoology (Molecular and Developmental Evolution)
The Biologist

Crítica

Recent Conferences Attended

"Representation in Biology," Instituto de Investigaciones Filosóficas, Universidad Nacional Autónoma de México, México, November 2004.

"Unification and Pluralism in Social Science," London School of Economics, London, U.K., September 2004.

"Filosofía de las prácticas científicas," Instituto de Investigaciones Filosóficas, Universidad Nacional Autónoma de México, México, June 2004.

"Complexities of environment and development in the Age of DNA," Marine Biological Laboratories, Woods Hole, MA, May 2004.

"Representing Genes," University of Pittsburgh, PA, February 2004.

Philosophy of Science Association, Milwaukee, WI, November 2002.

"From Embryology to Evo-Devo," Marine Biological Laboratories, Woods Hole, MA, May – June 2001. Graduate student discussion leader.

"Putting Humans Into Ecology," Marine Biological Laboratories, Woods Hole, MA, May – June 2000.

PERSONAL INFORMATION

Native speaker of English, Spanish, and Danish; working knowledge of French.
Danish Citizen.
Born in Aabenraa, Denmark on March 20, 1972.
Lived in Caracas, Venezuela from September 1972 until June 1990.
Lived in the USA from September 1990 until October 2003.
Currently living in Mexico City with a post-doctoral position at the Universidad Nacional Autónoma de México.

EDUCATIONAL DETAILS

Masters Thesis, Indiana University, IN. 10/04
Chair: Michael Wade
Committee: Curt Lively and Rudolf Raff
Title: A Molecular and Evolutionary Study of the Obligatory Endosymbiont *Wolbachia* in *Tribolium*.

Ph.D. Dissertation, Indiana University, IN. 10/03
Chair: Elisabeth Lloyd
Committee: Sander Gliboff, Victor Goodman, Frederick Schmitt, Michael J. Wade
Title: Formal Biology and Compositional Biology as Two Kinds of Biological Theorizing.

Masters Thesis, Stanford University, CA. 6/96
Supervisor: Peter Godfrey-Smith
Title: Constructions of Form in Biological Systems.

Undergraduate Honors Thesis, Stanford University, CA. 6/94
Supervisor: Peter Godfrey-Smith
Title: Charles Darwin's Views on Inheritance and Variation.

REFERENCES

Dr. Frederick Churchill (emeritus), History and Philosophy of Science Department, Indiana University,
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Dr. Michael Friedman, Department of Philosophy, Stanford University,
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Dr. Elisabeth Lloyd, History and Philosophy of Science Department, Indiana University,
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Dr. Susan Oyama (emeritus), Department of Psychology, City University of New York,
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Dr. Frederick Schmitt, Department of Philosophy, Indiana University,
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Dr. Michael Wade, Department of Biology, Indiana University,
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Dr. Michael Dickson, Department of History and Philosophy of Science, Indiana University,
midickso@indiana.edu (For teaching evaluation)