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Crabs, Flies, Yeast, Drip:
A Maggot's Take on Evolution and Adaptation

by

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ABSTRACT

In 1974, geneticist Bruce Wallace heard an address delivered by Hampton Carson describing three species of *Drosophila* that had independently adapted to live as obligate commensals of land crabs, spending at least the larval stages in the crab's nephritic patch. Wallace wondered if he could encourage flies to adapt in the laboratory by simulating the conditions of the nephritic patch (Wallace, pers. comm.). An artificial land crab was created: synthetic turf was inoculated with a soil-yeast solution and subjected to slowly dripping human urine (Wallace, 1978, and pers. comm.). A few *Drosophila virilis* managed to survive in the artificial crab, and after a year, there was a steady population of approximately 40 individuals. In an effort to clean the artificial crab, adults flies were removed and placed on standard media. The flies laid eggs that hatched, but the resulting larvae remained small and eventually died, apparently unable to feed on the food their ancestors had lived on for years in the university laboratory (Wallace, 1978).

In the current study, I reproduced Wallace's artificial land crab with the goal of examining previously unasked questions about the early stages of adaptation, especially changes in the growth, morphology, and behavior of the larvae.

INTRODUCTION

In 1974, Hampton Carson reported an intriguing discovery in his article “Three flies and three islands: parallel evolution in *Drosophila*”: three species of *Drosophila* had evolved on separate tropical islands to live part or all of their lifecycle as obligate commensals of terrestrial crabs, spending at least the larval stages glued to a patch that serves an excretory function for the crabs. There, they fed in a “microorganism-laden urinal” (Carson, 1974). Perhaps even more interesting is the fact that these species of flies arose from three independent phyletic lines. Carson ends by asking what genetic and evolutionary mechanism allowed this sort of innovation in lifestyle to occur in different genetic backgrounds?

In February 1974, Carson gave the presidential address for the annual meeting of the American Society of Naturalists, which subsequently became the basis for his published article (Wallace, 1978). In the audience was the population geneticist Bruce Wallace. After the address, Wallace approached Carson and asked why he did not keep the crabs and flies in a cage together and see if he could observe the adaptation to life on a nephritic patch first hand. To this, Carson replied, “Why don’t *you* try it?” And so, Wallace did.

Since Wallace did not have access to living land crab specimens, he created an artificial nephritic patch: he affixed a 3 x 5 cm piece of synthetic turf to the inside of a sealed Lucite box, inoculated in a water-soil-yeast solution, through which he dripped his own dilute urine (Wallace, 1978). In his original attempt, various species of *Drosophila* were introduced into the artificial land crab. Five days later, most of the 600 flies were dead; however a few *D. virilis* had survived. Wallace reintroduced just *D. virilis* into the artificial crab, and waited. After a year, a steady population of about 40 individuals had established itself. However, by that time, the artificial crab had become littered with empty pupal cases. In an effort to clean the apparatus, the artificial crab was disassembled and the residents were captured and placed back on standard *Drosophila* medium (Wallace, 1978).

Over the next few days, Wallace observed the adult flies lay hundreds of eggs, and from these eggs hatched hundreds of larvae. But these larvae remained small and eventually died, being apparently unable to survive on the food their ancestral stock had lived on for years in the university laboratory (Wallace, 1978).

What is described appears to be an example of very rapid adaptation (in the course of a year), whether this adaptive change also produced a new species was not determined. Speciation requires reproductive isolation from the ancestral population, but since all of the artificial crab population unfortunately died, reproductive isolation assays were not performed. Although several theories exist that suggest speciation events can and do occur “rapidly,” the term “rapid” often

means hundreds of years versus thousands or millions (Templeton, 1979b). In addition, while there are theoretical models for rapid speciation, there are few experiments and even fewer natural examples to support these theories. What follows is a discussion of some of the relevant theories and how they relate to Wallace's artificial crab and my own attempt to replicate his experiment.

The origin of species

It would seem almost offensive to write about evolution and *not* mention the work of Charles Darwin, so here begins my review. The title of Darwin's famous book may be On the Origin of Species (1859), but Darwin was not much concerned with events of speciation; rather, he presented a theory of adaptive change (modification by natural selection). In fact, in the 19th century, the concept of speciation as a process did not exist, and Darwin himself did not believe in species as a real unit of nature. He believed in a continuum from the individual variant to the genus, and that the "species" existed somewhere along this continuum.

Darwin believed that insensibly small adaptive changes would accumulate over many millennia by natural selection, and eventually some groups of organisms would look or behave distinct enough to be called a species. Under such conditions, one would expect to see in the fossil record a multitude of intermediate forms that gradually changed from form A to form B. However, this

is not the case. Darwin argues that the problem is not in his theory, but in the fossil record. He states the fossil record is “imperfect,” mainly because conditions rarely permit fossil formation (Darwin, 1859).

Punctuated equilibrium

What if the “gaps” in the fossil record are not only a result of its “imperfect” nature, but also a sign that something else, intrinsic to the organism, is taking place? This was the suggestion of Eldredge and Gould (1972) in their controversial essay “Punctuated equilibria: an alternative to phyletic gradualism.” They argued that populations spent the greatest portion of their existence in a state of relative stasis, fluctuating about a mean. Indeed, it does not make sense for a population to show consistent directional change over several million years when most conditions in nature fluctuate or cycle on a level of tens of thousands of years. For example Milankovitch cycles occur every 21,000 years. Eldridge and Gould proposed that the majority of evolutionary change occurs during speciation events. Because they believed these jumps occur fairly quickly and they knew proper conditions for fossilization are rare, the likelihood of catching speciation “in the act” is next to none. Additionally, still using the fossil record to support their theory and likely taking hints from their professor at Columbia, Ernst Mayr (who promulgated allopatric speciation by peripheral isolation) they proposed that

when speciation does occur, it happens in an allopatric manner (Figure 1). As Eldredge and Gould (1972) succinctly put it:

If new species arise very rapidly in small, peripherally isolated populations, then the great expectation of insensibly graded fossil sequences is a chimera. A new species does not evolve in the area of its ancestors; it does not arise from the slow transformation of all its forbears. Many breaks in the fossil record are real. (pp. 84)

Such a peripherally isolated population will become a new species if an “isolating mechanism” develops that prevents gene flow between the parental population and the isolated population even after the two species are given the opportunity to come into contact with one another and mate, that is, a period of secondary sympatry.

What made the theory of punctuated equilibrium so controversial was that it suggested that species form by jumps whereas Darwin always emphasized slow, insensible change. But, as I have mentioned, Darwin himself recognized that the fossil record was too incomplete to show gradual change. If it takes only 25,000 years for a peripheral isolate to become a new species, but conditions only allow fossilization to occur once every 100,000 years, the chances of capturing gradual evolution in the fossil record is very unlikely.

The really unexpected aspect of the theory of punctuated equilibria was the suggestion that species remain basically unchanged from the time they appear until their extinction some several million years later. Eldredge and Gould had found fossil lineages that did not change, and they claimed that stases are

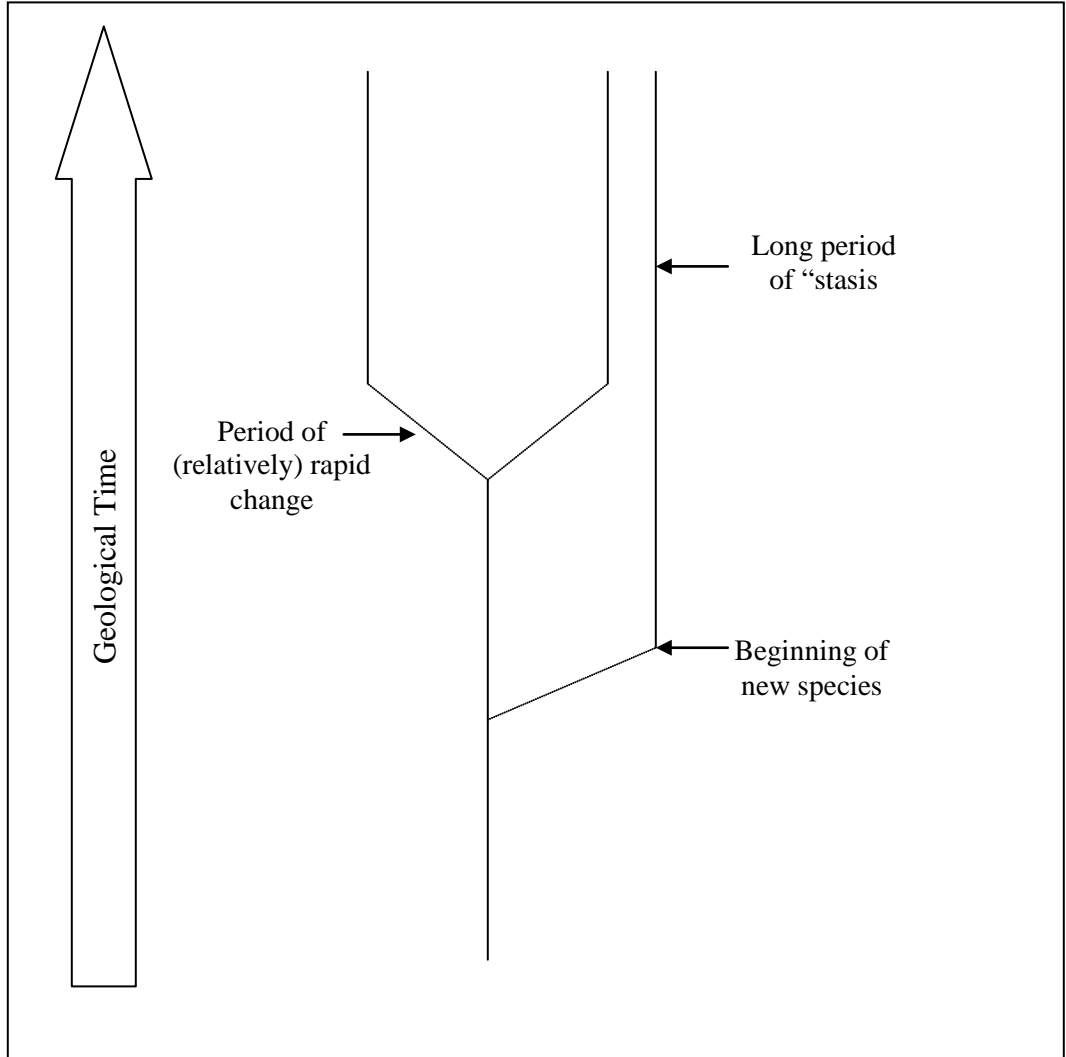


Figure 1. Schematic of punctuated equilibria. Long periods of relative stasis are “punctuated” by rapid (in comparison to how often populations are fossilized) speciation events. This model of allopatric speciation was first suggested by Eldredge and Gould in 1972.

data. But what mechanisms can explain the relative stability of species despite cycling climate change, extinction of old predators and competitors and the arrival of new ones, and by what process could relatively rapid change occur? As it so happened, Ernst Mayr was teaching at Columbia while Eldridge and Gould were graduate students there, and his theoretical view of species provided an answer for their empirical findings in the fossil record (Bock, 1994, Yoon 2002): a “genetic revolution” that produced incompatibilities between sister species (Mayr, 1954).

The species problem and the Biological Species Concept

What *is* a species and how should it be defined? A seemingly simple question that has a convoluted answer. The concept of a species is at the heart of much biological work: anatomy, ecology, development, and even molecular biology depend on knowing what species is being studied and how its idiosyncrasies, such as chromosome number and breeding season, will affect the methods and results of experimentation (Mayr, 1957). The definition of a species has remained elusive, and some have even argued that species are not real biological entities, but rather the cultural constructions of scientists in general and taxonomists in particular (Wilkins, 2009).

Despite these philosophical and definitional difficulties, a few generally accepted definitions of “species” have surfaced. Of particular interest to the current study is the Biological Species Concept (BSC), the work of Ernst Mayr.

Mayr (1942) states: “Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups” (pp. 120). This definition makes two important and related points: first, morphological change, ecological adaptation, or geographical isolation do not on their own signify a new species. Secondly, there must be some unbridgeable gap between the parental and daughter species that prohibits the exchange of genetic material (reproductive isolation). The major issue with this particular definition is that it requires sexual reproduction, but because the model organism for Wallace’s experiment were *Drosophila* that can only reproduce asexually under conditions produced in the lab and that rarely occur in nature (see a discussion of Templeton’s work below for an example of parthenogenesis in *D. mercatorum*) the BSC is applicable to the results of his experiment and my own replication of his work.

Following this definition, for there to be a speciation event a population must somehow become reproductively isolated from its ancestral population, and there are three general ways in which this can happen: 1) Behavioral: Preferential mate selection such that even when given the opportunity, two populations will not interbreed 2) Anatomical incompatibilities that prevent fertilization, for example, “lock and key” genital in a variety of insects (Shapiro and Porter, 1989). 3) Genetic incompatibility that results in hybrid offspring that are either infertile or not viable. But what is the origin of incompatibilities between newly separated species?

A genetic revolution: producing reproductive isolation

What exactly *is* a genetic revolution? The term evokes the image of RNA strands carrying muskets and waging war on the ribosomes, but I doubt that is what Mayr meant when he first introduced the term in 1954 when the path from DNA to RNA to protein was unknown. What the term is trying to describe is how a drastic change in the genetic (internal) environment can have significant effects on the evolution of organisms. Mayr (1954) cites examples of peripherally isolated populations that have greatly diverged from their parental population: while other biologists assumed that natural selection in slightly different environments or genetic drift could account for these changes, Mayr was doubtful either could make much of a difference in speciation and account for the significant ecological and morphological differences observed in peripherally isolated populations. Additionally, there were many known ecotypes that lacked reproductive isolation (Clausen, Keck, and Heiey, 1940). In general, Mayr did not believe geographical isolation or random drift to be valid mechanisms for isolation (Provine, 2004)

The explanation Mayr offers begins with the idea that the genome is not made up of genes that act in isolation from one another, but rather an integrated whole. He proposes that in a typical situation, genes that produce increased viability in a heterozygote are preferable to those that work best in a homozygous state and genes that act as “good mixers” (viable in a variety of genetic

backgrounds) will be under positive selection. To put it differently, a well-established population experiencing a normal amount of gene flow will tend toward heterozygosity, and thus selection favors alleles that function best in a heterozygous state. This system can quickly fall apart, however, when a few individuals become isolated from the rest of the population. A few flies getting blown or otherwise delivered to an island would be germane to this study.

Suddenly, a genome that was adapted to a situation where there was a relatively broad range of genetic variability finds itself in a quite different genetic environment; one of no gene flow and very little genetic variability. Since the rate of homozygosity will rapidly increase, selection will no longer favor “good mixers,” but instead the good “soloist.” Mayr (1954) summarizes “...that the mere change of the genetic environment may change the selective value of a gene considerably” (pp. 169). What Mayr meant by “genetic revolution” was a change in the genetic environment that results in the restructuring of the entire genome to accommodate the reversal of selective forces on alleles that function best as “soloists” instead of “mixers.”

The next question to address is what exactly are the conditions that are required for a genetic revolution to occur? Alan Templeton (1978b) provides a straightforward answer. First, there must be the isolation of one or a small number of individuals (a founder effect). This will cause significant genetic drift because all individuals carry a number of very rare alleles that will suddenly become common in the newly established population. Next, there must be a

drastic switch in the level of homozygosity as this small number of individuals interbreed and rare alleles that never before existed in a homozygous state in the parental population suddenly are. Other alleles become the only allele at that locus- in other words they become fixed. Lastly, the need to adapt to this novel genetic environment should result in a selective bottleneck. In some cases, a novel ecological environment will add additional selective stress.

Templeton (1978a, 1978b) conducted a series of experiments on *Drosophila mercatorum* in which he challenged individual virgin females to produce eggs parthenogenetically and therefore produce lines that were the result of an “ultimate founder effect” (descending from a single genome) (Templeton, 1978b, pp.1265). The mechanism of parthenogenesis requires the replication of a haploid egg pronucleus to produce a diploid state. While a female can produce genetically diverse offspring since meiosis and crossing over are maintained, once the process has begun, the result of parthenogenesis is complete homozygosity (Templeton, Sing, and Brokaw, 1976).

In these papers, Templeton (1978a, 1978b) builds on Mayr’s idea that the genome is an interconnected entity and suggests this is why a genetic revolution can so quickly cause changes in development, morphology, life history, and behavior. More specifically, the consequence of selective pressure on the structure of the genome itself (positive selection for good soloists since there is no out breeding) in the generation immediately after a founder effect is the production of a coadapted genome (Templeton, Sing, and Brokaw, 1976). A

change at a single locus can have reverberating effects on the entire genome, like a pebble dropped into a still pond creating ever-increasing ripples. One key feature that differentiates Templeton's results from Mayr's theory is that the flies showed evidence of a fortuitous coadaptation immediately after the switch from sex to parthenogenesis, whereas Mayr (1954) predicted coadaptation to evolve and build up over time. Instantaneous change must be the case for Templeton's flies since they either must be quick to make the switch to parthenogenesis or they are dead.

Most importantly, Templeton (1978b) found 1) the formation of an instantly coadapted genome acted to isolate the new population from its parental stock, since the structure of the new genome was partially incompatible with that of the parental line as evidenced by the decreased fitness value of crosses between the parental and parthenogenetic lines. 2) The different parthenogenetic lines were different from each other in terms of their coadaptation, reflecting genetics of the founder female for each line. 3) Isolation by the formation of coadapted genomes occurs rapidly after the founder effect. 4) The genes that seemed to be most sensitive to the circumstances of the genetic revolution and coadaptation were genes responsible for major developmental pathways, which can explain differences in morphology and life history. Disruption of major developmental pathways can also explain the observation that newly evolved populations are often developmentally and phenotypically unstable (Clarke and McKenzie, 1987). 5) Where Mayr hypothesized that a genetic revolution would result in

rearrangement of the entire genome, Templeton found heritable changes concentrated in certain types of genes, mostly those important for behavior, morphology, and life history, but not in isozyme loci that control “housekeeping” functions, for example, genes coding for enzymes of the Krebs cycle, as these enzymes remained unchanged in the parthenogenetic lines. It is important to realize that since all of these experiments were conducted before cloning, Templeton had no actual “developmental genes” (i.e. bands on an electrophoretic gel) to account for his observations of developmental and morphological abnormalities. This suggests not total rearrangement, but rather shuffling at a few key loci.

I should point out that one of the reasons Mayr’s model was so revolutionary was not so much that it suggested the possibility of rapid speciation, per se, but that it flew in the face of what Mayr liked to call “Beanbag genetics.” The central actors of the Modern Synthesis, mainly Fisher and Haldane, tended to treat the genetic material of a population as “a bag full of colorful beans” (Mayr, 1963). As such, their mathematical models of population genetics emphasized the concept that genes were shuffled independently from one another at every generation rather than being involved in complex arrangements. But Mayr, using his own findings in populations to support his ideas, was convinced that such a “beanbag” view was a meaningless over-simplification of the complex and highly integrated genome (see Mayr, 1963 for his full argument against reductionistic population genetics, and Haldane, 1964 for a counter argument). Mayer’s highly

integrated view of the genome would also suffice to explain the equilibrium of punctuated equilibrium (if either genome integration or equilibrium actually exist).

Wallace's artificial crab is a possible example of such a "genetic revolution," as it possesses all of the criteria outlined by Templeton (1979b): The process begins with a founder effect caused by the reduction of a large interbreeding population to a small number of individuals; in this case, the crash is in response to a novel environment. What results are an intense selective bottleneck driven by the environment and fixation dictated by the genes present in the founding individuals. This in turn leads to a change in the genetic environment from high levels of heterozygosity to high levels of homozygosity. Selection will now favor genomes that work in the newly established high level of homozygosity. The addition of needing to adapt to a very different external environment adds on an additional layer of complexity to Mayr's original theoretical and Templeton's experimental models.

The genetic revolution theory offers one mechanism of how reproductive isolation might occur; in Mayr's formulation it focuses mainly on changes in the internal environment. But might there also be a way to produce reproductive isolation that is centered on changes in the external environment?

You are what you eat (or what you smell)

Yeasts are a large group of diverse organisms, a grade of organization held together by the common feature of being fungi that exist predominately as single cells. As a diverse group of species, it is should therefore not be surprising that yeasts occupy a variety of specific ecological niches. For example, *Pichia insula* can only be found on rotting flesh of cacti on the Caribbean island of Curacao (Ganter, Cardinali, and Boundy-Mills, 2010), species of the genus *Candida* are the number one cause of blood stream infections in humans (Hazen, 1995), and I would not be surprised to find that there are specific yeast species associated with the nephritic patches of land crabs.

Individual species of yeast also have characteristic metabolic pathways that may be different from other species, even those living in similar environments. One of the consequences of different metabolic pathways is the production of diverse metabolites (Ganter, Cardinali, and Boundy-Mills, 2010). What this means for flies if they relocate (say, to an isolated Caribbean island) or switch food from yeast found in rotting plant material to yeast found on a terrestrial crab is that they will also likely be exposing themselves to the unique metabolic products of those yeasts.

Can a different type of food have a significant impact on the life of a fly or the evolution of a species? Recent research from Tel Aviv University suggests

that a simple switch in food source can support a speciation event in *Drosophila*. These researchers hypothesize that a change in diet causes a subsequent change in the types of symbiotic bacteria living in and on the flies and that the type of microbiota living with the flies influences mate choice (Milton, 2010). After just one generation of being raised on either starch media or molasses media, flies showed preferential mating to other flies that were raised on the same type of media (in other words, “molasses flies” preferred molasses flies and “starch flies” preferred starch flies). This effect lasted for 37 generations, but could be abolished by feeding the flies food supplemented with antibiotics, evidence in favor for the role of bacteria (Sharon *et al.*, 2010). The data suggest that the symbiotic bacteria influence mate choice by altering pheromone production (Sharon *et al.*, 2010). While preferential mating is not reproductive isolation, it is sliding in that direction and one could easily imagine how such an alteration in mate preferences could then lead to reproductive isolation after subsequent genetic drift. So an incipient bias in mating can act as an isolating barrier, reduce genetic exchange, and provide the possibility of sympatric speciation.

Another question to consider is why would a fly chose to switch food sources, other then cases of being forced to do so by evolutionary biologists in the lab? While terrestrial crabs can be found on almost every Caribbean beach, whether insular or continental, flies have only adapted to live on crabs on small islands where vegetation is limited (Carson. 1974). While the preferred egg-laying sites for *Drosophila* are fruit, leaves, slime flux and other rotting organic

matter that supports the yeast and other microorganism on which the flies feed (Oldroyd, 1964), a crab's nephritic patch is essentially a yeast infection that can provide microbial growth on which the flies can feed in the absence of the more traditional food sources. The fact that this curious change in food preference (even when given the chance to feed on typical fly media in the lab, *D. carcinophila* returns to feeding on a crab as soon as possible [Carson, 1974]) has occurred on three separate occasions in three different phylogenetic lines suggests that the mechanism behind the switch is simple, meaning that it is not genetically or molecularly difficult to pull off. Research on *Drosophila* olfaction provides one such simple mechanism. Ibba and colleagues (2010) suggests that a simple shift in the number of a single type of sensillum (insect chemoreceptor) can have drastic effects on a fruit fly's smell preference. So a change in food preference could easily be explained by a change in odor preference. The example of *D. sechllia* provides a naturally occurring instance of a switch in food preference being accompanied by the increase of a single sensillum type that causes the flies to be attracted to a scent that repels other closely related species. (Ibba *et al.*, 2010). It is possible that a similar olfactory adjustment, one that made the smell of ammonia irresistible, or at least bearable, initiated or reinforced the change in food selection.

Given these details regarding food selection, I can propose a model of how speciation might have occurred in the three species of crab-loving flies, as illustrated in figure 2.

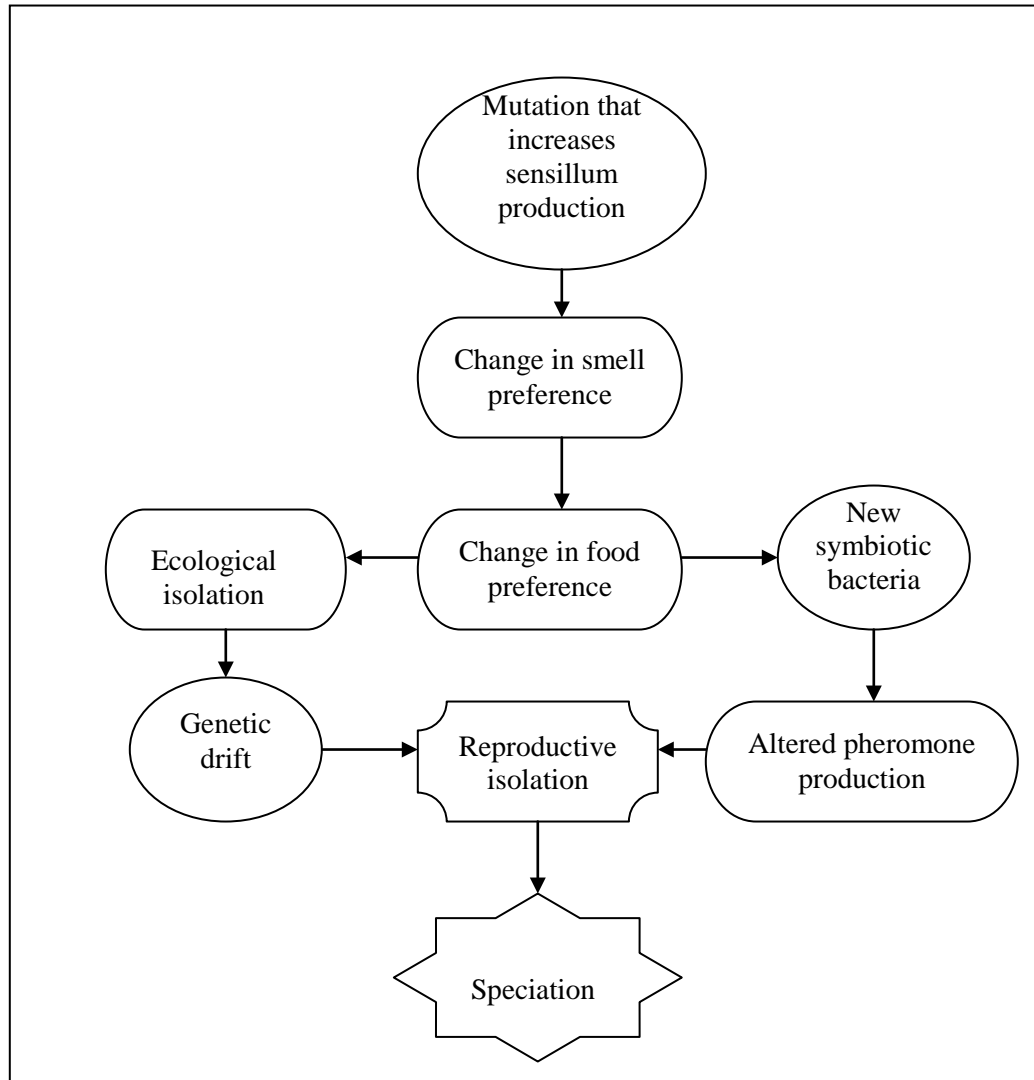


Figure 2. Illustration of modes of reproductive isolation leading to speciation. A change in food choice can lead to reproductive isolation without geographic isolation.

Nitrogenous waste of a land crab

A question related to the theme of smell and ammonia is “In what form do terrestrial crabs excrete nitrogenous waste?” This may at first seem like a trivial quandary, but the answer has real implications to the experiments at hand. A mechanism for getting rid of nitrogenous waste (resulting mostly from the breakdown of amino acids) is a necessary fact of life. In organisms that have transitioned from aquatic lifestyles to terrestrial or semi-terrestrial lifestyles in their recent evolutionary histories, the process of nitrogen excretion provides a challenge. The primary form of nitrogenous waste for aquatic crustaceans, and aquatic animals in general, is ammonia (NH_3) (Parry, 1960). NH_3 is the least expensive form of nitrogenous waste since no alterations are needed to modify it from its most frequent source: the amino group released as a result of protein catabolism. Ammonia is highly soluble in water, but it is also a highly toxic compound, so it must be excreted constantly into the water that surrounds an aquatic organism. Alternatively NH_3 can be excreted along with a copious amount of water. For the aquatic crustacean, this is not a problem since they are constantly surrounded by water. NH_3 excretion *does* become an issue when an organism moves to a terrestrial environment where it is no longer surrounded by water and where desiccation can be a major problem (Little, 1990). Many terrestrial animals deal with this issue by converting their nitrogenous waste into uric acid (often excreted as a paste) or urea, as both can be maintained in bodily

tissues at much higher concentrations than ammonia without toxic effects. The excretion of urea and uric acid require far less water than NH_3 for excretion (10 times less for urea and 50 times less for uric acid) (Wright, 1995), but they do require enzymatic pathways and energy to make the less toxic excretory product.

It would be logical to expect that terrestrial crustaceans have adapted to their new life on land by producing nitrogenous waste in the form of urea or uric acid. But for many terrestrial crustaceans this seems not to be the case. The primary form of nitrogenous waste for all crustaceans, aquatic and terrestrial, is NH_3 . For example, *Porcellio laevis*, a terrestrial isopod (a woodlouse) eliminates 57% of its nitrogen as NH_3 , 4% as uric acid, and none as urea (Parry, 1960). And this theme appears to hold true for terrestrial crabs, which you would expect to be even less adapted to land than other terrestrial crustaceans since most remain tied to the sea, returning to the water to reproduce.

So why would Wallace (and subsequently myself) use human urine for his laboratory simulation of a land crab if ammonia is the excreta of choice for land crabs? The first reason is one of practicality: terrestrial crab excreta is hard to come by whereas Wallace had a constant and copious supply of his own. Also, as it turns out, the system for eliminating and processing urine in land crabs is quite complex and deserves some attention here. Although the exact mechanisms have not been experimentally determined, I will present the current hypothesis as described by Wolcott and Wolcott (1991): Primary urine, which is isosmotic with the hemolymph, is eliminated to the exterior by the nephropores. The

nephropores of land crabs, such as *Gecarcinus lateralis* (one of the host species for *D. endobranchia* and belonging to the same genus as *G. ruricola*, the host to both *D. endobranchia* and *D. carcinophila*) are located behind the third maxillipeds. After primary urine exits the nephropore, it must enter the mouthparts. Wolcott and Wolcott (1991) hypothesize that in order to avoid ion loss and subsequent hemodilution, the primary urine is “reprocessed” when it comes into contact with ion pumps located in the gill membranes. It is at this point that NH_4^+ (ammonium) is added to the urine in exchange for the vital ions Na^+ and K^+ . Thus the final excretory product contains nitrogenous waste, not, for the most part, the primary urine.

It is now time to shift the focus back on the fly and how it fits into this picture. The three species that have made homes for themselves on land crabs spend different amounts of their total life span on the crabs and spend different periods of development in different locations on and in the crabs. Carson (1967) noted that adult *D. carcinophila* feed on the inner surfaces of the third maxillipeds and are otherwise observed around the eye stalks and antennae. The pupae of this species attach only to the under surface of the third maxillipeds, and younger larvae appear to be restricted to the filaments of the nephritic groove (beneath the nephropore). In contrast, *D. endobranchia* lay their eggs near the eyestalks of their host (either *Gecarcinus lateralis* or *G. ruricola*). The young larvae feed in the nephritic groove and then move to the gill chambers for the second instar larval stage, which has been observed to last several months. As third instar larvae they

relocate again, this time surrounding the crab's mouth opening. When it is time to pupate, *D. endobranhia* fall to the ground, returning to the crab after eclosion (Stensmyr, Stieber, and Hansson, 2008).

As can be seen by these examples, these two species of flies are exposed for different periods of time at different stages of development to various chemical conditions based on their location on or within the crab. Presumably, individuals located within the nephritic groove are exposed to the primary urine whereas individuals within the gill chambers are bathed in the excretory product as it is being modified by the crab.

So again I return to the question raised earlier, does it make sense to be using human urine for these experiments? Olfactory observation provides considerable evidence that after a period of being left at room temperature, urea in human urine begins to break down into ammonia as it percolates through the growth chamber of the experimental apparatus (or as the liquid used to mix instant media). Anyone who has ever used one of the public restrooms off a state highway on a holiday weekend will be familiar with this fact due to the distinct scent of NH_3 . Scientific analysis of the breakdown of human urine also backs up this olfactory observation (Kirchmann and Pettersson, 1995).

Urea and ammonia tolerance

Wallace was not the only person to expose *Drosophila* to urea or ammonia. In fact, a surprising amount of research has looked at whether fruit flies can develop a tolerance to urea and ammonia and the possible mechanism(s) behind this adaptation. First, it is important to note that both urea and NH_3 are not substances typically encountered by the adult *Drosophila* in large quantities, since their main form of nitrogenous waste is uric acid (Patton, 1953). Therefore, under natural conditions, urea and NH_3 are not substances for which adult flies have to evolve a tolerance. However, larval *Drosophila* do not have malpighian tubules and do not necessarily excrete uric acid. As mentioned, *Drosophila* typically lay their eggs in rotting organic matter, microenvironments that are semi aquatic, thus there is less selective pressure for the larval form to make the switch from NH_3 . Indeed, scientists have found that ammonia accumulated in *Drosophila* media over the life of a culture, which might indicate that NH_3 is the main form of nitrogenous waste for the larvae (Borash, *et al.*, 1998). Alternately, the NH_3 could result from the break down of uric acid, but if the former situation is the case, it could be that *Drosophila* are “preadapted,” so to speak, to developing resistance to NH_3 . By preadapted I simply mean that since larvae produce NH_3 they likely have some ability to tolerate it, which would give them a start on adapting to a higher concentration if they were to encounter it.

Borash and colleagues (2000) try to understand urea and NH_3 tolerance in the more general terms of toxin resistance. These authors propose that resistance to toxins that have wide-ranging effects, especially those affecting early development, are likely to require selection at multiple loci (polygenic) and therefore are likely to provide resistance to other toxins as well. An analogous example of cross-tolerance can be seen in the case of heat-shock proteins (Hsps). Hsps are not necessarily produced as a cellular response to chemical toxins, but to other environmental stressors such as heat, and could account for the evolution of cross-tolerance. Cross-tolerance does appear to occur in the case of urea and NH_3 . Flies selected for ammonia tolerance also displayed partial urea tolerance and flies selected for urea tolerance displayed partial tolerance to ammonia (Borash, Pierce, Gibbs, and Mueller 2000). These results are not all that shocking given the chemical similarity between ammonia and urea.

Studies have also been done that focused on determining the physiological mechanisms behind osmoregulatory adaptations present in *D. melanogaster* selected for urea tolerance (Pierce, Mueller, and Gibbs, 1999). Researchers found that larvae selected for urea tolerance accumulated less intracellular urea than their control counterparts. They suggested three possible mechanisms that could lead to this observed change 1) decrease urea up-take from the environment, 2) increase urea excretion, and 3) develop a way to metabolize urea into a less toxic compound. The actual mechanism or mechanisms that prevent the build-up of intracellular urea have yet to be determined. Any of the three proposed

mechanisms might also contribute to changes in morphology, life history, or development of the flies.

For example, Waddington (1975), as part of a series of experiments on *Drosophila*, observed morphological change in pupae that arose as an adaptation to living in media supplemented with high concentrations of sodium chloride, which, like urea creates a hyperosmotic environment. In successive generations of five stocks of flies, the amount of sodium chloride added to the media was adjusted so that of all the eggs laid, only 20-30% made it to adulthood. After 21 generations of selection, each of the five stocks was tested on various sodium chloride concentrations. With increasing salt concentration, Waddington observed an increase in the size of structures located on either side of the anus, known as anal papillae, which play a role in the larvae's osmotic regulation (Waddington used special strain of *Drosophila* carrying a gene that causes the anal area to be pigmented and papillae easy to measure). So perhaps there is a similar morphological change in flies selected or naturally adapted for urea and ammonia tolerance.

Canalization and the Inheritance of Acquired Characteristics

But the morphological change, though significant on its own, was not the only change Waddington (1975) noticed and is not sufficient to cause speciation. For each stock, he created a plot of the area of anal papillae versus salt

concentration. Waddington noted that these curves represent “a physiological function which we might call its ‘adaptability,’” and that the curves for the selected stocks were much steeper than those of the controls (Waddington, 1975, pp. 47-48). Thus by selecting for adaptation to a specific environmental condition (in this case high salt concentration), there was positive selection for a genotype that allowed for greater levels of genetic plasticity/“adaptability.” I wonder if this sort of plasticity could also play a role in selection for toxin cross-tolerance such as described by Borash, *et al.* (2000). By selecting for resistance to a specific toxin, such as ammonia, there is simultaneously selection for a more general genetic plasticity that would facilitate the generation of resistance to other toxins. In addition, genetic plasticity could be the first step toward the formation of new coadapted genomes, and maybe under some circumstances, reproductive isolation: it allows for further mutations or just environmentally induced changes in phenotype, some which provide a selective advantage. The overall outcome of this situation is the creation of an opportunity for a period of genetic change until the most advantageous genotype/phenotype becomes fixed by natural selection.

What I have just described in this toxin-tolerance vignette is a possible example of Waddington’s theory of the canalization and the “inheritance of acquired characters” (Waddington, 1941). Waddington (1975) offers his sodium chloride selected flies as an example of the inheritance of acquired characteristics due to the observation that the enlargement of anal papillae persisted even after the flies were returned to normal media.

While the term “inheritance of acquired characteristics” at first sounds Lamarckian, it is rooted in developmental biology and genetics (and Waddington’s desire to annoy biologists). The fundamental concept behind Waddington’s theory is that any phenotypic change induced by the environment must also at some level represent a meeting of ecology, gene action, and the constantly developing organism. All such interactions have a threshold at which the environment will cause an altered developmental outcome, and modification of that threshold can be selected for such that the trait is either more environmentally or more genetically determined. In such a case, it is easy to imagine how gene expression could be altered in such a way that that an advantageous phenotypic change is produced even in the absence of the original environmental stimulus; in other words it becomes genetically rather than environmentally determined. Thus, developmental reactions can fall under natural selection and the most effectual response becomes *canalized*. Canalization is the adjustment of gene expression by natural selection such that development produces the desired outcome regardless of environmental stimuli. The classic example of such a reaction is that of ostrich callosities.

It is easy to observe the formation of calluses in response to repeated friction or pressure on a particular area of skin in humans, and similar thickening of the skin occurs in other mammals as well as reptiles and birds. Ostriches have a peculiar matching set of callosities over their sternum and pubic bones, which are areas that rub against the ground when they crouch down. What is most

remarkable about these callosities, however, is that they are fully formed at the time of hatching (Duerden, 1920). Thus their formation is not under the control of the environmental stimulus of rubbing against the ground, but rather under the control of normal development. Waddington (1941) hypothesizes that the early ostrich ancestors were not born with sternal and pubic callosities but formed them in the normal fashion in response to friction and that individual ostriches varied in the responsiveness of their skin. Over time there was selection for the genotype that provided the optimum reactivity and presumably this resulted in a genotype that no longer required external stimulation to form the callosities (apparently enough baby ostriches died of infections because they did not have calluses on their undersides).

Returning to Waddington's (1975) work with *Drosophila*, the case of the enlarged anal papillae is much the same as ostrich callosities: the anal papillae first became enlarged as a response in some individuals to a hyperosmotic environment. This advantageous response came under positive selective pressure such that after 21 generations, the hyperosmotic environment could be removed and the larvae would still develop enlarged anal papillae. An alternate possibility is that the enlargement of anal papillae is part of a stress response that is already canalized and ready to be activated by the right environmental cue. Geist (1978) has gone as far as to argue that epigenetic adjustments, if they are themselves well canalized, negate the need for actual genetic change.

One criticism of this work is that Waddington only measured the anal papillae of larvae after 21 generations so we are left with no information about what happened in those most likely very crucial first generations. Also, of personal interest, if this is a case of genetic adaptation and not epigenetic modification, does it have enough of a developmental effect to reproductively isolate the resulting population? Since developmental effect and pleiotropy are in essence the same idea, it is conceivable that a genetic change in a developmental system can tug at a web of other developmental and physiological processes. These questions helped to guide my own experimental goals.

Experimental goals

Can speciation happen rapidly by Darwin's process of adaptive change (phyletic gradualism without the gradualism)? This is one of the questions Bruce Wallace wanted to answer when he embarked on his experimental adventure (Wallace, pers. comm.) and a lead I wanted to follow in my own attempt to replicate his experiment. My first goal, therefore, is to see what happens to a population of *D. virilis* in my own version of the synthetic crab.

I compared the larvae of both the parental population and flies living in urine to see if there were any observable morphological or behavioral differences that might offer a clue as to how they are able to adapt to this novel environment. It has been suggested that one of the advantages of first entering the world as a soft and fairly immobile larvae is that the larval stage represents a plastic stage in

development that can adapt to a variety of environments (Oldroyd, 1964). However, this hypothesis may be out of date and Lamarckian (and not in the way that the inheritance of acquired characteristics is seemingly Lamarckian). It is certainly the case that larvae are essentially single-minded feeding machines and they can afford to be so because the mother has carefully selected the site of oviposition so that the larvae need be concerned only with feeding and not with problems of protection or dispersal. If so, experimental conditions that include a drastic change in larval conditions are a severe test. Therefore it still makes sense to carefully observe the larval forms. It will be particularly telling to observe any changes in glands that play a role in osmotic regulation, such as the anal papillae observed by Waddington, and excretion, as these are closely linked to diet, metabolism, and the general environment.

I was also interested in the early changes of this “adaptive transition” and wanted to know how many adult flies are involved and how many viable offspring they produce. In addition, I wanted to see if there are any detectable differences in the life history of the resulting population in terms of generation time, fertility rate, and developmental timeframe, as well as any behavioral and morphological differences of the adult flies.

METHODS AND MATERIALS

Animals and Stock Cultures

All flies used were derived from a single vial of *D. virilis* obtained from Connecticut Valley Biological Supply. The stock population was maintained at room temperature in standard culture bottles containing a 5.0 cm layer of instant fly media (Black Jungle Terrarium Supply) prepared according to the manufacturer's instructions. Bakers yeast (Fleischmann's) was added as a food source. Cultures were transferred to fresh medium every few weeks, as necessary.

Urine

The urine used in all of the experiments was my own: female, age 22, on no medications or supplements. It was stored in one-liter glass jugs at 4°C.

Synthetic crab

My synthetic crab was constructed based on the one described in Wallace's 1978 paper (Figure 3). For my own synthetic crab instead of artificial turf (which was unavailable to me), I used plastic grid-work used in aquarium filters. This provides the surface area for the flies to lay their eggs. The plastic grid fit snugly inside a specially constructed Lucite box that allowed one centimeter of clearance on one side of the grid. The lid of the box was removable by way of four screws and had a small hole, into which was inserted the tip of a burette. The burette was in turn connected to a reservoir, which sealed closed with a lid. A drain in the bottom of the Lucite box led to a waste-collection bin and the Lucite box sat on top of the waste bin. The whole apparatus was built in modules so that the individual parts could be removed and cleaned as necessary. The Lucite box that housed the flies could be removed from the rest of the apparatus and placed under a dissecting microscope so that I could observe the development and behavior of the flies during the course of the experiment (Figure 4).

To inoculate the apparatus, I used a solution with five spoonfuls of garden soil (obtained from outside of Clapp) in 600 ml of distilled water that had been poured through a fine mesh to filter out large particles. This solution was then poured over the plastic grid-work. In addition, I added one packet of

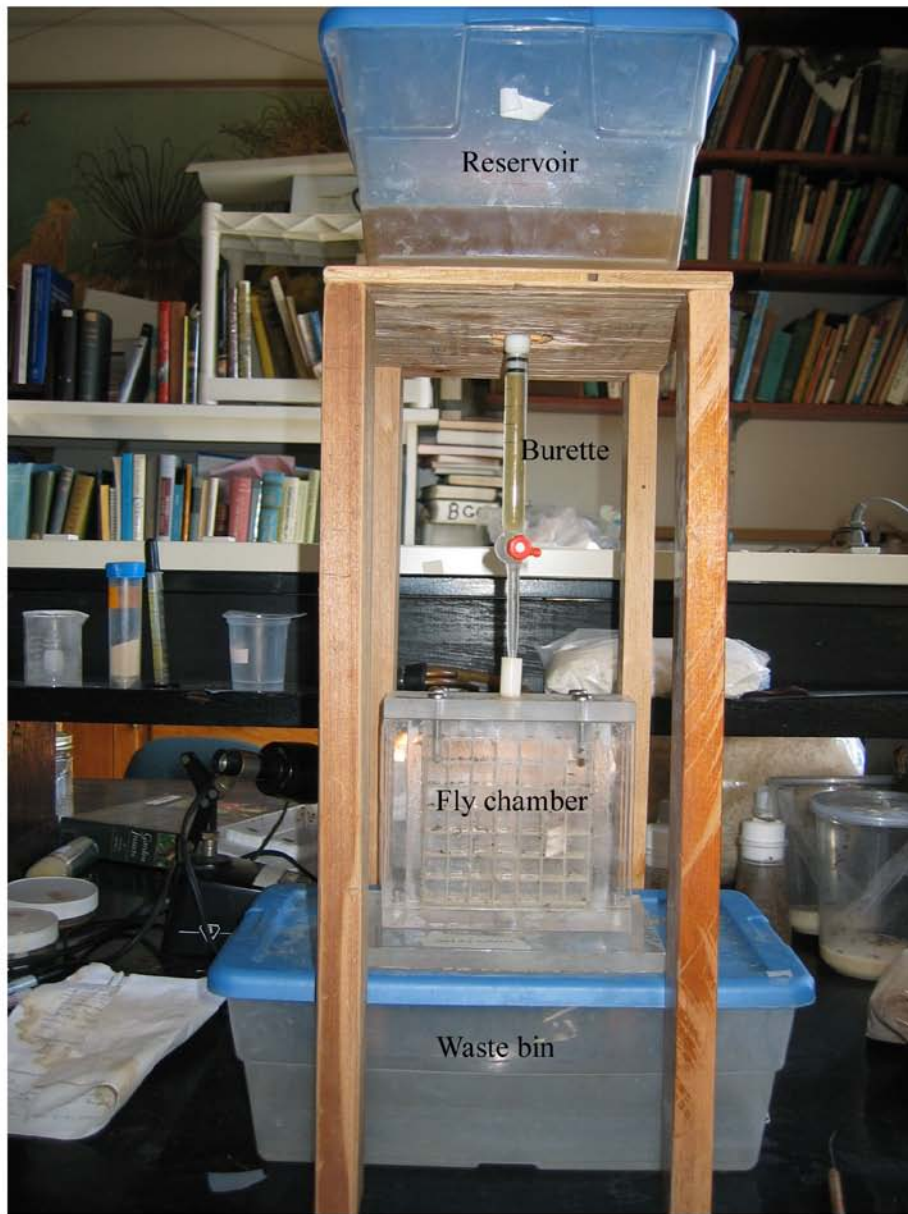


Figure 3. The synthetic crab. My crab was designed based on Wallace's (1978) description. The burette drips urine from the reservoir into the Lucite fly chamber. Waste drains into a collection bin below.



Figure 4. The synthetic crab fly chamber. The fly chamber is a square 9.5cm plastic grid inside a closed Lucite box. The chamber is detachable from the rest of the apparatus so that it can be placed under the microscope.

Fleischmann's yeast to 600 ml of warm water and poured this solution over the plastic grid-work.

To the reservoir I added undiluted urine. The drip rate, which could be set by adjusting the burette's stopcock, was 1mL/min, or approximately 1.5 liters every 24 hours. Old urine was replaced with a fresh supply once every week. A biofilm formed, but it was rinsed out frequently enough to allow flow through the system. Flow definitely slowed down and on occasion clogged points in the apparatus with small diameters.

At the beginning of the trial, approximately 50 adult flies were introduced to the apparatus. These adults had been previously living under standard culture conditions. The behavior and development of the flies was monitored throughout their life.

Urea tolerant population

To explore the possible effects of developing in the presence of urea, I modified the methods of previous studies that have also been concerned with the effects of culturing drosophilids in the presence of ammonia or urea. For example, Pierce, Mueller, and Gibbs (1999) were interested in determining the type of physiological mechanisms employed by *D. melanogaster* selected for urea tolerance. In another study, Borash, *et al.* (2000) asked whether flies (again, *D.*

melanogaster) selected for urea tolerance also developed tolerance, or partial tolerance, for ammonia and vice versa. Both studies produced tolerant strains by raising larvae on media supplemented with ammonia or urea. The adult flies, however, were never exposed to either toxin.

I wished to explore if I could produce a urea tolerant population of *D. virilis* where the adults, as well as the larvae, were subjected to the urea-supplemented media. In addition, I wanted to observe any possible morphological or life history differences between flies raised on standard media and those raised on urea-supplemented media and compare both of these groups to flies raised in the synthetic crab.

A urea challenge was produced by transferring stock flies to a culture bottle containing instant media prepared with urine diluted 50% with water. One week later, I transferred the resulting population to a new culture bottle, only this time the media was mixed with undiluted urine.

In order to observe fly development and capture images under a dissecting microscope, I prepared a Petri dish with a thin layer of urea-supplemented media or regular media for the control. I replaced the glass Petri dish cover with a plastic lid into which many punctures had been made. I also inserted a piece of filter paper that fit snugly into the underside of the lid. These modifications were made because previous attempts to culture flies in a Petri dish using the regular lid had resulted in an overly wet environment in which flies drowned in puddles of

condensation. Whenever the media appeared dry, I added a few drops of urine or water to return the media to its original consistency.

Ten adult flies were placed into the Petri dish for 24 hours and then released. Everyday after, I captured images using a camera (pixeLINK) attached to an Olympus dissecting microscope.

Pupal measurements

To determine whether there was a difference in pupal size, or if there was a greater variation in size between any of the populations, I measured pupal cases from two culture bottles of the stock population, two culture bottles of the challenge population, and all pupal cases that were successfully extracted from the synthetic crab at the end of the study. Size was defined as the longest anterior-posterior line and was measured using Image J software. I used Levene's Test to compare variance and three pair-wise independent sample t-tests (with adjustments made for samples which did not meet the equality of variance assumption where necessary) to compare means.

Media smears

I wanted to see if there was any variation in the microbiota living in the three different conditions. I therefore smeared a small amount of media and urine-supplemented media from active cultures onto glass slides and then covered

the smear with a glass cover slip. I created a third slide by taking scrapings from various areas inside the synthetic crab and smearing them on a slide. Since this smear was much dryer than the others, I added a drop of urine from the reservoir before topping with a cover slip. I observed all three slides with light and phase-contrast microscopy (Olympus).

RESULTS

Developmental timeframe

I was able to produce populations of flies in all three conditions (stock, challenge, and synthetic crab populations); however, the time course of development was different for each of the conditions (Table 1). The stock population developed the fastest, taking 15 days to progress through an entire generation. The synthetic crab population had the longest generation time of 27 days and the challenge population fell in the middle with a generation time of 23 days.

Interestingly, all three populations took the same six-day time span to go from the parental adult to the third instar larval form, but the similarity stops there: the time between the third instar larval stage and pupation was three days, eight days, and ten days for the stock population, challenge population, and synthetic crab population respectively. The time period from pupation to adult was six days, nine days, and eleven days for the stock population, challenge population, and synthetic crab population respectively.

Fertility rate and survival rate

The stock and challenge population trials followed identical protocols minus the media composition; therefore their fertility and survival rates can be directly compared (Table 2). The stock population resulted in 56 pupae, 45 of which eclosed. In contrast, the challenge population resulted in only eight pupae. From these eight pupae, five adults emerged. Thus the fertility rate (fertility rate = pupae/parental adults) for the stock population is 11.2, where as the fertility rate of the challenge population is 1.6. The survival rate [survival rate = (adults/pupae) x100] for the stock population is 80% and the survival rate for the challenge population is 63%.

While the protocol for the synthetic crab population was much different from the other two conditions, mainly the trial began with 50 rather than five adults and the adults were left in the culture until they died, it is still worth noting their fertility and survival rates. The fertility rate for this condition is 0.12 and the survival rate is 33%.

	Adult – Third Instar	Third Instar – Pupa	Pupa – Adult	Adult - Adult
Stock	6 Days	3 Days	6 Days	15 Days
Challenge	6 Days	8 Days	9 Days	23 Days
Synthetic Crab	6 Days	10 Days	11 Days	27 Days

Table 1. Timeline of developmental stages of three fly populations. Stock flies were raised on media prepared with as according to manufacturer's instructions. Challenge flies had been living for three generations on media prepared with 100% urine instead of water. Stock flies were introduced into the synthetic crab to create the synthetic crab condition.

	Fertility Rate	Survival Rate
Stock	11.2	80%
Challenge	1.6	63%
Synthetic Crab	0.12	33%

Table 2. Fertility rate and survival rate of three populations. Fertility was calculated as the ratio of pupae to parental adults and survival rate was calculated as the percentage of pupae that made it to adulthood

Morphology

The most striking difference between the stock population (Figure 5) and the two experimental conditions was the color difference of the pupae. Pupae from the challenge population (Figure 6) and synthetic crab population (Figure 7) tended to have much darker pupal cases than pupae from the stock. In addition to the dark pupal cases, pupae from the synthetic crab developed what appear to be melanotic tumors (Figure 8) as described by Barigozzi (1958). There was also a great deal of variation in the size of pupae in the synthetic crab, especially in comparison to the relatively uniform size of pupae from the stock population. This result will be discussed in further detail later.

In contrast to the notable difference in melanization of the pupae, larvae from the synthetic crab often appeared quite transparent (Figure 8 and 9) in comparison to larvae from the stock population (Figure 10). There was no coloration difference between stock larvae and those from the challenge population (Figure 11).

Individuals in the synthetic crab died at all different stages of development: some died as first or second instar larvae, others died shortly after pupation, and still others seem to have undergone the transformation into the adult form, but failed to eclose successfully.



Figure 5. Pupa from the stock population. This image is from the first day this pupa was observed, nine days after the culture began. Ecdysis for this individual occurred six days later. Dissecting microscope Magnification 40x.

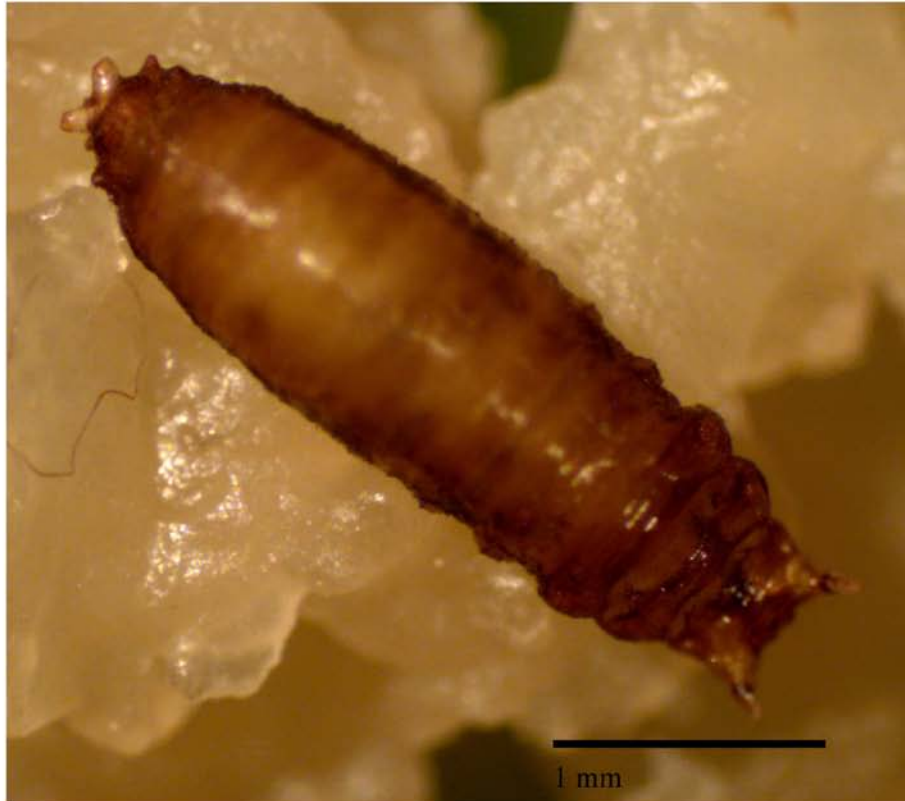


Figure 6. Pupa from the challenge population. This image was taken the first day of pupation, fourteen days after the start of the culture. This individual successfully eclosed 8 days later. Pupae of the challenge population were darker than the stock pupae but not as dark as the synthetic crab pupae. Dissecting microscope. Magnification 40x

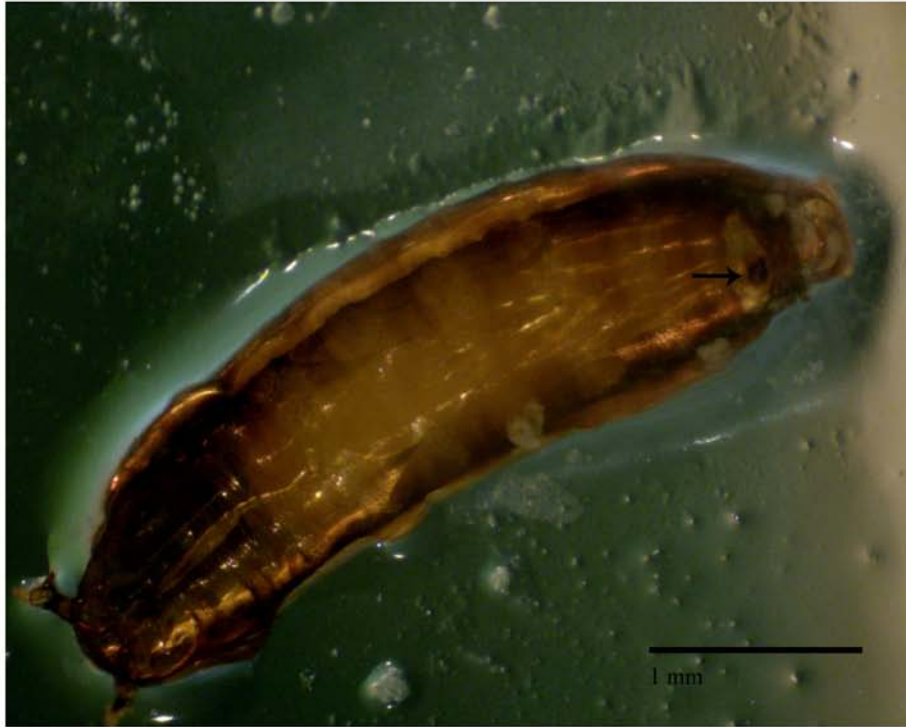


Figure 7. Pupa from the synthetic crab. This image was captured on the first day of pupation, 16 days after adults were introduced to the synthetic crab. The black arrow indicates the location of the melanotic tumor. This individual successfully eclosed four days later, but then died within 24 hours. Dissecting microscope. Magnification 40x.

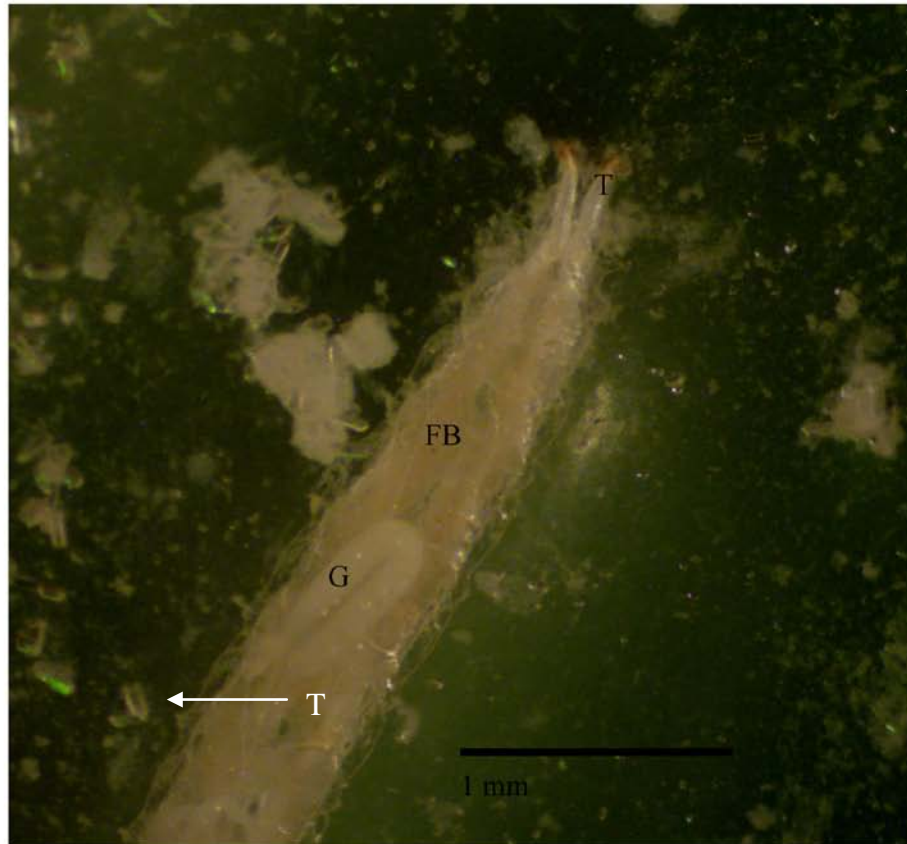


Figure 8. Posterior end of a larva from the synthetic crab. The transparent nature of the larva provides a clear view of the gut (G), fat bodies (FB), and the posterior end of the tracheal system (T). The elongated appearance of this individual is likely do to stretching as it moved across the Lucite surface. Dissecting microscope. Magnification 40x.



Figure 9. Third instar larva from the synthetic crab. Larva from the synthetic crab appear more transparent than larva from the stock and challenge populations. The mouth hooks (MH) are visible at the anterior and the end of the tracheal system (T) is visible at the posterior. Dissection microscope. Magnification 40x.



Figure 10. Third instar larvae from the stock population. Larvae from the stock population are less transparent than larva from the synthetic crab. Dissecting microscope. Magnification 35x.

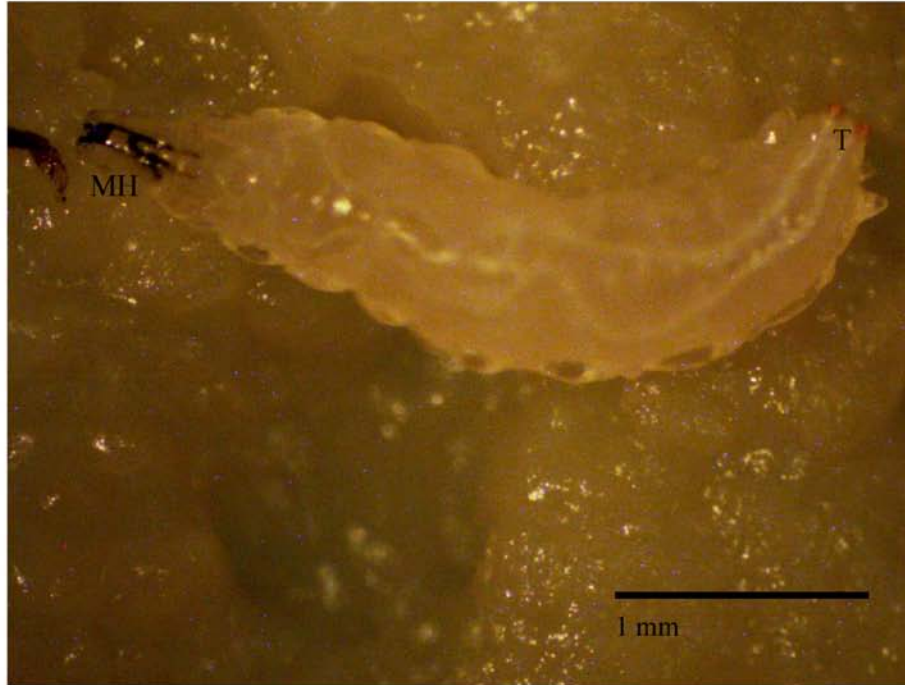


Figure 11. Third instar larva from the challenge population. Larva from the challenge population are similar in color to larva from the stock population. They are less transparent than the larvae from the synthetic crab, but the mouth hook (MH) and posterior end of the tracheal system (T) is still visible. Dissecting microscope. Magnification 40x

Pupal length

I found no significant difference in the mean length of pupae from the three populations (stock $M=3.55\pm 0.24\text{mm}$, challenge $M=3.47\pm 0.27\text{mm}$, synthetic crab $M= 3.56\pm 0.48\text{mm}$). However, the synthetic crab population was significantly more variable than the stock population ($F=18.150$, $p<.001$) and the challenge population ($F= 13.884$, $p<.001$) as determined by Levene's test, which tests the null hypothesis that population variances are equal. In addition to being the most variable, the synthetic crab population was also responsible for the most extreme individuals, with the smallest pupa measuring 2.76mm and the largest measuring 4.23mm (Figure 12).

Microbiota

Saccharomyces cerevisiae (baker's yeast) was the only species observed in both the stock and challenge media smears (Figures 13A and B) as confirmed by J. Knight. *S. cerevisiae* as well as other unidentified micro species were present in the sample taken from the synthetic crab (Figure 14).

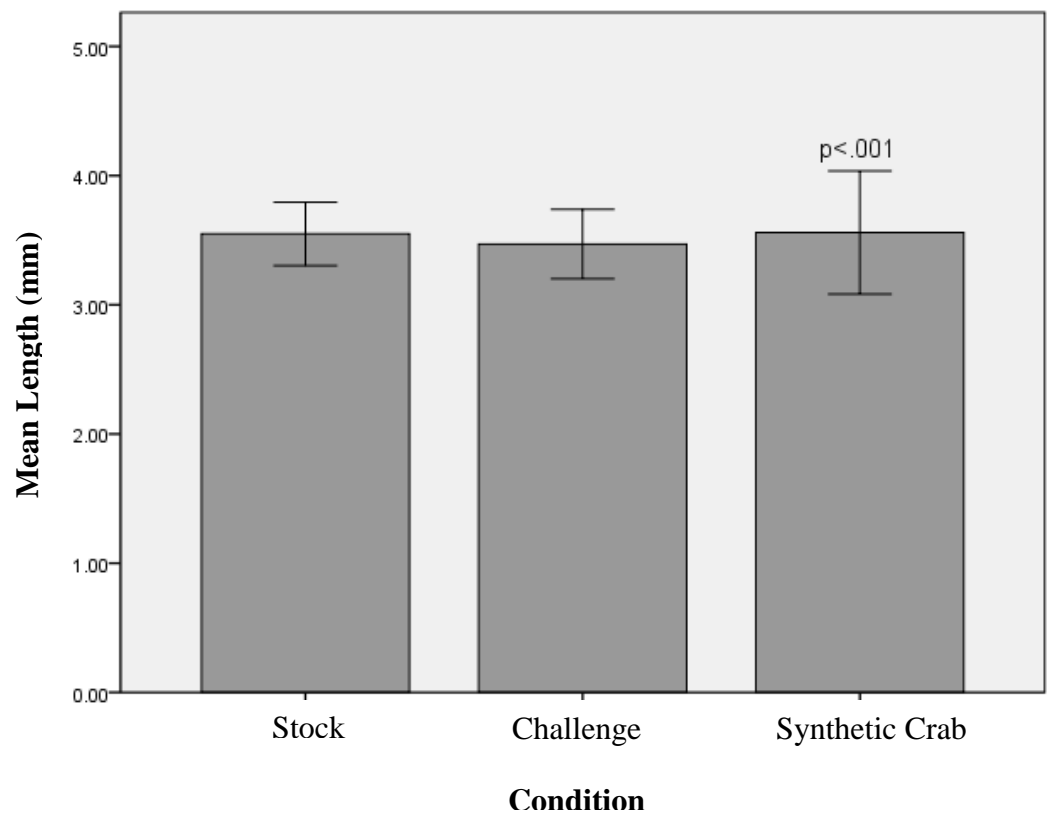
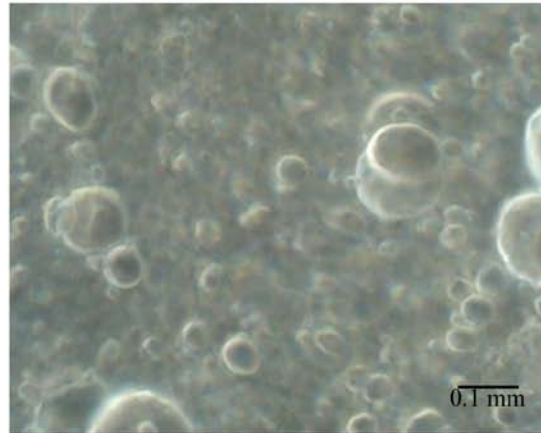
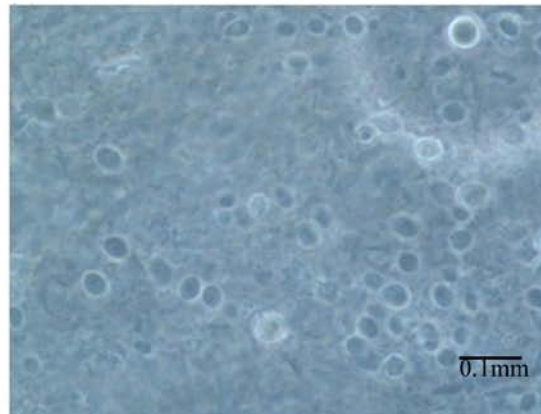


Figure 12. Pupal case length. There is no significant difference in the mean length of the pupae between the three groups as measured by three pairwise independent t-tests (Stock vs. challenge: $t=1.373$, $p=.174$; stock vs. synthetic crab: $t=-.081$, $p=.936$; challenge vs. synthetic crab: $t=-.688$, $p=.501$). The synthetic crab population is significantly more variable than both the stock population ($F=18.150$, $p<.001$) and the challenge population ($F= 13.884$, $p<.001$). There is no significant difference in variation between the stock population and the challenge population ($F=.092$, $p=.762$). Error bars are equal to ± 1 SD.



A



B

Figure 13. Microbiota from the stock and challenge media. *Saccharomyces cerevisiae* appears to be the only species living in both the stock (A) and challenge (B) media. The cells in the challenge media do appear smaller; however, it is unclear whether this is significant. Phase contrast. Magnification 1000x, oil immersion.

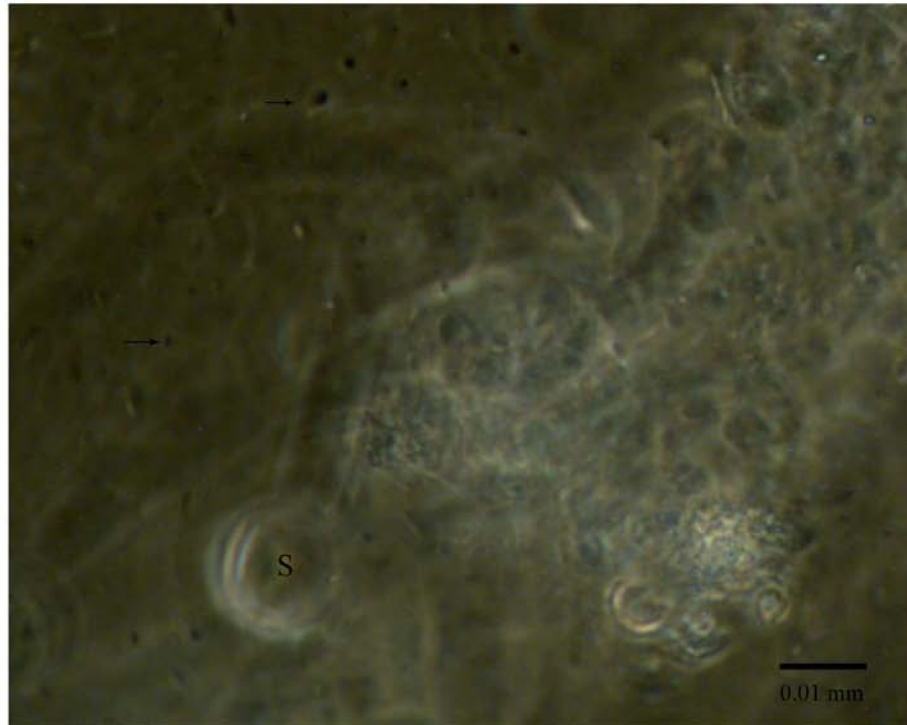


Figure 14. Microbiota from the synthetic crab. In addition to *Saccharomyces cerevisiae* (S), several other microorganisms (species not identified), indicated by arrows, appear in this sample taken from various locations within the synthetic crab. Phase contrast Magnification 1000x, oil immersion.

DISCUSSION

One of the reasons this study is such an interesting system in which to study various theoretical models of evolution is because it is in many ways a “natural” experiment. The flies were not subjected to selection regimes that were completely artificial. An example of purely artificial selection is Waddington’s experiment using heat shock, after which he bred individuals with the cross-veinless phenotype and killed all the rest in order to create a stock that was cross-veinless even without the heat shock so it had become a cross-veinless genotype (Waddington, 1975). Another example is the selective breeding of domesticated animals to produce desirable traits. The reasons that I say my study was a “natural” experiment are twofold: first, the methods were modeled on a known evolutionary event (flies adapting to the nephritic patch of a land crab), and secondly, flies were selected solely on their ability to survive under the experimental conditions; they were not secondarily removed from the population based on their expression of certain traits. In conducting this “natural” experiment I was hoping to gain a better understanding of the processes of evolution, how the processes of adaptation may or may not lead to reproductive isolation and speciation, and how these events actually occur in nature, not how

they occur during a contrived laboratory experiment. I wanted to see what happened rather than direct results to a specific end.

This is not to say that mathematical and theoretical models are of no use. On the contrary they are quite necessary! However, it should be noted that Mayr (1959) himself questioned the value of mathematical models, using the term “beanbag” genetics in a derogatory way to describe the work Haldane, Wright, and Fisher. But without models one has no frame of reference, nothing with which to compare her own observations (Wallace, 1968). Models can be seen as being similar to the rules of grammar: they provide the basics for communication. You must first understand the rules and be able to apply them properly before you can break them in intentional or meaningful ways. Likewise, a biologist must understand the simplified models to begin to understand the complexity of data from real populations that do not perfectly conform to the model. A problem of models comes when they are assumed to always be true or when they become the primary basis for new theories. Theories should be built upon scientific evidence; when theories are built on theories, what you get at the end is a house of cards. Sometimes these metaphorical houses withstand the test of time and are strengthened by experimental evidence, but other times experimental data ends up removing one of the cards, and the whole house comes tumbling down. The hypotheses presented by Darwin in On the Origin of Species are an example of a collection of inter-connected theories (many with obvious flaws) that has managed to stand for 150 years. The theory of a geocentric universe and the

convoluted mathematics used to describe the orbits of the sun and the other planets around the earth have collapsed.

As I anticipated, there were many microorganisms other than baker's yeast (*S. cerevisiae*) living in the synthetic crab, though none were identified, just as several of species of nematodes, mites and microbes can be found in the nephritic patches of land crabs (Carson, 1967). The microbes offer a unique food source to flies on both the real and the synthetic land crab. As discussed earlier, an alteration in diet can lead not only to ecological isolation, but even to reproductive isolation and, thus conceivably, to speciation. Sharon *et al.* (2010) point to the role of symbiotic bacteria in this process, so an interesting and informative follow-up to my work would be an examination the "hologenome." The hologenome theory as proposed by Zilber-Rosenberg and Rosenberg (2008) recognizes the genome of the host and its most closely associated symbionts as a single evolutionary unit. In other words, a host and its symbiotic species co-evolve. Zilber-Rosenberg and Rosenberg (2008) offer the example of microsymbionts that exist in the gut and mouth of humans and the advantage these bacteria provide. The importance of these and other symbionts of humans have spurred the funding of a "Human Microbiome Project" by the National Institutes of health (For more information see <http://commonfund.nih.gov/hmp/>). In the case of the urine-bathed fly, I would be particularly interested in bacterial contributions to the hologenome, since it is the bacteria that have the potential of

changing the flies' metabolic processes and altering pheromone production and subsequently influencing mate selection.

My findings related to food have implications beyond the role of the hologenome in the processes of evolution. The effects of nutritional deficits and starvation altered development. After just a single generation in the synthetic crab or living on urea-supplemented media I observed morphological and life history changes in *D. virilis*, and many of my observations, such as longer developmental time and decreased viability, are consistent with past research on the effects of stress, and starvation in particular, on life-history. Rion and Kawecki (2007) point out that many natural populations of *Drosophila* seem to have significant variation in regards to the trait of starvation resistance. These authors also suggest that starvation resistance is likely to be plastic (i.e. responsive to environmental conditions) as a result of the high trade-offs associated with increased starvation resistance. The number of trade-offs result from the integral role of food in providing energy and raw materials in every aspect of the organism's functioning. Populations of *Drosophila* selected for starvation resistance are characterized by reduced pre-adult survival and viability (Chippendale, Chu, and Rose, 1996). Starvation resistance is also correlated to low fecundity, extended developmental time, and overall longer lifespan (Rion and Kawecki, 2007). These findings are really just common sense: if food is scarce, an easy way for a fly to get the nutrition it needs to pupate and then make it to adulthood is to prolong the larval feeding stages. In addition, if a female is

struggling to simply maintain homeostasis, it is doubtful she will expend the massive amount of energy required to produce dozens of eggs when the population can survive with her only producing just a few. Slowing down the metabolic rate is an easy way to deal with less food and decreases the rate of development. It has been demonstrated that decreasing the metabolic rate not only increases longevity, but it also increases stress resistance (Hoffmann and Parsons, 1989).

In the terms introduced by Valerius Geist (1978), conditions of severe starvation could trigger a switch into what he calls “maintenance mode.” In maintenance mode, animals suppress all that are not necessary for reproduction and survival, tend to be smaller, and lethargic in order to reduce energy expenditure. In contrast, in “dispersal mode,” when there are plenty of resources available, the animals maximize their ability to colonize a new area and they often show increased reproductive output, increased activity and mobility, and a larger body size.

The idea behind maintenance and dispersal modes is based on the pretence that it is advantageous to an organism to have alternate phenotypic pathways “built in” to the genome that can deal with different environmental conditions (such as resource limitation versus resource abundance) and environmental cues can act as the trigger that will initiate development of one phenotype over the other. In this way the organism can adapt to changing conditions in a single generation via an epigenetic system and thereby not requiring natural selection on

actual genes*. Geist (1978) makes it a point to say that if these epigenetic systems are themselves well canalized and well adapted to the conditions an organism is likely to face, natural selection will no longer take place, except, perhaps, to refine the alternative phenotypes. If it is the case that all that needs to occur is an epigenetic switch into maintenance mode, there is no reason to expect to see reproductive isolation and speciation. In terms of Carson's conceptualization of "open" and "closed" genetic systems, it is possible to imagine an organism with two or more closed systems. "Open" systems appear to be relatively plastic and available ("open") to the process of natural selection, suggesting their mode of action occurs later in development, while other genetic components are highly canalized and "closed" to natural selection given their early and fundamental role in development and producing essential phenotypic traits (Carson, 1975). Therefore the switch from one phenotypic mode to another would be controlled by the epigenetic activation of one closed system and suppression of the others.

Additionally, it is interesting to note the similarities between starvation resistance and Geist's maintenance phenotype. The major difference between them is theoretical: Starvation resistance is an acute response to stress whereas the maintenance phenotype is an epigenetic syndrome with wide-ranging physiological and behavioral responses to an ecological situation. In both cases a successful response, one that increases survival and successful reproduction, does

* Geist (1978) also reminds us that a change in behavior is another way to adapt to the environment without natural selection and genetic change.

not result in natural selection because the response is already an integrated part of the organism's repertoire.

It seems that the effects of starvation can explain many of my results, however, increased melanization and the formation of melanotic tumors was not reported in any of the reviews on starvation resistance. But my observations should not come as much of a surprise to those familiar with entomology and insects' response to stress. Since the 1800's naturalists have noticed the frequent occurrence of insect melanic polymorphisms and ecotypes, with the perhaps the most famous example being that of the peppered moth which went from a white form peppered with melanic dots to a predominately dark form in the span of just 50 years (Kettlewell, 1955). The strongly supported hypothesis explaining this phenomenon points to the role of industrialization and the ability of the moths to camouflage themselves in a forest full of trees blackened with soot*. The

* One of the points that struck Kettlewell (1973) was the fact that in over 90% of the species that exhibited industrial melanism, the melanic form is dominant. This surprises him because the leading belief of the time was that most new mutations are recessive in nature and need to "evolve" dominance by way of "modifier genes" (Fisher, 1931). If this were true, then industrial melanism could not be a new mutation. Therefore Kettlewell developed the (most likely false) hypothesis that melanism is a "recurring necessity" (indeed, this is the subtitle of his book). Kettlewell cites the fact that 10,000 years earlier with the advent of the last glacial period, coniferous forests replaced the majority of European and North American deciduous forests. The trunks of pines are not only usually devoid of lichens, but they are also naturally darker than the trunks of deciduous trees. Moths would have previously evolved a dominant melanic form under these conditions. Kettlewell (1973) argued that both pathways are already present and a rare form merely replaces the common one under conditions that favor the switch. However, with our current understanding of molecular genetics, it is easy to see

selective advantage of improved camouflage is readily apparent, but the mutation or alteration of gene expression must appear in the population before natural selection can take action. The answer seems to lie in how insects generally react to environmental stress (such as pollution) that can become internal stress (disruption of developmental pathways for example). The examples of changes in melanization as a response to stress are numerous: many ladybird beetle species have multiple melanic ecotypes, with increased melanism occurring in cold humid regions (Majerus, 1998). Similarly, Parkash, Rajpurohit, and Ramniwas (2008) demonstrated that in wild populations of *D. melanogaster*, darker individuals were found at high altitudes and colder climates. The darker flies were also more resistant to desiccation than their lighter-colored counterparts. Melanin also plays a role in the *Drosophila* immune response, with melanotic masses forming at infection sites much as a mollusk might form a pearl (Tang, 2009). Similar melanotic tumors can also be observed in transgenic flies without infection (Woodard, pers. comm.), likely as a result of internal stress. In some of these cases, increased melanization offers a selective advantage, in other cases it appears to be non-adaptive or a byproduct of the activation of a stress response. Additionally it is possible that a change in melanin synthesis has its own side effects. For example, melanin is primarily the amino acid tyrosine, which is important in the biosynthesis of, among other things, dopamine and

how a gain of function mutation, such as one that causes the production of melanin everywhere when previously it was confined to a minimal number of cells, would instantly be dominant.

norepinephrine, so changes in melanism could produce changes in neurotransmitter synthesis and have subsequent effects on behavior. It is interesting to note that in artificially selecting for docile behavior, as in the domestication of animals, changes in behavior caused by altered neurotransmitter levels have been correlated to changes in coat color, specifically, localized depigmentation (Trut, Plyusnina, and Oskina, 2004).

But in all of the examples of increased melanization in insects, it can be seen as an index of recent exposure to stress and activation of a stress response, whether the stress originates internally or externally. In this context I can understand my own observations of increased melanization in the challenge and synthetic crab populations, and melanotic tumors in the synthetic crab population, as an indication of stress. Looking for fluctuating asymmetry in adult flies in the future would be a sensible next step; in the current study, I had too few adults from the synthetic crab to analyze and larvae are too soft-bodied to detect accurately fluctuating asymmetries.

The activation of a stress response has in some cases been known to “unleash” otherwise “hidden” variability. Heat-shock proteins (Hsp's) are a class of molecules that typically act as chaperones to stabilize other proteins, especially cell cycle and developmental regulators (Rutherford and Zuker, 1994). Hsp's can be seen as part of the buffering process since they permit a variety of genotypes to produce the same phenotype. But during a stress response, Hsp's are diverted to restore function to other denatured proteins in the cell. Thus, beyond the primary

damaging effects of a stressor on an organism, there is an increase in morphological variation due to losing Hsp's as developmental buffers (Queitsch, Sangster, and Lindquist, 2002). It is possible that the increased melanism in the flies exposed to urine is a visible indication of a more global stress response that could in turn lead to loss of buffering and increased variability. And indeed, I do see increased variability in the synthetic crab population.

The increased *variability* of pupa size can then be understood as evidence of stress resulting in a disruption of developmental pathways. Because of the canalization of developmental systems, well-established populations tend to be rather homogeneous in phenotype. However, populations with a newly evolved phenotype are likely to be much more variable since the old system has been destabilized and the new form has not yet been exposed to the generations of stabilizing selection required for canalization (Maynard Smith, et al., 1985). Researchers taking advantage of this observation have used fluctuating asymmetry (random deviations from symmetry in a fundamentally symmetrical structure) as a measure of developmental stability or as evidence of recent environmental or genetic stress (Clarke and McKenzie, 1987). While increased variability in size is not exactly the same as fluctuating asymmetry, I would argue it is also likely to be a sign of developmental destabilization.

It is noteworthy that while the variability of flies in the synthetic crab increased, the mean was statistically the same for all three conditions. While a change in the mean value of any morphological trait might be the end product of

evolution, it is variation that is the raw material for natural selection, and without it, there is nothing to be sorted by selection. Additionally, an instantaneous increase in variability is more likely to result from the release of latent variability by stress (which is what I was dealing with since none of the flies in the synthetic crab went on to produce subsequent generations).

Also, how can I make sense of the fact that the synthetic crab population was significantly more variable than the stock and challenge populations, but the challenge population was no more variable than the stock population? While both were subjected to urine, the synthetic crab population also had to contend with nutritional stress. Since the synthetic crab produced the largest as well as the smallest pupae out of all pupae measured for analysis, it is clear that the effect of nutritional stress is not just that of stunted growth and it is also somehow contributing to the destabilization. The change in food availability is also surely increasing the intensity of the selective bottleneck already produced by toxin exposure. It would be interesting to see if starvation or changes in food quality on its own is enough to cause the destabilizing effects seen in the synthetic crab population, or if it is the unique combination of toxin exposure and nutritional stress that produced the increased variability.

Another possible explanation could be that the challenge population was gradually acclimated to urine exposure. Instant media was first prepared with urine diluted 50% with water and then one week later, the resulting population was transferred to media made with 100% urine. In contrast, the synthetic crab

flies were immediately subjected to 100% urine without any media that additionally diluted the urine. The experiments Waddington described in 1975 are similar in that flies were gradually selected for sodium chloride tolerance: the salt concentration was adjusted each generation so that 30% of the eggs laid made it to eclosion. The survival rate for my challenge population was 63%; however, the calculation was based on larva-to-adult survival. I did not count the number of eggs laid, so I cannot determine the embryo-to-adult survival rate, but I would guess it to be less than 63% and probably closer to Waddington's 30%. At this rate of survival, Waddington saw morphological changes (enlarged anal papillae) and a moderate level of adaptation (the selected flies became somewhat more tolerant of sodium chloride than unselected stock) and these effects remained even after the selected flies were returned to media with a normal sodium chloride concentration. Waddington (1975) offers this as an example of the inheritance of an acquired characteristic by way of genetic assimilation. This requires canalization. Canalization is developmental buffering; the opposite of the developmental destabilization that I presume occurred in the synthetic crab population and caused the increased variability. Since canalization decreases phenotypic variability (while perhaps allowing for the increase of genetic variability), it would make sense that the variability of the challenge population was statistically no different from the stock. So while gradual selection can lead to morphological and adaptive change by genetic assimilation, unless the change

directly affects mating, it is unlikely it would lead to reproductive isolation and as such, is not likely to be significant at the macroevolutionary level.

With the difficulties perfecting the protocol and the constraints of time I was unable to produce enough flies in the synthetic crab to perform any type of genetic or behavioral analysis to establish reproductive isolation. Wallace was similarly unable to conduct further experiments on his synthetic crab flies. If it had not been for their unfortunate death, he would have set up side-by-side cultures flies from the synthetic crab and control flies to see if they exhibited selective mating, and if they produced hybrid offspring, would these offspring be viable (Wallace, per. comm.)?

What my results do indicate is the disruption of developmental pathways, which is the first step in producing a genetic revolution. As mentioned above, some genetic components appear to be relatively plastic and “open” to the process of natural selection, while other genetic components are stable and “closed” to natural selection (Carson, 1975). But if something big enough occurs to cause changes in these closed elements, you would expect to see (if the system is still at all viable) a shift in all subsequent pathways, a rearrangement of essentially the entire genome (Waddington, 1941), which begins to sound a lot like Mayr’s (1954) description of a genetic revolution*. Or, more in line with Templeton’s

* This system also has similarities to Goldschmidt’s “hopeful monsters.” Goldschmidt (1940) proposed that a single mutation that affected an early embryonic process could cause drastic morphological changes and produce a new type or species in a single step. Ironically, Mayr considered Goldschmidt his nemesis.

findings, change isolated in these “closed” systems, which he suspects are developmentally important genes, is enough to cause the formation of new coadapted gene complexes that move populations in the direction of reproductive isolation (Templeton, 1978b). However, it is important to note that I had absolutely no evidence of *genetic* change in response to stress. All I have is *phenotypic* response to stress

As I have mentioned, I battled many issues while conducting this study and in the end was unable to produce the kind or amount of data that I had been hoping for at the outset. My response to this situation (homologous to the stress response of my flies, perhaps) was to take a broader approach, pulling in knowledge from all areas of biology, to try and make sense of my limited results, which did not lend themselves to the type of pointed and reductionistic analysis typical of scientific research today. People do not approach research as simply a biologist; they are an evolutionist, an ecologist, a developmental biologist, a comparative anatomist, or a molecular geneticist. But I had to take a lesson from the meaning of the word “biology” (“bioes” being Greek for “life” and “logoes” meaning “to study”): Biology is the study of life, and life, an organism, is continually influenced by all of these specialized areas of research. While dividing up the field of biology into increasingly specialized areas of study is beneficial in that it allows for in-depth understanding, it is my belief that this particular way of approaching science can sometimes lead us to miss the forest for

the trees and it becomes too easy to miss the contextual and general meaning of a discovery. As Theodosius Dobzhansky (1942) noted:

This extreme compartmentalization of biological knowledge provided fruitful in that it led to an enormous accumulation of factual information; it has been deleterious in so far as it resulted in a lack of understanding between the representatives of the various disciplines and a consequent lowering of the efficiency of biological research. (p. vii)

It is not my intent to suggest that biologists abandon the detailed research of their specialized field; however, I would like to offer that there is an alternative and equally rewarding approach to research. It is an important lesson in humility to take a step back and notice the complexity of the entire forest, to return to the previous metaphor. If biologists take this more conceptual approach every once in a while, they may well be surprised at what connections they discover when they see the world in a maggot living in their pee.

APPENDIX

Part of understanding the evolution of organisms is recognizing that evolutionary theory itself has evolved. To follow the evolution of thought and theory, it is in this case helpful to ask the question “How many degrees separate this player from Theodosius Dobzhansky?” (Figure 15)

Zero degrees of separation: Theodosius Dobzhansky

Dobzhansky was a Russian-born naturalist who immigrated to the United States in 1927 to learn genetics in the Thomas Morgan’s famous fly lab at Columbia University, which moved to California Institute of Technology the following year. While working in Morgan’s lab, Dobzhansky’s primary focus was the genetics of natural populations but was also influenced by the work of geneticists George Beadle and Boris Ephrussi who were the stars of the lab in the 1930’s (Kohler, 1994).

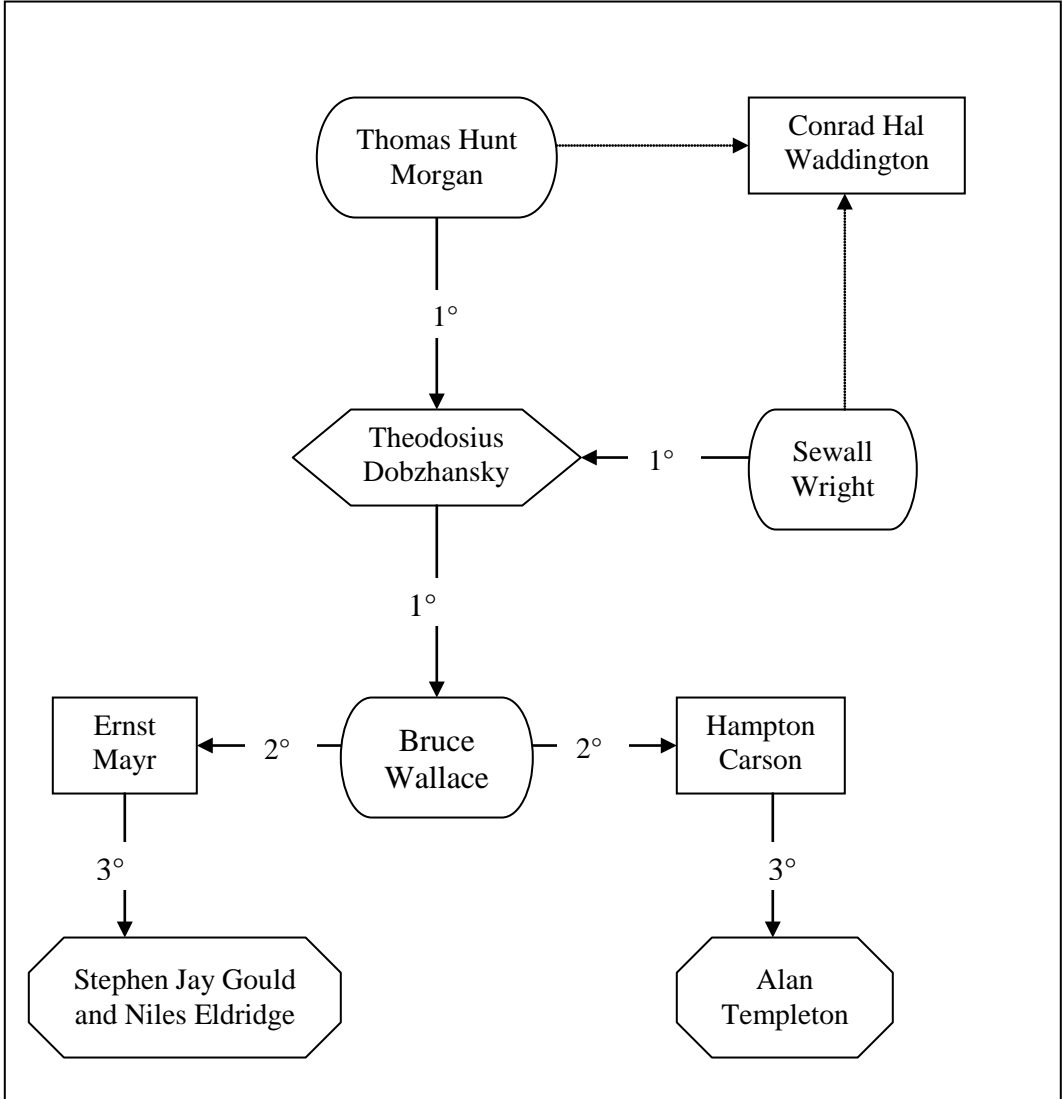


Figure 15. Degrees of separation from Theodosius Dobzhansky. The evolution of theory illuminates the evolution of organisms. This chart indicates the flow of ideas from teacher to protégé (and the parallel universe of Waddington) using Dobzhansky, the founder of evolutionary genetics as we know it, as the central point of reference.

Despite the fact that Ernst Mayr usually receives credit for the Biological Species Concept, it was Dobzhansky in his seminal book, Genetics and the Origin of Species (1937), who first suggested that a biological concept of the species was more useful than a species concept based on morphological differences and that reproductive isolation was the key to understanding speciation (Dobzhansky, 1951). It is no coincidence that Mayr's main source for understanding genetics for his own book Systematics and the Origin of Species (1942) where he presents his own definition of the Biological Species Concept, came from reading Dobzhansky (Provine, 2004).

A few years later, in 1940, Dobzhansky left Morgan's lab to return to Columbia University. For many years he and his students tracked differences in natural populations of *Drosophila pseudoobscura*. He used acetocarmine preparations of salivary chromosomes to look for mutations, especially translocation polymorphisms to characterize the populations. In this way Dobzhansky was able to merge the field studies of natural population genetics with theory (Kohler, 1994).

**One degree of separation: Morgan, Wright, and Wallace
(and the parallel universe of Waddington)**

Thomas Hunt Morgan

Thomas Hunt Morgan started out as an embryologist and ironically, early on in his career Morgan was adamantly opposed to the work of Gregor Mendel. But later, along with his extraordinary students, Bridges, Muller, and Sturtevant, Morgan demonstrated that Mendelian genes were real, physical entities located along the chromosomes (Dobzansky, 1980). Dobzhansky arrived in Morgan's lab in 1927 after earning a fellowship from the Rockefeller Foundation (Ayala, 1985).

Sewall Wright

In 1940, Dobzhansky left Morgan's lab to return to Columbia University and continued a long-term, long distance relationship with Wright (who was a professor at the University of Chicago from 1925 until 1955): Wright provided the foundation for theories, Dobzhansky did the field work and data collection, and Wright followed up with the mathematical analysis (Kohler, 1994).

Wright considered himself a developmental geneticist and whose first love was the guinea pig, and published a remarkable number of papers dealing with the complex gene interactions that are responsible for the coat color in guinea pigs. This led Wright to believe in the importance of gene interaction and he pushed the idea that selective forces on a gene can change based on its interaction with other genes and the genetic background in which it exists (Lewontin, 1980)

Bruce Wallace

Bruce Wallace was one of Dobzhansky's first graduate students, earning his PhD from Columbia in 1949. While he was still a student, Wallace acted as research associate at Cold Spring Harbor Laboratory and stayed there as resident geneticist and later assistant director until 1958. During the summer of 1950, in an effort to learn more about the current state of genetics, Ernst Mayr went to Cold Spring Harbor. He had previously recognized the importance of reproductive isolation in the process of speciation, but he had no genetic interpretation of how isolating mechanisms would evolve. Both men fondly remembered their time together that summer (Provine, 2004).

In his early years, following in the tradition of Dobzhansky, Wallace continued research in the area of population genetics using *Drosophila* as his model. For example, from 1949-1950, Wallace produced several strains of irradiated *D. melanogaster* in order to study the relationship between rate of mutation and fitness that Haldane had previously expressed as a mathematical equation (Wallace, 1968). Ecology and environmental science were always of interest to Wallace, but they became more of a primary focus in his later years. Perhaps his synthetic crab experiment in 1978 is representative of his shift toward more ecologically minded work.

Wallace was elected to the National Academy of Sciences in 1970, the same year he acted as president for the American Society of Naturalists with Hampton Carson as his vice president (American Society of Naturalists, 2008).

The parallel universe of Conrad Hal Waddington

Waddington does not fit neatly into Dobzhansky's direct lineage, but his theories make an important appearance in this study, and he can be seen as existing in a universe parallel to that of Dobzhansky. His early work with fossil brachiopods convinced him that the evolution of organisms is intrinsically intertwined with the evolution of developmental systems (Waddington, 1975). Therefore, where Morgan's lab took a populational and genetic approach to evolution, Waddington took an individual and developmental approach. But one theory of Morgan's was of particular interest to Waddington: that the only meaningful way to discuss genes was in terms of their activity, and gene activity resides in the world of epigenetics (Waddington, 1975).

Waddington's conceptualization of the "epigenetic landscape" is a reflection of his individual and developmental views on the evolutionary process, and was also likely influenced by Wright's "adaptive landscape." Wright's adaptive landscape is a visual representation of the adaptive fitness of a population based on the selective values of all possible gene sets and by natural selection, a population will reach an "adaptive peak" of optimal fitness given the gene sets available (Provine, 1986). Waddington essentially flips this

populational genetic landscape upside-down, turning adaptive peaks into valleys of normal development; this course of development is seen as a coordinated interaction between genotype and environment (Waddington, 1941).

Two degrees of separation: Mayr and Carson

Ernst Mayr

When asked what his most important contribution to evolutionary theory was, Ernst Mayr replied that it was the “genetic revolution” (Provine, 2004). Mayr presented his theory of “genetic revolution” in Animal Species and Evolution in 1963, *after* his genetics lesson over the summer of 1950 with Wallace (it is because of Wallace’s central role that I count Mayr as having two degrees of separation and not one from Dobzhansky). Before 1950, Mayr’s knowledge had come from reading Dobzhansky’s “distilled version” of Wright’s theories on population genetics and evolution and came to believe that Wright’s theory was primarily one of random drift and genes acting in isolation (Provine, 2004). But in fact, Wright’s shifting balance theory is based on his belief that the genotype to phenotype relationship is very complex with many cases of interaction and pleiotropy (Wright, 1982). It was most likely Mayr’s incorrect interpretation of Wright that led Mayr to call him a “beanbagger” in 1963. The genetics Mayr learned from Wallace seemed to be of a different type: one that emphasized the role of the genetic environment and gene interaction in

determining the selective value of a gene and included a concept of a highly integrated, coadapted genome (Provine, 2004). Once Mayr was introduced to this “new genetics,” it is not hard to see how he took the next step in conceptualizing the genetic revolution.

Hampton Carson

Hampton Carson earned his undergraduate and doctoral degrees from the University of Pennsylvania. A pivotal moment in his studies came in 1941 when Dobzhansky came to speak at the University laboratory, which at the time was headed by C.W. Metz (Carson, 1980). But it is my belief (which may be slightly biased) that his own conceptualizations of the processes of evolution did not occur after his interactions with Bruce Wallace at Cold Spring Harbor Laboratories and Pavia in the 1950’s (Figure 16) and their presidencies at the American Academy of Naturalists.

In 1963 Carson left for the University of Hawaii, Honolulu where he combined techniques of cytogenetics (similar to those used by Dobzhansky), his abiding interest in the natural history of fruit flies endemic to the Hawaiian Islands in relation to the geological history of the islands, and a theoretical commitment to the role of coadaptation in the origin of species. His own theory of open and closed genetic systems unites all of his interests (Carson, 1975).

Three degrees of separation: Gould, Eldridge, and Templeton

Stephen Jay Gould and Niles Eldridge

When Mayr first came to the United States, it was to work as an ornithologist at the Museum of Natural History in New York. He eventually also took a position as an adjunct professor at Columbia University (Bock, 1994). In the mid 1960's, both Stephen Jay Gould and Niles Eldridge were graduate students at Columbia. It was during their time together at Columbia, exposed to Mayr's theories of allopatric speciation by peripheral isolation, that the two began formulating their own ideas about speciation, which solidified into the theory of "punctuated equilibria" in 1972 (Yoon, 2002).

Alan Templeton

After earning his PhD from the University of Michigan, Alan Templeton spent several years (1974-1977) as a postdoctoral fellow in the lab of Hampton Carson in Hawaii. Templeton used Carson's concept of open and closed systems and Mayr's theory of genetic revolution to explain what was happening to his parthenogenetic flies.



Figure 16. Bruce Wallace and Hampton Carson, Pavia 1953. Photograph source are the Isadore Michael Lerner papers. Retrieved from <http://cdm.amphilsoc.org/u/?genetics,253>.

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