Waddington's Widget: Hsp90 and the Inheritance of Acquired Characters

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Abstract

Conrad Waddington published an influential model for evolution in his 1942 paper, *Canalization of Development and Inheritance of Acquired Characters*. In this classic, albeit controversial, paper, he proposed that an unknown mechanism exists that conceals phenotypic variation until the organism is stressed. Recent studies have proposed that the highly conserved chaperone Hsp90 could function as a "capacitor," or an "adaptively inducible canalizer," that masks silent phenotypic variation of either genetic or epigenetic origin. This review will discuss evidence for, and arguments against, the role of Hsp90 as a capacitor for morphological evolution, and as a key component of what we call "Waddington's widget."

Keywords: Hsp90, Evolution, Morphological Development, Canalization

1. Introduction

According to Webster's Online Dictionary, a "widget" is "an unnamed article considered for purposes of a hypothetical example." In 1942, Conrad Waddington (1905-1975) published his classic paper, *Canalization and the Inheritance of Acquired Characters,* in which he argued that an unnamed article regulates phenotypic expression of several, apparently acquired, developmental characters. Waddington cited several examples of apparently acquired characteristics that have useful purposes in adult organisms, but little or no function in fetuses, such as the callosities on the knees of fetal ostriches and the thickening soles of the feet of fetal humans [1]. In Waddington's paper, he proposed the existence of "adaptively inducible canalizers" (Meikeljohn and Hartl's term [2]), or "evolutionary capacitors" (Rutherford and Lindquist's term [3]), that reveal phenotypic variation in times of stress. Waddington proposed that, when variation is selected in subsequent generations, "canalization" (stabilization) of the new phenotype occurs so that the phenotype can become expressed even in the absence of stress [1].

In later studies, which Waddington interpreted as confirming his 1942 hypothetical model explaining the apparent inheritance of acquired characters, he showed that an unnamed article concealed the *crossveinless* [4] and *Ubx* phenotypes [5] in *Drosophila.* When the unnamed article was removed by stress (heat shock or ether exposure), selection of the exposed phenotype occurred, and, after several generations of selection, the phenotype was "canalized," *i.e*., expressed even in the absence of stress. Unfortunately, few scientists paid attention to Waddington at the time because his scientific program was suspect, presumably because it appeared

Lamarckian [6]. We will discuss this issue in more detail later, but it is fair to say that Waddington's body of work has recently undergone a resurgence of interest.

Because of the mysteriousness of the unnamed article that hides phenotypic variation, we call it "Waddington's widget." The molecular mechanism of Waddington's widget remained a mystery until 1998, when Rutherford and Lindquist presented evidence that Hsp90 fulfills the requirements for being a likely component [3]. In this paper, and in a similar study using *Arabidopsis* [7], Lindquist and colleagues showed that genetic or pharmacological inactivation of Hsp90 exposed previously hidden phenotypic variation, and that this phenotypic variation can be selected and eventually canalized or fixed in the population [3].

In apparent contrast to the papers from the Lindquist laboratory, work from our laboratory provided unique evidence for an epigenetic mechanism for the capacitor function of Hsp90 [8]. Recently, several reviews have described the possible genetic [9-14] and epigenetic [15-17] roles of Hsp90 in morphological development and evolution. The latter three reviews argue that both genetic and epigenetic mechanisms likely explain the evolutionary capacitor function of Hsp90. In this review, we discuss the evidence in favor and the arguments against the proposed capacitor function of Hsp90, and other possible uses of Hsp90 in development and evolution. Foremost, after a short historical perspective on potential mechanisms of evolution, we address the question, "Is Hsp90 Waddington's widget?"

2. Inheritance of acquired characters – an abridged historical perspective

Jean Baptiste Pierre Antoine de Monet, Chevalier de Lamarck (1744-1829) was an influential French naturalist and evolutionary theorist. Lamarck proposed a theory of evolution in his book *Zoological Philosophy* (1809) that maintains that animals acquire useful characteristics during their lifetimes, and that they can pass on these acquired characteristics to their offspring [18]. In this controversial book, contentious even at the time it was first published, Lamarck stated, "continued use of any organ leads to its development, strengthens it and even enlarges it, while permanent disuse of any organ is injurious to its development, causes it to deteriorate, and ultimately disappear if the disuse continues for a long period through generations" [18]. Lamarckian theorists of the 19th Century, known at the time as "naturalists," famously maintained, for instance, that a giraffe first develops a long neck by stretching to reach tall trees, then passes this characteristic to its young [19].

The Lamarckian theory of inheritance of acquired characteristics was replaced, at least for the majority of 20th Century evolutionary biologists and geneticists, by Charles Darwin's (1809-1882) theory of natural selection that he first published in *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life* (1859) [20]. The main reason, of course, for the continued and further increasing popularity of Darwin's theory of natural selection in the 20th Century was that a mechanism for selection of discrete units or genes was provided in the laws of genetics proposed by Gregor Mendel (1822-1884) in *Experiments in Plant Hybridization* (1865) [21].

Thomas Hunt Morgan (1866-1945), with his small group at Columbia University, including A. H. Sturtevant (1891-1970), C. B. Bridges (1889-1938), and H. J. Muller (1890-1957), further drove the nail in the coffin of Lamarckism with their pioneering genetics research with *Drosophila*, starting in 1908 [22]. In 1915, Morgan, Bridges, and Sturtevant published *The Mechanism of Mendelian Heredity*, a book that established *Drosophila* as an excellent model system in genetics [23]. In 1928, with the exception of Muller, Morgan moved his group to Caltech where they remained the remainder of their careers [22]. Ironically, before moving to Columbia University and beginning his research with *Drosophila*, Morgan was skeptical of Darwinism, which he perceived to be "too speculative and not grounded in observable phenomena" [22]. Also, at this time early in his career, as was the fashion among many well-respected developmental biologists, Morgan was critical of Mendelism and the chromosomal theory of heredity [22].

Despite the disrepute the majority of 20th Century biologists held (and still hold) for the idea of Lamarckian evolution, T.D. Lysenko (1889-1976), who became an influential agronomist in the Soviet Union during the Stalin years, was a firm adherent [24]. Lysenko discovered that the germination characteristics of winter wheat could be made to mimic spring wheat by the Lamarckian-appearing practice of exposing seeds to moisture and cold. His simple process of "vernalizing" wheat held out the prospect, misplaced as it turned out, of improving wheat yields in the harsh weather of Siberia. Lysenko's experiments were poorly controlled and never subjected to peer review, but they were accepted nonetheless by a political establishment that viewed environmental malleability of biological attributes as consonant with Marxist ideology.

Large-scale application of Lysenko's practices, combined with the brutal collectivization of agriculture ordered by Stalin in the early 1930s, contributed to severe famines that killed an estimated 10 million Russians. Despite this abject failure, Lysenko was elevated in 1937 to membership in the Supreme Soviet and he became the head of the Institute of Genetics of the Soviet Academy of Sciences. In 1948 he delivered an impassioned address denouncing Mendelian thought as "reactionary and decadent" and declared such thinkers to be "enemies of the Soviet people" [24]. It was due to Lysenko's efforts that many scientists, those who were geneticists or who rejected Lamarckism in favor of natural selection, were imprisoned, executed or exiled [24]. With Stalin's death in 1953, Lysenko's influence began to fade, although a complete repudiation did not occur in the Soviet Union until well over another decade had passed.

The debacle that was Lysenkoism irreparably tarnished the image of Lamarckian evolution for a great majority of scientists. Yet vernalization is a real botanical phenomenon that, ironically, eventually proved tractable to Mendelian genetic experimentation. Studies in the last decade have identified the mechanisms by which photoperiod and exposure to cold regulate the timing of flowering in *Arabidopsis*, wheat, oats and barley, among other plant species. The processes governing cold-stress response and cold tolerance are much less well understood than those involved in oxidative stress or heat shock, but make use of signal transduction cascades involving gibberellins, MADS-family transcription factors, lectins, and epigenetic modification of the plant genome by DNA methylation (reviewed in ref. [25]).

3. Waddington and 20th Century Biology

Early in the 20th Century, before the calamitous Lysenkoism events described above, Lamarckian evolution was still in vogue, at least by the "naturalists" who believed in the inheritance of acquired characters. The "naturalists" fought pitched battles with the "geneticists" who, using Darwinian principles, believed in the inheritance of genetic variants by means of natural selection. In 1942, Waddington tried to mediate the battle between the "naturalists," who were beginning to wane in influence, and the "geneticists" [1]. In his classic paper, he proposed a genetic mechanism for the apparent, but some people argue not genuine (see below), inheritance of acquired characters. According to Waddington, "Once the developmental path has been canalized, it is to be expected that many different agents, including a number of mutations available in the germplasm of the species, will be able to switch development into it. By such a series of steps, then, it is possible that an adaptive response can be fixed without waiting for the occurrence of a mutation" [1]. According to Waddington, canalization is mediated by "Developmental reactions [that] are adjusted so as to bring about one definite end-result regardless of minor variations in conditions during the course of the reaction" [1].

In evolutionary biology, the concept of canalization only became important after Waddington demonstrated genetic assimilation of environmentally induced phenotypes [4, 5]. For example, Waddington showed that a *crossveinless* phenotype is induced in a small percentage of offspring when parental *Drosophila* are heat shocked, and that selection of progeny with this environmentally induced phenotype for several generations leads to assimilation of this phenotype in nearly 100% of the progeny, even

in the absence of heat shock [4]. Waddington performed similar experiments with the *Ultrabithorax* (*Ubx*) phenotype induced by heat shock, a *Ubx* "phenocopy" in the jargon of the geneticists, and found that this phenotype can also be assimilated into nearly 100% of the selected population [5]. Subsequently, assimilation experiments were also performed in several laboratories on the *extra cross-veins* phenotype, the *dumpy* larval phenotype, and the *large anal papillae* phenotype in wild-type strains of *Drosophila* (reviewed in [26]).

Another approach that several laboratories have used in assimilation experiments is to select for extreme phenotypes in an already mutant background. For example, wing vein length was selected in ci^D mutant flies, and vibrissae number was selected in *Ta* mutant mice. Again, fixation of an extreme phenotype of either mutation occurs after 10-20 generations of selection. Other examples include selection of facet numbers in *Bar*-mutant *Drosophila*, wing-vein interruptions in *Hairless*-mutant *Drosophila*, and bristle number in *ocelli-less* mutant *Drosophila* (reviewed in [26]).

A. J. Bateman, a graduate student of Waddington, proposed three models to explain the increase in frequency of the *dumpy* phenotype: 1) a shift in the mean of the distribution; 2) a shift of the threshold; or 3) an increase of its variance [27]. She interpreted her data as supporting model 2, that selection of the *dumpy* phenotype shifts the threshold such that the new genetic makeup of the selected population favors this phenotype [27]. In a later section, we incorporate Bateman's three models to explain epigenetic canalization (Fig. 1).

According to Scott Gilbert in his popular textbook *Developmental Biology*, "Waddington's work was misinterpreted as supporting the inheritance of acquired traits"

[6]. Gilbert's view is surprising because Waddington himself used the phrase "inheritance of acquired characters" in the title of his classic paper described above [1]. Nevertheless, Gilbert espoused this argument because, "While Waddington's results look like a case of 'inheritance of acquired characteristics,' there is no evidence for that view. Certainly, the *crossveinless* phenotype was not an adaptive response to heat. Nor did heat shock cause the mutations. Rather, the heat shock overcame the buffering systems, allowing preexisting mutations to result in mutant phenotypes rather than wild-type phenotypes." Gilbert was evidently striving to revitalize Waddington's reputation by distancing Waddington's own views from Lamarckian evolution. As discussed below, perhaps this was not necessary.

4. Hsp90 as a capacitor for morphological evolution

Hsp90 is unique in its functions as a major heat shock protein. Unlike the other proteins in this class of stress-induced proteins, Hsp90 is not required for the maturation or maintenance of proteins in general. Rather, most of the identified cellular targets of Hsp90 are involved in signal transduction and chromatin organization (reviewed in [14, 28]). Several cell cycle and developmental regulators have been shown to form non-functional conformations in the absence of Hsp90, and Hsp90 activates these signaling pathways by stabilizing their alternate, functional, conformations [28]. It has been postulated that low-affinity interactions of Hsp90 and its targets keeps the signaling proteins poised for activation until they are activated by post-translational modifications from upstream signaling molecules [28]. For example, several members of steroid-hormone receptors, cyclin-dependent kinases, and Src-family kinases have been shown

to be substrates for Hsp90 [28]. Recently, Hsp90 itself has been shown to be associated with the chromatin in complexes that destabilize the estrogen receptor transcriptional activation complex [29].

Rutherford and Lindquist showed that reduction of Hsp90 reveals previously concealed phenotypic variation in *Drosophila* [3]. They showed that when Hsp90 activity is reduced by mutation or pharmacological inhibitors, phenotypic variation of nearly every adult structure of the fly is induced. As Waddington had done with the *crossveinless* phenotype, Rutherford and Lindquist observed that selection for 10 or more generations of a particular adult structural abnormality leads to the fixation of the new phenotype in the population. Also as Waddington had seen with the *crossveinless* phenotype induced by heat shock [4], fixation of the phenotypes in the populations selected by Rutherford and Lindquist occurs after the restoration of normal levels of Hsp90.

Evidence that this is primarily a genetic rather than an epigenetic phenomenon is that adult structures are differentially altered in strain-dependent manners in laboratory strains and wild populations. Also, in what Lindquist and colleagues later called a “litmus test for the genetic basis of traits” [17], they show that outcrossing the selected flies with high trait penetrance to unselected lines heterozygous for the Hsp90 mutation results in only the Hsp90-mutant progeny having the selected phenotype [17]. Their interpretation is that, “Should the trait have been epigenetically inherited, it would not have disappeared in outcrossed progeny with wild-type Hsp90 levels” [17].

Our experiments did not pass the “litmus test” for genetic traits, that is we still saw the enhanced Kr^{If-1} phenotype when we outcrossed selected flies to unselected

flies with the same *iso-Kr*$^{lf\text{-}1}$ background ([8] and data not shown). However, we can imagine situations where an epigenetically inherited trait disappears in outcrossed progeny. For example, the outcross strain might have a higher than usual concentration of chromatin inhibiting proteins, such as the histone H3 lysine 9 methyltransferase, Su(var)3-9, that plays a central role in heterochromatic gene silencing [30]. Also, strictly speaking, these authors could not exclude an *additional* contribution due to heritable epigenetic, or chromatin-conformational, variation.

5. Hsp90 is a capacitor for cryptic morphological variation in plants

Levels and patterns of genetic variation differ greatly between outbreeding species such as *Drosophila* and self-fertilizing species such as the plant *Arabidopsis thaliana*. One might speculate that inbreeding species, because of their nearly isogenic genomes, would have much less phenotypic variation, at least within an isolate, than outbreeding species. However, Queitsch *et al.* show in their recent paper that *Arabidopsis thaliana* isolates can have dramatic phenotypic variation when Hsp90 activity is pharmacologically reduced [7]. As Rutherford and Lindquist showed in *Drosophila* [3], Queitsch *et al.* showed that reducing Hsp90 function produces an array of morphological phenotypes in *Arabidopsis* accessions and recombinant inbred lines [7]. Interestingly, the induced phenotypic variations are dependent on underlying genetic variation, despite the fact that very little phenotypic variation was present prior to abrogating Hsp90 function and subsequent selection.

Since different *Arabidopsis* isolates develop different phenotypic alterations, in a strain-specific manner, Queitsch *et al.* argued that the effects of reducing Hsp90 are

genetic rather than epigenetic in nature [7]. However, as with their *Drosophila* experiments, they did not specifically rule out an additional epigenetic contribution to the effects that they observed [7].

6. Evidence that Hsp90 functions as a capacitor for morphological evolution in an epigenetic manner

We reported evidence that Hsp90 affects development by altering the chromatin [8]. Our intent was to determine whether Hsp90 could function as a capacitor for morphological development by an epigenetic mechanism in a sensitized system. In 1957, Bateman attempted to perform a canalization experiment with the *crossveinless* phenotype in an isogenized strain [31]. However, this experiment failed, possibly because she did not use, as we did, a sensitized strain [31]. The reason we wanted to test this "chromatin hypothesis" is because we had isolated mutations in both Hsp90 and several Trithorax Group (TrxG) genes as maternal enhancers of the $Kr^{lf\text{-}1}$ "developmentally sensitized" eye phenotype [8]. The most efficient of the maternal enhancers of the $Kr^{lf\text{-}1}$ phenotype was a mutation in the TrxG gene *verthandi* (*vtd*), which showed over 90% expression of an eye-bristle phenotype in the male progeny, and an overall 50% expression of the phenotype [8]. The other enhancers only had 5-15% expression of the enhanced phenotype in both male and female progeny.

Surprisingly, we found that the enhanced $Kr^{lf\text{-}1}$ phenotype was transmitted in several subsequent generations, even in the absence of the initiator vtd^{3} mutation [8]. Also, the penetrance of the phenotype increased in a selection experiment in the

absence of the *vtd*^{3} mutation [8]. While we acknowledged that a genetic mechanism could explain this result, the fact that TrxG proteins affect chromatin structure, generally in manners that promote transcription, suggested the likelihood of an epigenetic mechanism [8].

The way that we sought to test the epigenetic capacitor hypothesis was by removing, as much as possible, all sources of genetic variation in a sensitized *Drosophila* strain, *iso-Kr*$^{lf\text{-}1}$ [8, 32]. We enhanced the *iso-Kr*$^{lf\text{-}1}$ phenotype by feeding these flies the potent and specific Hsp90 inhibitor geldanamycin [8]. The heart of Waddington's canalization hypothesis is that genetic variation must be present for selection of a novel phenotype after an environmental stress. Nevertheless, we still observed an enhanced *Kr*$^{lf\text{-}1}$ phenotype, whose penetrance increased in a 13-generation selection experiment [8]. Further evidence that what we were observing was an epigenetic phenomenon was that the enhanced *Kr*$^{lf\text{-}1}$ phenotype was unstable, even after 13 generations of selection, and that the penetrance was never greater than 70% [8]. The instability of the enhanced *Kr*$^{lf\text{-}1}$ phenotype is further illustrated by the fact that only two generations of selection against the phenotype were sufficient to restore it to background levels [8].

We believe that such a negative selection experiment can be a useful litmus test for epigenetic phenomena because of the unstable nature of epigenetic alterations in phenotypes compared with genetically assimilated phenotypes. The stability of genetically assimilated phenotypes is demonstrated in Hirsch's selection of the geotaxis phenotype in *Drosophila* published in 1959 [33]. The high and the low geotaxis strains were selected for over 20 generations, and then propagated for over 40 years without

further selection for these phenotypes. Remarkably, the phenotypes were as stable as they were 40 years earlier [34], thus supporting a genetic assimilation mechanism, rather than a transient epigenetic assimilation mechanism.

In studies to date with *iso- Kr*$^{lf\text{-}1}$, we did not rule out the existence or importance of cryptic genes, but instead we showed that the existence of cryptic genes is probably not necessary to explain at least some of our observations. We believe that the existence of cryptic genes is also probably not necessary to explain some of the results of Waddington [4, 5], Rutherford and Lindquist [3] and Queitsch *et al.* [7]. For example, Queitsch et al. showed that some of the Hsp90-inhibitor induced phenotypes in *Arabidopsis* were due to genetic variation, but others could not be propagated in isogenic lines [7]. Because of their instability, it is possible that epigenetic effects caused some of the non-propagatable phenotypes.

While it is true that other laboratories have shown that epigenetic states can be inherited, that the frequency of an epigenetic state within a population can be selected to increase by breeding individuals with these states, and that the propensity for individuals to adopt certain epigenetic states is influenced by that organism's genotype and environment [15], we believe that our contribution to evolutionary theory is that Hsp90 can function as a capacitor for morphological development in an epigenetic manner. Rutherford and Lindquist [3] and Queitsch *et al.* [7] did not address the possibility that Hsp90 might function in an epigenetic manner. According to Massimo Pigliucci, a prominent evolutionary theorist, "This [Sollars *et al.*] is one of the most convincing pieces of evidence that epigenetic variation is far from being a curious nuisance to evolutionary biologists, but may play a fundamental role in adaptation to

rapidly changing environmental conditions, side by side with standard genetic variation" [15].

7. Models for the epigenetic function of Hsp90

Sangster *et al.* recently proposed a speculative model for how vtd^{3} enhances the $Kr^{lf\text{-}1}$ phenotype [17]. These authors propose, since *vtd* maps to a region near the centromere of chromosome 3, a region with few unique DNA sequences, that *vtd* is not a gene that encodes a protein, but rather a chromatin regulatory locus [17]. One of the few protein-encoding genes in the region containing *vtd* is the *alpha-catenin* gene, a key component of the Wingless-signaling pathway [17]. They hypothesize that the vtd^{3} mutation causes a "spread in the nearby heterochromatin" to the *alpha-catenin* gene, and that this inactivation is heritable, "if one invokes that heterochromatic spread due to loss of one *vtd* element may be transmitted to an intact homolog by a *trans*-silencing mechanism" [17]. This model is consistent with our observation that ectopic Wg signaling was one of the epigenetic causes of the enhanced $Kr^{lf\text{-}1}$ phenotype ([8], and additional data not shown).

However, we feel that the model of Sangster *et al.* [17] is highly speculative. For instance, attempts to identify the *vtd* gene in the sequenced *Drosophila* genome might have failed because *vtd* could have small exons interspersed over a large region of heterochromatin. There are examples of genes on the Y-chromosome that resist annotation attempts because they are very large, and have small exons interspersed in the heterochromatic regions [35]. Also, while reduction of *alpha-catenin* expression via a heterochromatic mechanism would be expected to increase the efficacy of Wg

signaling, it would probably not increase the amount of Wg protein itself. However, we observe that maternal reduction of *vtd*^{3} increases Wg expression because a *wg-lacZ* reporter is expressed in the peripodial membrane of the wing imaginal disc coincidently with the enhanced *Kr*$^{lf\text{-}1}$ phenotype [8]. Furthermore, Wg expression is not in a positive-feedback loop in any of the known Wg signaling pathways [36].

We prefer a model in which Wg chromatin itself, or the chromatin of a gene that encodes an activator of Wg expression, such as Hedgehog (Hh), is activated by the *vtd*^{3} mutation. Of possible relevance is the finding from Schubiger's laboratory that stress, caused by cutting off pieces of wing imaginal discs, activates Hh expression in the peripodial membrane of 2nd instar larval discs [37, 38]. Ectopic Hh expression could be causing the ectopic Wg expression that we observe in the peripodial membranes of *vtd*^{3}-mutant 3rd instar larval eye discs [8]. It is possible that the different maternal enhancers of *Kr*^{lf}, such as *vtd* and *Hsp90* mutations, could be functioning through independent chromatin-regulatory mechanisms.

In Figure 1, we present three, non-mutually exclusive, models for the epigenetic function of Hsp90. These models are similar to those proposed by Bateman to explain the assimilation of genetically canalized phenotypes – (1) a shift in the mean, (2) a shift in the threshold, and (3) an increase in the variance of the phenotype [27]. In a *Nature News and Views* article discussing our paper, Rutherford and Henikoff support Model 1, in which the mean for the enhanced *Kr*$^{lf\text{-}1}$ phenotype is shifted by a reduction in Hsp90 [16]. Bateman preferred Model 2, a shift in the threshold, for her genetic assimilation experiments [27]. We are leaning towards Model 3, an increase in the variance of the phenotype, because recent results with lead-acetate fed flies from our laboratory [39],

and our collaborators' laboratories [40, 41], support this view. These results are discussed in more detail in a later section.

8. Lamarckian evolution revisited

Whereas the papers on Hsp90 by Rutherford and Lindquist [3] and Queitsch et al. [7] reaffirm Gilbert's contention, quoted above, that there is no evidence to support the idea that they were observing "inheritance of acquired characterstics" [3], we believe that our paper does support aspects of Lamarckian evolution [8]. Whereas neither the *crossveinless* phenotype observed by Waddington [1], nor the ectopic eye bristles that we observe in $Kr^{lf\text{-}1}$ flies are adaptive responses to stress, we believe it is likely that some Hsp90-induced chromatin alterations are an adaptive response to stress. For example, chromatin alterations of the heat shock genes could, at least partly, explain the acute tolerance to stress observed during repeated heat shocks [42, 43].

While stress probably does not cause mutations in our epigenetic system, it evidently causes heritable chromatin alterations [8]. We cannot rule out the possibility that geldanamycin-induced stress is causing new mutations in our *iso-* $Kr^{lf\text{-}1}$ strain, as stress reportedly causes "adaptive increases in mutation rates" in mutator strains of bacteria [44]. Below, we present a hypothetical Lamarckian-type example for our epigenetic model for evolution. However, without further evidence for the generality of epigenetic effects on evolution, one may take such models, as suggested by Pigliucci, with "a grain of salt" [15].

A giraffe that cannot reach the upper leaves of a tree would undergo starvation stress, and this type of stress would cause a decrease in Hsp90, and all other proteins

for that matter, because of a limited amino acid pool [45]. A reduction in Hsp90 levels would likely lead to low-Hsp90-induced chromatin changes, as we observe in *Drosophila* [8]. The chromatin changes could potentially cause a range of morphological phenotypes in the progeny giraffe, some with long necks, and some with short necks, and some with any number of other morphological alterations. The long-necked progeny would be the ones who survive to reproductive age, since they could presumably reach the tops of the trees, and a combination of epigenetic [8] and genetic [3] selection could occur to canalize the new phenotype in subsequent generations. While we do not agree with many of the concepts of Lamarckian evolution, we argue that many of them should be revisited. Kenneth Weiss more elegantly stated this is his paper discussing the pros and cons of Mendelian genetics [46]. In this paper, he said, “Like the famous princess, we seem to think that we can always detect a Mendelian pea no matter how many layers of environmental and other influences may lie over it” [46].

9. Other roles for Hsp90 in an organism – sleep deprivation and survival

Sleep is controlled both by a circadian pacemaker system and by a “homeostatic drive” that increases in strength when an organism is awake [47-49]. While an organism sleeps, the homeostatic drive dissipates until it weakens sufficiently and the organism awakes. Since this is a relatively new area of research, little is known about the identities of the unnamed components of the homeostatic drive, *i.e.*, its widgets. The supreme importance of sleep is demonstrated by the fact that a few days of sleeplessness invariably causes death in all animals tested (reviewed in [50]). In

Drosophila, mutations in circadian oscillator genes, such as *period* (*per*), *timeless* (*tim*), *clock* (*clk*), and *cycle* (*cyc*), cause death after only 10 hours of sleep deprivation [48]. Unlike the other circadian mutations, mutations in *cyc* showed a reduction in expression of stress-response genes, such as Hsp90. However, activating heat shock genes before sleep deprivation rescued the *cyc*-mutation lethality induced by sleep deprivation [48].

Interestingly, mutations in Hsp90 also cause an exaggerated homeostatic response to sleep deprivation. Like flies with circadian mutations, flies with Hsp90 mutations died after only 10 hours of sleep deprivation [48]. Evidently, as with the "evolutionary capacitor" [3], Hsp90 is also a widget for the "homeostatic drive" for sleep. While the mechanism of Hsp90 protection against sleep deprivation has not yet been determined, it would be worthwhile determining if this is influenced by both genetic and epigenetic mechanisms.

10. Evolutionary implications of the capacitor function of Hsp90

One criticism of Hsp90 as an "evolutionary capacitor" is that all of the phenotypes are severely deleterious, and it is hard to imagine how individuals that have had these genetic or epigenetic variants revealed would have any advantage over buffered individuals [3, 7, 8]. For example, Meiklejohn and Hartl state in their review of canalization, "Over evolutionary time, the frequency with which a phenotypically revealed allele provides a selective advantage greater than the negative consequences of removing environmental canalization is likely to be extremely small" [2]. Waddington

made a persuasive response to this criticism in his 1953 paper on the assimilation of the *crossveinless* phenotype [4]. Waddington said, “There is, of course, no reason to believe that the phenocopy (the *crossveinless* phenotype) would in nature have any adaptive value, but the point at issue is whether it would be eventually genetically assimilated if it were favored by selection, as it can be under experimental conditions” [4]. Similarly, there is little reason to believe that the ectopic outgrowths in the eyes of $Kr^{lf\text{-}1}$ flies that we observe would in nature have any adaptive value, but the point of the experiments was to determine, as a proof of concept, whether they could be epigenetically assimilated if it were favored by selection. Another argument in favor of evolutionary capacitors was made by Gilbert, who said, “The developmental genetics approach to evolution concerns more the *arrival* of the fittest than the *survival* of the fittest” [6].

One could imagine a situation where the ectopic outgrowths have an adaptive value, as is apparently the case with stalk-eyed female flies preferring males having long eye stalks as a form of mate selection [51], or with other *Drosophilidae* with unusually shaped head capsules and eyes [52]. However, Darwin himself treats sexual selection as a special case, and oftentimes an exception to his “survival of the fittest” model [20].

Rutherford and Lindquist proposed that the capacitor function of Hsp90 has been selected during evolution [3]. However, it would seem impossible for a group-level trait such as a morphological capacitor to be selected since it would only benefit distant generations and not the immediate generation. Again, to quote Meikeljohn and Hartl, “However, if it is beneficial to constrain the phenotype against mild environmental

perturbations, it must be still more beneficial to buffer against more extreme environmental perturbations" [2].

Indeed, the capacitor activity of Hsp90 is likely a perfect example of a "spandrel," a term that Stephen J. Gould (1941-2002) borrowed from architecture to designate "the class of forms and spaces that arise as necessary byproducts of another decision in design, and not as adaptations for direct utility in themselves" [53]. The capacitor function of Hsp90, such as it is, is likely a spandrel of its everyday function as a chaperone for developmentally important signaling molecules.

However, calling the capacitor function of Hsp90 a "spandrel" should not diminish the importance of this activity, as the Meiklejohn and Hartl imply [2]. Gould points out the evolutionary importance of spandrels by saying, "These sequelae – spandrels in the terminology of this paper – arise nonadaptively as architectural byproducts but may regulate, and even dominate, the later history of a lineage as a result of their capacity for co-optation to subsequent (*and evolutionary crucial*) utility" [54] [emphasis added]. What better gene to be co-opted for an evolutionary crucial utility than a chaperone for numerous signaling pathways, such as Hsp90? Co-optation of a regulator of regulators such as Hsp90 allows the generation novel structures, such as eye appendages, in a single generation – a feat that cannot be accomplished by the co-optation of any individual signaling molecule.

11. Is the intron in the Hsp90 gene a governor for its activity?

Why isn't it the activity of Hsp90 more inducible than it is? Perhaps the "purpose" of the evolutionary conserved intron in Hsp90 is to act as a governor so that its activity does not get too high during stress. Indeed, saying that a gene's buffering properties are reduced during times of environmental stress seems to make little sense for a gene whose expression is upregulated under stressful conditions, such as Hsp90. Similarly, Meiklejohn and Hartl's view on this issue is, "The inability of Hsp90 to buffer against a wider range of environmental conditions than it does is therefore more likely to be a coincidental feature of its mechanism of action than an adaptive trait" [2].

The presence of an intron in the 5' untranslated region of almost all Hsp90 genes from *Drosophila* to humans (with an interesting exception, see below) suggests that the introns are there for a reason – their presence would likely reduce the maximal expression of Hsp90 during stress than if there were no intron. Lindquist's laboratory had shown over a decade ago that mRNA splicing is disrupted during heat shock [55, 56]. Recent studies have shown that heat shock induces partial disassembly of certain snRNPs that participate in pre-mRNA splicing [57, 58], and Hsp-mediated reassembly [57, 58], restores normal pre-mRNA splicing [55-59].

We speculate that organisms that lose the intron in Hsp90 might benefit in the short term by having increased Hsp90 function, but in the long term they have a reduced ability to undergo further morphological evolution because the capacitor function of Hsp90 is likely eliminated by the removal of the intron "governor." To our knowledge, and after an extensive GenBank search, the only organisms so far identified that apparently have lost the intron in Hsp90 are the *Lepidoptera* (butterflies) [60]. Perhaps the capacitor activity of Hsp90 has also been lost in butterflies – Hsp90 activity

is probably not reduced by stress because splicing does not occur on Lepidoptera Hsp90 pre-mRNA. Since morphological signaling pathways have been co-opted to make spots in butterfly wings [61], one might predict that loss of Hsp90 activity during stress could lead to unwanted outgrowths where the ectopic expression of the developmental genes occur, as we observe with $Kr^{lf\text{-}1}$ flies with reduced Hsp90 function.

Another possibility for why Hsp90 needs a governor is that the presence of excess Hsp90 might be what is deleterious to some aspects of morphological development. Several lines of evidence suggest that this may be generally true. First, cells rapidly clear the excess heat-induced hsp's following cessation of heat shock [45]. Second, hsp induction is suppressed during developmental stages where protein synthesis is already rate limiting and intensive [45]. Third, evolutionary tradeoffs between thermotolerance and fitness have been noted [45, 62, 63].

12. Other fields that might benefit by invoking the capacitor function of Hsp90

Ecologists study how environmental factors, such as pollutants, affect the development of organisms in the wild. Bateman's Model 3, discussed above, whereby an environmental insult increases the variance of the expression of a phenotype, is supported by many ecological studies. For example, John Graham refers to the amount of variance in a phenotype as a measure of "developmental stability" [64, 65]. Developmental stability, like canalization, refers to the ability of an organism to produce a consistent phenotype in a given environment [64, 65]. Conversely, developmental instability is measured by ecologists as within-individual variance or by deviation from

perfect bilateral symmetry [64, 65]. Another ecology term, “fluctuating asymmetry,” refers to the random variation between right and left parts of a bilaterally symmetrical structure, and is widely used as a measure of developmental stability [64, 65]. It would be interesting to determine whether Hsp90 and other components of “Waddington’s widget” regulate these effects.

Environmental health scientists could also benefit by incorporating models of Hsp90 function in toxicological studies. Recent data from the Lnenicka laboratory, which collaborates with our laboratory and the Hirsch laboratory on the effects of the heavy metal neurotoxin lead acetate on *Drosophila*, suggests that Bateman’s Model 3, an increase in the variance of a phenotype (Fig. 1c), might be correct for some gene-environment interactions [41]. Lnenicka’s laboratory shows that there is usually a significant correlation between the number of synapses on a larval muscle fiber and the size of the fiber [41]. However, when flies are reared in the presence of as little as 2 ppm lead acetate in their food, this correlation is no longer significant [41]. The CDC cutoff point for high blood lead level is 0.1 ppm (15 mg/dl) [41]. Interestingly, in lead-acetate fed *Drosophila* larvae, the mean number of synapses on a particular muscle fiber does not change, but the variance greatly increases. In other words, this data supports Model 3 because, while lead-treated larvae generally have the same number of synapses as unleaded larvae, there are a greater number of abnormal muscle fibers with either fewer or more synapses, in approximately equal numbers [41].

Lead does not always affect a fly’s physiology in the same manner because our laboratory, and our collaborator’s laboratories, have shown that an equivalent amount of lead acetate (2 ppm) in the food of adult females is an aphrodisiac because it decreases

the mean time it takes females to mate with males [39]. We interpret our results as supporting Model 1 (Fig. 1a) because there was a shift in the mean in distribution of times that it took females to respond to males, but no significant increase in the variance [39]. We are currently attempting to determine whether both genetic and epigenetic mechanisms function in the response of *Drosophila* to lead acetate, and whether the effects of lead acetate are mediated through Hsp90, as we suspect.

13. Future Prospects

In a speculative methods paper, we have recently described epigenetic mapping experiments that we are pursuing to follow up our published epigenetics research [8, 32]. We also have recently described how modern multi-generational epigenetic-mapping techniques can be used in the fields of cancer and obesity research [66]. The excitement of the resurgence of the field of epigenetics is summarized by Pigliucci, who said, “Nonetheless, it seems that genetic assimilation and epigenetics, after decades of neglect, are finally back on the center stage of evolutionary research. Perhaps they will remain in the spotlight long enough to be incorporated in mainstream evolutionary theory” [15].

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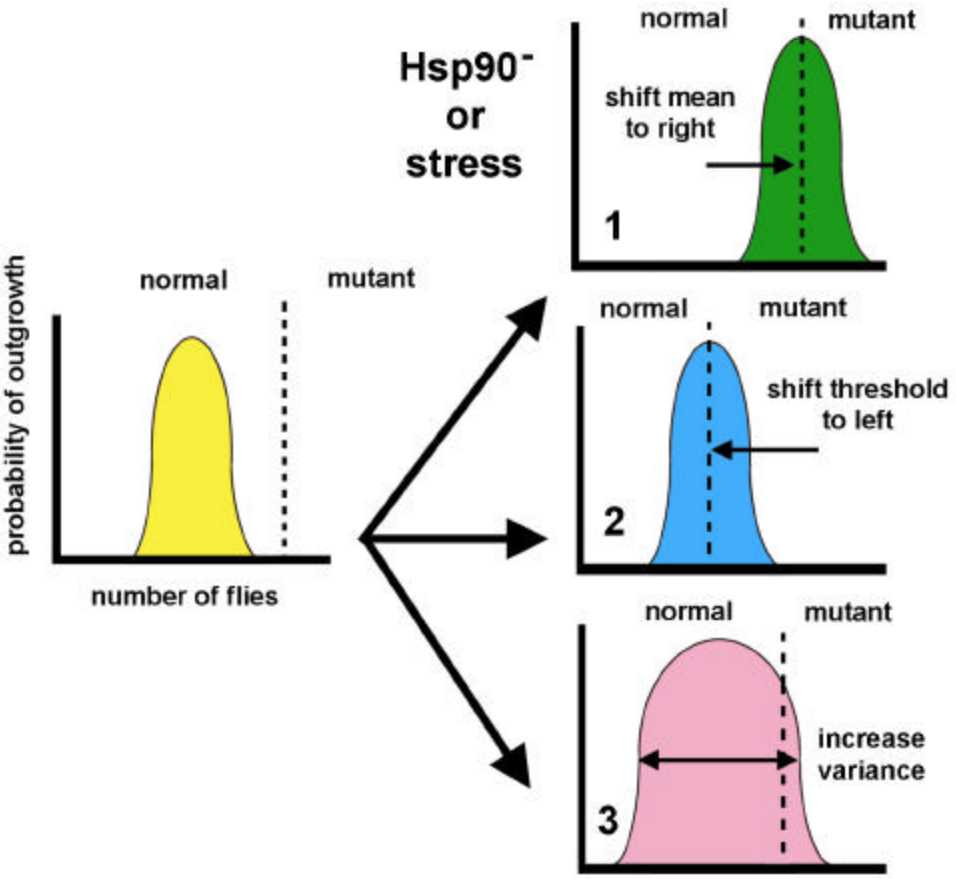
Figure 1. Three models for the epigenetic function of Hsp90. Model 1 is that selection of the epigenetically-induced phenotype shifts the mean of the distribution towards the threshold. Model 2 is that the threshold is shifted towards the original mean. Model 3 is that the variance of the distribution of the phenotype is increased (see text).


Hsp90⁻
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