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International Journal of Bioinformatics & Biological Systems (IJBBS)

A Systems View of Waddington's Genetic Assimilation

Research Article

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Abstract

Lamarck believed that traits acquired during an organism's lifetime could be passed onto the next generation. Although the idea of the inheritance of acquired characters was discarded due to lack of experimental evidence, Conrad H. Waddington realized its significance. In 1953, he showed that *Drosophila melanogaster* (wild-type) flies that were heat-shocked produced a Crossveinless (cve; disrupted posterior crossveins) trait. Through repeated selection of this trait with heat-shock, he not only increased its frequency in the population, but also found that individuals, from the untreated stock, showed the phenotype. This apparent inheritance of an acquired character is important to evolutionary theory, because it provides a mechanism whereby the environment may influence future evolutionary change. Despite the long history of this experiment, genetic assimilation remains elusive. The main aim of this work was to examine genetic assimilation and understand it as an evolutionary theory. Revisiting the experiment indicated that there is much that remains unclear. We have shown that production of cve is strain specific, with the white-eyed lines being vulnerable and the wild-type not. Though the frequency of the cve allele increased in every generation, there was a fitness cost for acquiring crossveinless. Assimilation of cve was found to be heritable but, unlike Waddington's classic work, it did not tend towards fixation; appearing more like a transient, low penetrance effect.

Keywords: Crossveinless, Genetic Assimilation, heat-shock, *Drosophila.*

Introduction

Are environmentally induced or acquired traits passed on to future generations? Does inheritance of acquired traits play any role in shaping the evolution of organisms? Waddington's work on genetic assimilation [1] provided one of the first experimental proofs for the theory of acquired inheritance. Since then, many have tried to explore the possible implications genetic assimilation might have as an alternate evolutionary theory.

Canalization, a term first coined by Waddington himself, refers to development buffering machinery by which variability is minimized and an invariant phenotype produced in every generation. Such ability ensures that phenotypic deviants do not cross a certain threshold barrier, and become expressed (Figure 1). This barrier is high under normal environmental conditions. During a sudden change in the environment, the functioning of this barrier can become impaired. This impairment may allow the expression of all those otherwise curbed, hidden accumulated variations to be expressed in the population. Forceful artificial selection (as in the case of Waddington's experiment and ours) in the presence of the triggering stress increases the frequency of that particular polymorphism in subsequent generations. Remarkably, within a few generations the population reaches a point where it is no longer dependent on the stress, and freely expresses the cryptic trait. In other words, the trait is fixed and incorporated into the genetic makeup of the organism. This is what Waddington referred to as Genetic Assimilation [2-4].

The Waddington Experiment

For the experiment, Waddington selected pupae of the Edinburgh strain- S/W5, because they had a higher tendency to show posterior crossvein defects. Waddington found that the pupae of *Drosophila melanogaster* Edinburgh strain-S/W5, when heat shocked (17-23 hour old pre-pupae; at 40°C for 4 hours) produced flies

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Received: January 11, 2016
Accepted: March 30, 2016
Published: April 13, 2016

Citation: Nair A, Dearden PK (2016) A Systems View of Waddington's Genetic Assimilation. *Int J Bioinform Biol Syst.* 1(1), 10-17.

that failed to make proper cross-veins (the crossveinless trait; Figure 3). The premise of the experiment involved the generation of two selection lines; an "upward selection line", including flies with disturbances in their posterior crossveins, and a "downward selection line", with normal crossveins. By selecting flies with crossvein defects, Waddington showed an increase in the frequency of crossveinless individuals. The exact opposite was seen in the downward selection line. During the experiment, the virginity of the female flies was not ensured. As the selection lines kept on progressing, by generation 16, it was discovered that approximately 1-2 % of flies from untreated pupae, in the upward selection line, showed the posterior crossveins defect without the stimulating heat-shock. At this point, according to Waddington, the particular phenocopy had become incorporated into the genotype.

Motivation and Hypothesis: why repeat a 60-year-old work?

While the Waddington experiment is a key experiment in Evolutionary Biology, the mechanism by which it works is, as yet, unknown. Recently it has been postulated that heat-shock protein 90 (HSP90) may provide a mechanism (Queitsch, Sangster et al. 2002) as it seems to be able to maintain the activity of proteins even when they are mutant, thus providing a store of variation ready to appear when conditions become stressful and HSP90 function is titrated away. While this is an attractive theory, it is yet to be proven that HSP90 is responsible for Genetic Assimilation. In the present study, treating Drosophila prepupae (both wild strain and the white eyed mutant) with Geldanamycin (which is a potent inhibitor of HSP90) did not generate crossveinless flies (see S2 Table A.26 for specifics).

By experimenting with a simple phenocopy, such as the "crossveinless" (cve; Drosophila flies with a disrupted posterior crossvein in their wings), Waddington concluded that repeated selection of the trait, under heat-shocking conditions, the population reaches a point where individuals no longer need the initial stimulating factor (heat-shock) to express the unique phenocopy (Waddington C.H., 1953). Although this was ground breaking work, no further work has been done to understand the implications Genetic assimilation and the selection experiments might have on the population;specifically, with regards to the fitness costs associated with acquiring crossveinless. Thus the Initial aim of the present study was to revisit Waddington's work and have a better understanding of genetic assimilation. But it became apparent to us that it much more than the mere acquisition and fixation of a singular, non-adaptive trait.

Materials and Methods

Fly Stocks

Two strains of Drosophila melanogaster were used in the experiment: the wild-type Canton-S flies from Bloomington Drosophila stock center at Indiana University (BDSC) and the local laboratory white-eyed w^{1118} stock.

Fly incubation

Fly lines were maintained at 25°C in a P Selecta HOTCOLD-C (2101502) incubator. For preparation of fly food, proper maintenance of stocks, and the standard method of periodic mass transfer of adults to fresh food, standard protocols were followed [16]. The number of flies during each transfer was kept between 50 and 100. This made sure that there was adequate food for the adults but more importantly there wasn't any competition among larvae (distinct juvenile form many animals undergo before their metamorphosis into adults) for food.

Waddington experiment setup

The Waddington experiment requires an extremely simple setup. However the two key physical requirements are as follows:

A platform to heat-shock prepupae

As per the general methodology adopted in Waddington's selection experiment (see chapter 3 and 4 for details), the vials containing the prepupae were heat-shocked in a programmable water bath (Contherm Scientific Ltd., 350-380 Series of high Temperature Digital Water Bath) was used for the heat- shock treatment. The water bath was carefully calibrated before setting it to the desired temperature for heat-shock.

A method to score and classify heat-shocked flies

When the flies enclosed, they had to be scored as either those that showed the expected trait (crossveinless) or those that did not (non-crossveinless). However before the flies could be counted, they had to be in a position where they could be easily handled. This was achieved by anesthetizing flies under light CO_2 using a BOC gas, fitted regulator (set to ~5000 kPa (kilo Pascal)), flow meter (adjusted to ~7 l/min) and a Porvair filtration fly pad. Once the flies were anesthetized (usually within 5 seconds of exposure to CO_2), they were physically handled with a pair of sharp tweezers and a fine, soft paintbrush (size 3, flattened tip). Lastly, to observe flies, a cold light stereomicroscope (Leica L2; Leica Microsystems) was used.

Basic tabulation and statistical methods were used to score and classify flies (cve and non-cve). Contents of the table used in every generation included: the total number of pupae collected over several time points (usually 12-15), number of cve males and females scored, number of non-cve males and females scored and the total number of flies that died. The tables A1-22 in the supplemental data (S1) do not show that the sum of numbers of cve, non-cve and dead flies equals the total pupae collected. They merely lists out the following: (i) the total number of pupae collected (over several time-points) for heat shock in a particular generation, (ii) the number of cve and non-cve males/females scored following eclosion, (iii) and the number of pupae that failed to enclose. Apart from this, in every generation there were many flies that expressed alternate phenotypes, different from cve. This is because heatshock used in the experiment despite having specific parameters, is still a generalized stress as opposed to a directed mutation. Hence, it is quite likely that other phenotypic deviants would be produced in every generation.

To calculate the percentage of cve in a particular generation, the total number of cve scored was divided by the sum of the total number of cve and the total number of non- cve i.e. %cve= ncve/(ncve+nnon-cve), where n is the total number.

Results

Repetition of the Waddington experiment

The wild *Drosophila* Canton-S flies (Wt), when heat-shocked, never gave disturbed posterior crossveins while the white-eyed mutant (w^{1118}) strain did, allowing the trait to be selected for in the following generations. The heat- shock conditions used in the original work [1] proved to be lethal on both strains, and thus parameters were altered to induce the crossveinless trait. Initially, wild strains of Drosophila were used in the experiment. Around 1380 pupae were collected for heat-shock from the parent generation.Following eclosion, flies were scored for the presence of the crossveinless trait. Surprisingly, though the conditions of the original experiment were replicated, not a single crossveinless fly was recovered. Following rigorous optimization, which involved altering the heat-shocking parameters like, the duration of the heat- shock, the temperature of heat-shock and the age of the pupae before the heat-shock, the ideal heat-shocking conditions were chosen: heat-shocked after 24 hours of incubation (at 25°C) at 40.5°C for 45min (SI2). The upward selection line (Supplementary Information, S1; Figure 3), as expected, showed an increase in the occurrence of the crossveinless phenotype. In the initial generations, this increase in the number of cve individuals is irregular. Fifteen % of flies obtained by heat-shocking the first batch of pupae, showed the crossveinless response. From there, the upward selection line showed an increase in the percentage of cve, responding strongly to the heat-shock treatment. By generation F8 the percentage of crossveinless rose to 94%; in other words, almost all of the flies in that generation had posterior crossvein defects. However, further heat-shocking and selection did not produce an increase in the frequency of the trait as it did in the earlier generations; instead the percentage stabilized around 94%. As expected the Downward Selection Line (Figure 3) displayed a constant decrease in the proportion of crossveinless flies; the decline was however not linear.

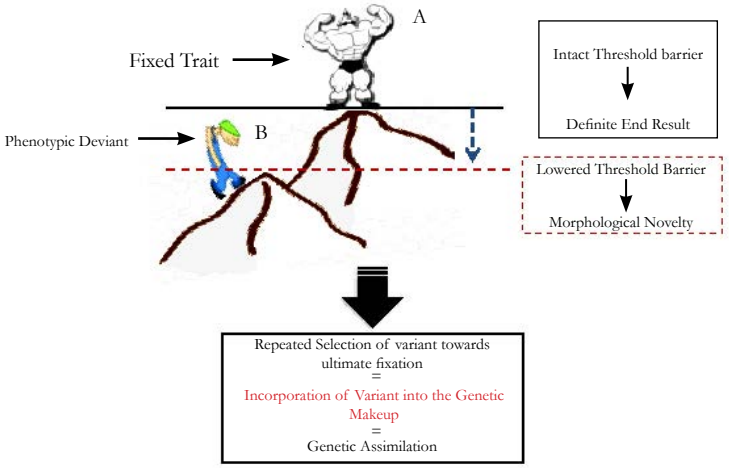
True breeding crossveinless lines

Generation F4 in the heat-shocked upward selection line started producing cve flies in the untreated population, though in small amounts. Nonetheless, it showed that the character had been assimilated by this generation. Although one could have started scoring and selecting these true breeding flies from F4, it seemed prudent to carry out the collections when the numbers improved (i.e. by 2% in F6; see Figure 4). Ten generations of true breeding flies were raised. But unlike, the original work, where after 7 or 8 generations of true breeding assimilated flies, Waddington almost reached fixation (~99% cve in most of the lines), percent cve in genetically assimilated lines, steadied around 20 and 40%; withF10 dipping further to a staggering 10% (Figure 3). The results above suggest that genetic assimilation might be a short-lived phenomenon. However, the efficient inheritance of crossveinless suggests that a plausible epigenetic transgenerational effect might be underlying genetic as similation.

A genome-wide polygenic system with no fixed number of combinations to produce crossveinlessness

There are multiple genes that influence the fly's ability to make posterior crossveins and these are distributed over the main chromosomes of Drosophila melanogaster (Milkman R.D., 1960b). Having said that, it would be an exaggeration to say that there are dedicated genes for this response. It is true that there are specific experimental parameters along with critical developmental time frames within which crossveinless is produced, but the conditions are certainly not exclusive just for this response (cve). The fairly consistent appearance of alternate phenotypes in the heat-shocked cve lines shows the pleiotropic nature of this response (figure 5.1). Earlier works (Milkman R.D., 1959) have reported the links between defective crossveins and other phenotypes. Many single mutant genes are believed to interfere with posterior crossvein formation. So the specific heat-shocking conditions under which crossveinless is normally produced, four fly types may be

Figure 1. Canalization and the assimilation of stress induced phenotypic variants.



Cartoon A is analogous to a trait fixed over generations and cartoon B is equivalent to a phenotypic deviant. Canalization in the form of a threshold barrier (shown as horizontal bars) buffers the fixed trait from all phenotypic variations that arise spontaneously, during the development of an organism. During stress, this barrier is compromised and lowered, allowing phenotypic deviants to manifest. According to Waddington, repeated selection of such variants under stress could ultimately fix some of these variants in the genetic make up of the organism; in other words become genetically assimilated.

Figure 2. Gradation in the expression of crossveinless

(left) Posterior crossveins following appropriate heat-shocking are either disturbed with three-fourths missing (left), almost absent in the wings (center). Non-cve fly with fully formed crossveins (right).

Figure 3. Line Graph Line graph presenting an overall increase and decrease in the percentage of crossveinless over ten generations in the Upward and the Downward Selection Line. The black dots represents the upward selection line and the grey dots indicates the percent of cve in the downward selection line.

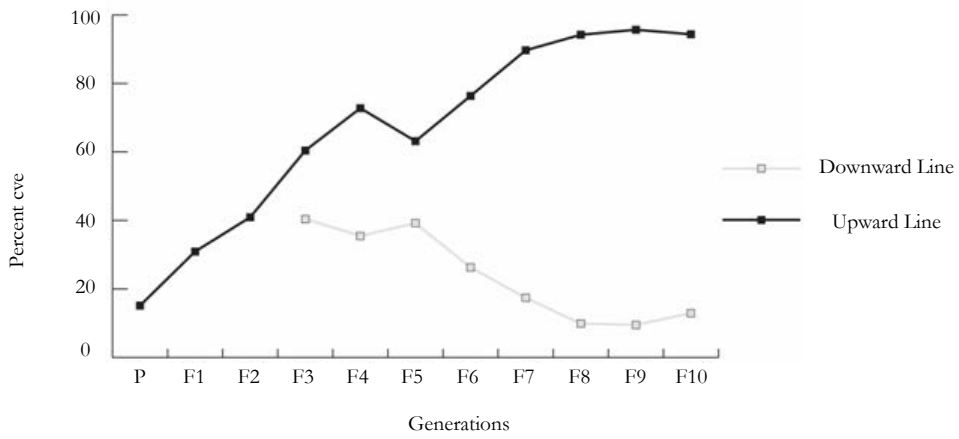
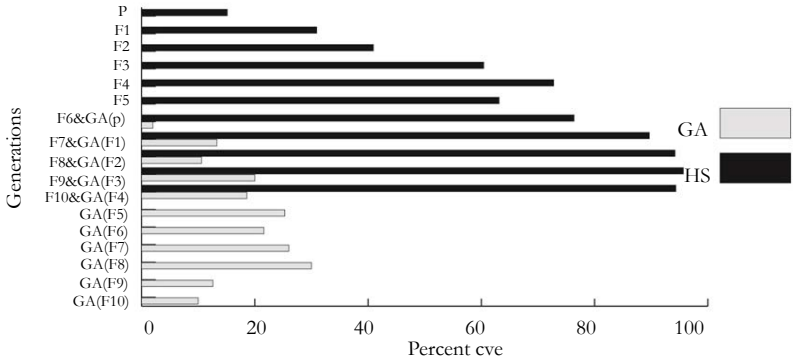


Figure 4. Bar graph explaining the generation and selection of genetically assimilated (GA) cve lines (10 generations) from the heat-shocked-upward (HS) selection line.



seen in the progeny: a fly which is strictly crossveinless; a non-crossveinless (normal) fly; a crossveinless fly with alternate phenotypes; and a non- crossveinless fly with alternate phenotypes (S2; Table A24).

Discussion

Crossveinless is perhaps built on an unstable epigenetic-landscape

The concept of robustness is straightforward and is a system's ability to maintain function or performance in the face of internal and external perturbations [5-7]. Homeostasis is a state where an organism (living system) steadily maintains all of its physiological processes. In so doing it achieves stability or equilibrium with its environment. Initially it appears that homeostasis and robustness are the same. Upon closer inspection it is clear that homeostasis deals with the state of the system, and robustness maintains the system's function. Robustness may help a system carry out its functions, even when it transits from one steady state to the next most optimal steady state or an unstable state (see Figure 5 below). For example, the HIV-1 virus, despite having an inherent high mutation rate, continues to resist therapeutic interventions

[8]. So it appears that homeostasis and stability are subclasses within a more general class i.e. robustness. To understand the line between robustness and stability, and the concept of fragility, an analogy can be drawn. For example, the manners in which lattices are packed to give a stable crystal structure. A lattice is an array of points, spheres or crystals in a regular configuration throughout a given space [9]. The arrangement of points is such that it gives rise to the most stable lattice. So, as shown in the figure, if we start with an array of spheres (the blue layer), the best way to arrange them is by packing them as closely as possible so that no more spheres will fit into the given area. A second layer of spheres is placed on top of the first, so they nestle into either the left or right pointing holes of the layer below. Placing the second layer of spheres in any of the holes would produce two stable lattices with the only difference being in their overall arrangement.

Assuming that the lattice created by filling up the left pointing holes represents the initial robust-steady state 1, a known perturbation (which the system's robustness is prepared for) may only affect the system slightly so that it might drive the steady state 1 towards an alternate but yet stable robust state 2. State 2, in this case, would be a lattice that has been created by filling up the right pointing holes (Figure 5.A). However, disturbances, for which the system is least equipped, would create a fragile state (Figure 5.B). A fragile state would be one where a few basal layer (blue layer) spheres are missing, and addition of secondary or tertiary layers would further weaken the stability of the structure. Thus, during unexpected perturbations, fragility of the system increases in successive generations. Although the machinery by which robustness sustains a system's function is quite sturdy, there are biological trade-offs that link robustness to components like fragility, resource demands and system performance. A system is only designed to be robust against known and predictable disturbances. This allows opportunities for conditions such as phenotypic plasticity and expression of cryptic traits to manifest. So, a system that is corrected for specific perturbations will be fragile against uncertain ones. Any further attempts to enhance robustness against these sudden disturbances would not only lead to a proportional increase in fragility, but also increase resource demands. This would dramatically degrade system performance. An analogy would be to imagine a case where having a full system backup would increase robustness against data loss during a component failure, but it will demand more resource and will eventually lower the net performance of the system [10, 11]. In other words, by compromising a system's performance, and by adding more resource, a simultaneous increase in robustness and decrease in fragility can be achieved. Conversely, one may maximize performance by giving up on robustness against various perturbations.

Figure 5. An Analogy That Illustrates How Stability, Homeostasis and Robustness Function in a Biological System.

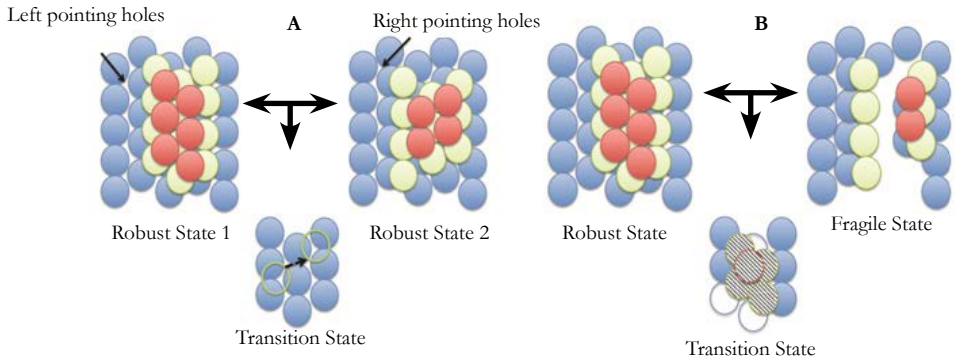


Figure 6. Effect of Heat-Shock on Fly Viability. A Scatter Plot Showing Drastic Decline in The Number of Pupae Collected Over Ten Generations.

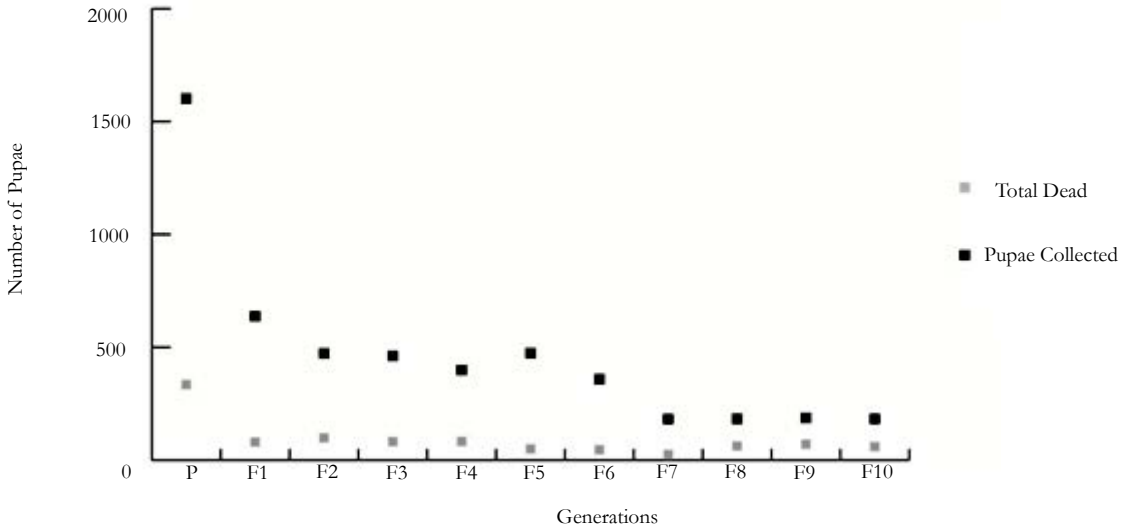


Figure 7. Interaction between gene and environment, and its effects on the fate of a phenotype in an epigenetic landscape. Adapted from Waddington's epigenetic landscape [2].

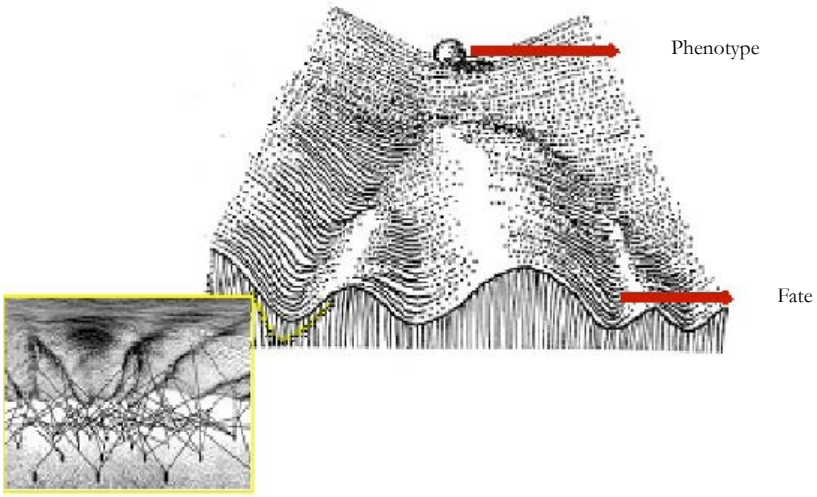
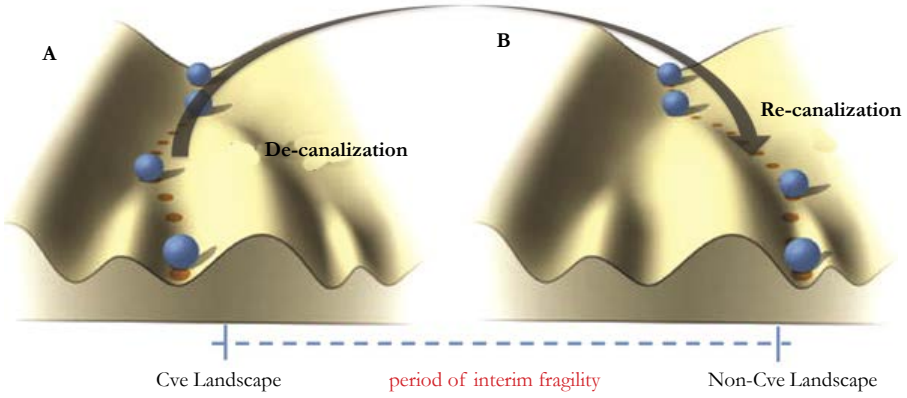


Figure 8. cve (A) versus non-cve (B) epigenetic landscape. Adapted from Waddington's epigenetic landscape [2-4].



This, however, increases the fragility in the system. Therefore, it is hard to find a formalism to provide a single, clear-cut definition for robustness and its biological trade-offs.

Nevertheless, understanding robustness allows us to hypothesize as to what may be happening in the classic genetic assimilation experiment. Initially, in Waddington's selection experiment, the system (referring to the non-heat- shocked flies in this case; Figure 1) is not prepared for the unexpected heat-shock at a critical developmental time. The system fails to maintain its function, leading to the appearance of traits like crossveinless. The shock enables these otherwise contained phenotypes to overcome the impaired threshold barrier (Figure 1). To tackle the stressed state, the system tries to improve the overall robustness. All this does is cause a simultaneous increase in the fragility towards the heat-shock. This might explain the increase in the percent of cve in successive generations - the additive nature of crossveinless. As long as the stress is present, there is no way that this demand for additional resource could be met, eventually worsening the system's performance. This could explain the deleterious effects seen in cve flies, such as compromised viability (Figure 6). To maintain interim homeostasis (in this case it would be to retain energy for vital processes), the system may give up robustness against perturbations. But the status-quo might change once the flies assimilate crossveinless and are unable to make crossveins even in the absence of the triggering heat-shock.

Fixation of a trait, in a population, is a threshold phenomenon. The biological system contains a buffering mechanism that ensures that some traits are expressed in the population and some are not. The reason behind such discrimination is to eliminate variability and maintain consistency as much as possible in future generations. The mechanism that does this was first termed by Waddington as Canalization (Figure 1). Until typical conditions are altered (by induced stress), canalization sets a threshold barrier high enough to preclude phenotypic deviants from occurring in the population. A trait is stabilized by fixation on the grounds that it will be either adaptive or will be at least maintained without compromising a population's fitness. In the case of the crossveinless trait produced by the heat-shock, it is not only non- adaptive and insignificant, but also it impairs the population's fitness considerably.

Waddington's Epigenetic Landscape

Waddington was the first to physically illustrate the relationship between gene, environment and the resultant phenotype. He called this the Epigenetic- Landscape. According to this concept, a trait is like a ball that rolls down a landscape with ridges and

valleys (see figure 7 below). The depth of the ridge alters the path taken by the ball, and thereby determines the ultimate fate of the phenotype in terms of its manifestation. So, the deeper the groove, the more stable the trait is going to be, and thus more consistent in future generations. Altering an existing landscape can change the fate of a phenotype. According to Waddington, the landscape can be shifted by numerous strings (Figure 7 below) of interacting proteins to represent genetic variation that can be tightened or loosened by stress. The epigenetic-landscape created for crossveinless, as a result of repeated artificial selection, does not seem to be robust enough considering the diversity of the trait in terms of its morphological appearance, its polygenic nature and the extent of damage on the population's survivorship.

The 'magnetizing an iron nail' analogy

In order to generate an explanation for the apparent, brief transmission of crossveinless, the creation of an ordinary nail-magnet can be used as an example. Repetitively rubbing a magnet along an iron nail aligns some of the magnetic domains in the nail in a common direction. This will have an effect of making the nail into a magnet. The nail will not be a strong magnet but it will come away with some magnetic properties. This particular analogy is similar to Waddington's Genetic Assimilation experiment [1]. In the classic experiment, pupae were repeatedly heats-hocked, in every generation, to produce flies (*Drosophila*) with the crossveinless response. After a few generations (F14) of selection (in the upward selection line), some of the untreated flies started to show the response. It is at this point, as claimed by Waddington that the acquired trait gets incorporated into the genetic makeup of the organism, and would be transmitted independently into future generations. This is Genetic Assimilation. Just like the nail-magnet, constant selection with heat-shock creates a temporary crossveinless- landscape in the so-called assimilated lines. It is assumed to be temporary because the parameter on which this landscape is created does not seem to be stable enough. It is hard to imagine the stabilization of a trait that is morphologically inconsistent, offers no benefit to the fly whatsoever, and also weakens the fitness, at different stages, of the organism's development. Therefore, as the assimilated lines move through a generations without being heat-shocked, genetic buffers via canalization would try to stabilize the most appropriate phenotype; one that is both invariant and beneficial to the population. Thus, following a couple of generations with an absence of any trigger from the typical heat-shock, genetically assimilated flies would perhaps undergo a restructuring in their epigenetic landscape. They would de-canalize the cve landscape followed by the re-canalization of the normal non-cve landscape (see Figure 8 below). This might explain the transient transmission of the true breeding crossveinless and an incomplete fixation of cve in the population (as shown in Figure 4).

This transition makes sense in many ways. Firstly, because there is no more pressure from the stressful heat-shock, so there is no extra demand for additional resources. This would in turn make sure that there is nothing affects the system's performance. Secondly, there are no fragile perturbations (crossveinless response via heat-shock) that would leave the system unprepared. Keeping the aforesaid factors in mind, it seems logical to assume that the system's robustness should consequently stop any further continuance of this trait (crossveinless). De-canalization would push crossveinless out of the homeostasis or equilibrium, and then there might be an interim state of fragility or vulnerability towards disturbances. To counter this period of disorderliness new thresholds, via re-canalization, are set up. This time, since there is no compromise on the system performance via external stress, reactivation of system's robustness fixes the old, but optimum trait (i.e. non-cve or proper crossveins) in the future generations. This might explain the transient transmission of the crossveinless response followed by a reversion to the normal state.

Assimilation doesn't necessarily mean fixation

Explanations gave possible reasons as to why the assimilation of cve might be a transient effect, but one might argue why the trait still continued to express itself in the population. The answer to this might come from the fact that crossveinless is an additive trait [12-14]. Selecting cve individuals in successive generations ensured the maintenance of cve alleles in the population. In other words more and more crossveinless flies in following generations. Therefore, selecting true breeding cve flies in every generation ensured the maintenance of crossveinless, regardless of a low frequency. However, as the assimilated flies were not under the selection pressure created by the heat-shock, the genetic buffers that mediate canalization (genetic buffers act as capacitors of morphological evolution by regulating the storage and release of genetic variation) were perhaps no longer impaired enough. This would follow that the system would make sure that a non-adaptive trait such as crossveinless does not get fixed (exemplified by the fact that assimilated flies in the present study failed to achieve fixation of cve). After all, canalization only prevents phenotypic deviants from getting fixed; it does not remove them from the population. The Mutation-Selection Balance theory [15] offers a conceptual backing to the aforesaid claims. According to this theory, a cryptic variant that might be deleterious will not necessarily disappear immediately from a population. Its frequency in the population might float up and down for a while before returning to zero. In a hypothetical large population, with additional mutations, the frequency might not return to zero at all. The variant will reach equilibrium or a balance between mutation (that is pushing the frequency forward) and selection (which is pushing it down). Selection fails to eliminate deleterious phenotypic variants because they are frequently created anew through recurrent mutation. This is perhaps why crossveinless wasn't completely eliminated from the true breeding lines in the absence of heat shock. The mutation-selection balance offers a simple model for understanding how variations such as the crossveinless might continue in natural populations.

Acknowledgements

We would like to thank GRAVIDA, New Zealand and the University of Otago for providing funding and research facilities that was key to the successful completion of this work.

Supplementary Information & Tables

S1 Table A.1 to A.11: Waddington experiment selection data. Upward selection line data for ten generations. Table A.12 to A.19: Downward selection line data for ten generations.

S2 Table A.20-22: Tables showing collection and scoring of cve flies from pupae heat-shocked (at 40°C for 45min) for 23, 24 and 25 hours a.p. In order to get the ideal heat-shocking conditions, three parameters were altered: the heat-shocking duration, the

heat-shocking temperature, and the age of the pupae before heat -shock. Flies were first turned five days prior to pupation then collected in four sets at 12 time points. Following the collection, pupae were incubated for 24 hours at 25°C. They were then heat-shocked at 40°C for four different exposure times: 45min, 2hrs, 3hrs, and 4hrs. Pupae were collected for 5 days. Flies were scored after 5 days of pupation. The second round of optimization involved collecting pupae for 5 days and heat-shocking them at four different temperatures: 40°C, 41°C, 41.5°C, and 42°C respectively for a period of 45 minutes. Flies were scored after 5 days. The third round of optimization involved varying the time of incubation. This involved running the experiments in triplets. The temperature and the duration of heat-shock were kept constant at 40°C for 45 min, while the incubation period was altered. Pupae were heat-shocked, after three incubation times: 23 hrs, 24 hrs and 25 hrs respectively.

https://scidoc.org/articlepdfs/IJBBS/IJBBS-01-102_Supplementary.pdf

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