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Abstract

In the process of morphological evolution, the extent to which cryptic, preexisting variation provides a substrate for natural selection has been controversial. We provide evidence that HSP90 phenotypically masks standing eye size variation in surface populations of the cavefish *Astyanax mexicanus*. This variation is exposed by HSP90 inhibition, and can be selected for, ultimately yielding a reduced-eye phenotype even in the presence of full HSP90 activity. Raising surface fish under conditions found in caves taxes the HSP90 system, unmasking the same phenotypic variation as direct inhibition of HSP90. These results suggest that cryptic variation played a role in the evolution of eye loss in cavefish and provide the first evidence for HSP90 as a capacitor for morphological evolution in a natural setting.

A longstanding question in evolutionary biology is the extent to which selection acts on pre-existing, "standing variation" in a population as opposed to *de novo* mutations. Recent studies have indicated that both mechanisms have contributed to morphological evolution. (1, 2). Thus, while *de novo* mutations may exist and contribute to phenotypic evolution, repeated use of standing variation has played an important role in the evolution in these fish. However, these observations also raise a critical question: How is genetic variation maintained in a population if it is not adaptive prior to new selective conditions?

Conrad Waddington proposed that developmental processes are quite robust and produce the same phenotype regardless of minor genotypic variation, a phenomenon he termed

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"canalization" (3). In such conditions, cryptic variation can accumulate and can be maintained without consequence. He further proposed that under certain environmental conditions this property could be lost ("decanalization") resulting in expression of the cryptic variation upon which selection could act (4).

More recently, Lindquist demonstrated that HSP90 (heat shock protein 90) provides a molecular mechanism for buffering genetic variation and releasing it in response to environmental stress (5–10), The HSP90 chaperone assists in the folding of protein that are metastable signal transducers, such as kinases, transcription factors, and ubiquitin ligases. HSP90 is normally present at much higher concentrations than needed to maintain these proteins, allowing it to act as a buffer, protecting organisms from phenotypic consequences that would otherwise be caused by genetic variants of these proteins. Because protein folding is so sensitive to environmental stress, changes in the environment can exhaust the chaperone buffer, unmasking vulnerable polymorphisms. And because multiple variants can be unmasked at the same time, this system provides a mechanism to create complex traits in a single step (11).

Besides changes in the activities of kinases, phosphatases, transcription factors, and ubiquitin ligases, other, distinct mechanisms have been reported by which changes in HSP90 function can lead to changes in phenotype (5, 10, 12–16)

Evidence strongly suggests this mechanism has operated in microbial populations (7, 8), but its relevance to the evolution of natural populations of higher organisms remains highly controversial. Thus far, examples of HSP90-mediated canalization in multicellular eukaryotes have been limited to lab strains of various model organisms. Moreover, with the exception of some phenotypes in *Arabidopsis* the phenotypes of HSP90 released canalization in higher organisms is not obviously adaptive. Finally, it has been unclear how a heat shock or other environmental factor would feature in the normal context of natural selection.

If a long term environmental stress were to drive the course of phenotypic evolution, it would plausibly arise when species are confronted with a completely foreign set of conditions. Such circumstances are met when organisms, such as the cavefish *Astyanax mexicanus* are inadvertently introduced into a cave environment.

Cavefish display many phenotypic differences from their surface conspecifics. We chose to focus on the dramatic loss of eyes in the cave morph, a trait that has been shown to be influenced by at least 14 mapped quantitative trait loci. Moreover, genetic evidence suggests that eye loss is very likely to be adaptive (17, 18). Loss of eyes could have had direct adaptive significance, for example in the energetic cost of maintaining eyes in an environment where they lack utility and/or could have been selected indirectly through the pleiotropic need to expand other sensory systems (see Supplementary Text). It is possible that neutral effects could have also contributed, to some extent, to the process by which eyes were lost in the cave populations of *Astyanax*.

To reduce the capacity of HSP90 chaperone activities during development of the fish (mimicking the effects of environmental stress) (5, 6, 7, 19, 20) we used the well-

characterized and highly specific inhibitor Radicicol. When developing *Astyanax mexicanus* were treated with 500nM Radicicol, this resulted in a strong increase in expression of two marker genes for HSP90 inhibition, *BAG3* and *HSP27*(*HSPB1*) as well as HSP90 itself (Figure 1a and Supplementary Figure 1), consistent with impairment of chaperone activity. The treated fish displayed an array of different low penetrance phenotypes in surface, cave and F2 populations (Supplementary Figure 2). Similar to previous studies, most of the low penetrance phenotypes were not obviously adaptive and either not viable or transient. Radicicol was not teratogenic for eye development *per se*. When an inbred laboratory strain of zebrafish was reared under similar conditions of Radicicol exposure (using a concentration which elicited a similar transcriptional response of target genes), no difference was observed in eye size or morphology (Supplementary Figure 3). In contrast, when *Astyanax mexicanus* surface fish were raised in the presence of the drug, we observed unusually large variation in eye size in larval fish (Figure 1B).

We initially characterized the effect of HSP90 inhibition on adult stages in an F2 population derived from a cross between a surface fish and a cavefish, allowing us to examine eye size in the simultaneous presence of surface and cave alleles. Indeed, we observed both larger eyes and smaller eyes in the treated fish, leading to a statistically significant increase in the standard deviation of the treated fish (+58% compared to DMSO treated control groups; two sided F-test: p=0.0004; Bartlett's test: p=0.001; Levene's test: p=0.03) (Figure 1 C,D). These experiments were conducted in the dark because Radicicol is slightly light sensitive; however, raising *Astyanax* embryos in the presence or absence of light had no effect on eye size (Figure 1C). Thus, inhibition of HSP90 permits the expression of cryptic variation in eye size present in the *Astyanax* populations.

If the cryptic variation we observe in eye size played a role in the evolution of the reduced eye trait within the caves, one would expect this variation to be present in the modern river population of surface fish (similar to the ancestral form), but to be less prevalent in the cave populations that have undergone selection for this trait. We observed a statistically significant increase in eye size variation (SD +83% compared to DMSO treated control fish; two sided F-test: p=8.1E-6; Bartlett's test: p<0.001, Levene's test: p<0.001) in the parental surface populations, again including both larger and smaller eyes than are ever seen in untreated broods (Figure 2A). The same result was seen whether measuring the eye itself or the infraorbital bones surrounding the eye socket or "orbit" (SD +108%; two sided F-test: p=3.4E-6; Bartlett's test: p<0.001, Levene's test: p<0.001) (Figure 2B, Supplementary Figure 4).

A very different result was observed, however, when we conducted parallel experiments on fish from the Tinaja cave population. Cavefish do not possess visible eyes, but still retain a cavity in the skull where the eye would be located (Supplementary Figure 4), so in this case we focused on the orbit size. We detected no increase in the variation in orbit size in the inhibitor treated cavefish (SD −12%; two sided F-test p=0.239), suggesting that some of the alleles have been selected for in the cavefish population (Figure 2C). However, we detect a statistically significant decrease in the orbit size in the inhibitor treated individuals (two tailed t-test: p=0.002) (Figure 2C). This shows that the alleles that have been selected for in cavefish evolution are alleles that, at least in part, are dependent on and responsive to

HSP90. Moreover, the HSP90-dependent alleles remaining in the cave population are specifically those contributing to reduced eye sockets. These data are consistent with the possibility that HSP90 played a role in the evolution of eye size in Tinaja cave-fish.

To test whether the cryptic variation in eye size uncovered by HSP90 inhibition can be genetically assimilated, we treated a population of embryonic surface fish with Radicicol. We then selected for smaller eye size by inter-crossing fish from this treated brood whose eye size was smaller than any of the untreated fish raised in parallel. The resultant F2 fish, raised in the absence of the drug, all had eyes and orbit sizes at the lower end of the range of sizes observed in the parental fish, and included many individuals with eyes and orbits smaller than any seen in the untreated surface fish and comparable to the smallest of the treated fish (Figure 3A–B). Thus, the individuals that develop the smallest eyes in the presence of HSP90 inhibition contain alleles that can contribute to inheritance of small eye size in the absence of treatment.

A critical question, however, is whether a river fish finding itself suddenly trapped in a cave environment would experience an HSP90-related stress response. To characterize abiotic factors that differ between the cave and river environments, we measured pH, oxygen content, temperature, and conductivity in the Tinaja cave and a nearby surface fish habitat in the Sierra de El Abra (Figure 4A, Supplementary Table 1). The biggest numerical difference we detected was the much lower conductivity present in the cave water. Further sampling of additional caves revealed conductivities as low as 230 μS (Sabinos cave), compared to the 1300 μS conductivity in adjacent river environments. Low conductivity can elicit a heat shock like stress response in fish (17), making it a good candidate for a cave specific stress factor.

To investigate, we raised surface fish at the lowest measured conductivity from the Sabinos cave (230 μS). When fish embryos develop under such conditions they up-regulate HSP90, showing that they are indeed in a state of physiological stress response, and, moreover, activate the same heat shock response genes that are up-regulated with HSP90 inhibition by Radicicol (Figure 4B and Supplementary Figure 5). Thus, the environment encountered by these fish during their evolutionary transition from surface to cave, stresses the protein homeostasis mechanisms of the organism in a manner similar to a specific stress on HSP90 chaperone activities.

Adult river fish placed in low conductivity during larval development displayed statistically significant increases in eye and orbit size variation of 50% (two-sided F-test p=0.0018; Bartlett's test: p=0.006, Levene's test: p=0.005) and 58% (two-sided F-test p=5.9E-4; Bartlett's test: p=0.001, Levene's test: p=0.01), respectively (Figure 4 C–D). This demonstrates that a cave-specific environmental stress can elicit similar changes in morphological eye development as biochemical inhibition of HSP90.

We detected an increase in variation in eye size in fish treated with Radicicol or in the presence of environmental stresses, representing cryptic variation present in the untreated population. We further demonstrated that in cavefish populations the alleles that are responsive to HSP90 have undergone selection in the transition from surface to cave forms,

leaving only alleles that produce a smaller orbit when released from their normal HSP90 interactions. This strongly suggests an involvement of HSP90 in cavefish evolution and provides an actual case in nature for Waddington's classic theory of the role of canalization in evolution. It is worth noting that not all cave-specific traits appear to have relied on HSP90-canalized cryptic variation for their evolution. We examined several other traits and found, for example, that there is no cryptic variation in body size (Supplementary Figure 6) or in neuromast number (Supplementary Figure 7) uncovered by HSP90 inhibition in the populations we examined.

It is also reasonable to assume that the change in conductivity is only one factor contributing to the stress response surface fish might experience after colonizing the caves (e.g. lower oxygen levels or starvation). However, such environmentally induced stress is likely to have been only transient as the cavefish would have adapted to these new conditions over subsequent generations. In this context it is worth noting that cavefish have higher basal HSP90 levels than surface fish (22), potentially rendering them more stress resistant. However during the transition period when the fish were adapting to the cave conditions, the HSP90 dependent standing variation in eye size we observed in the surface population of Astyanax mexicanus would have helped potentiate a rapid response to the cave environment.

Of course, the extreme environment of the cavefish is exceptional in many ways. Yet environmental challenges are likely to be a driving force for many other adaptations. For example, temperature increases are extremely common in nature and even simple starvation affects Hsp expression in European Sea Bass (23).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References and Notes

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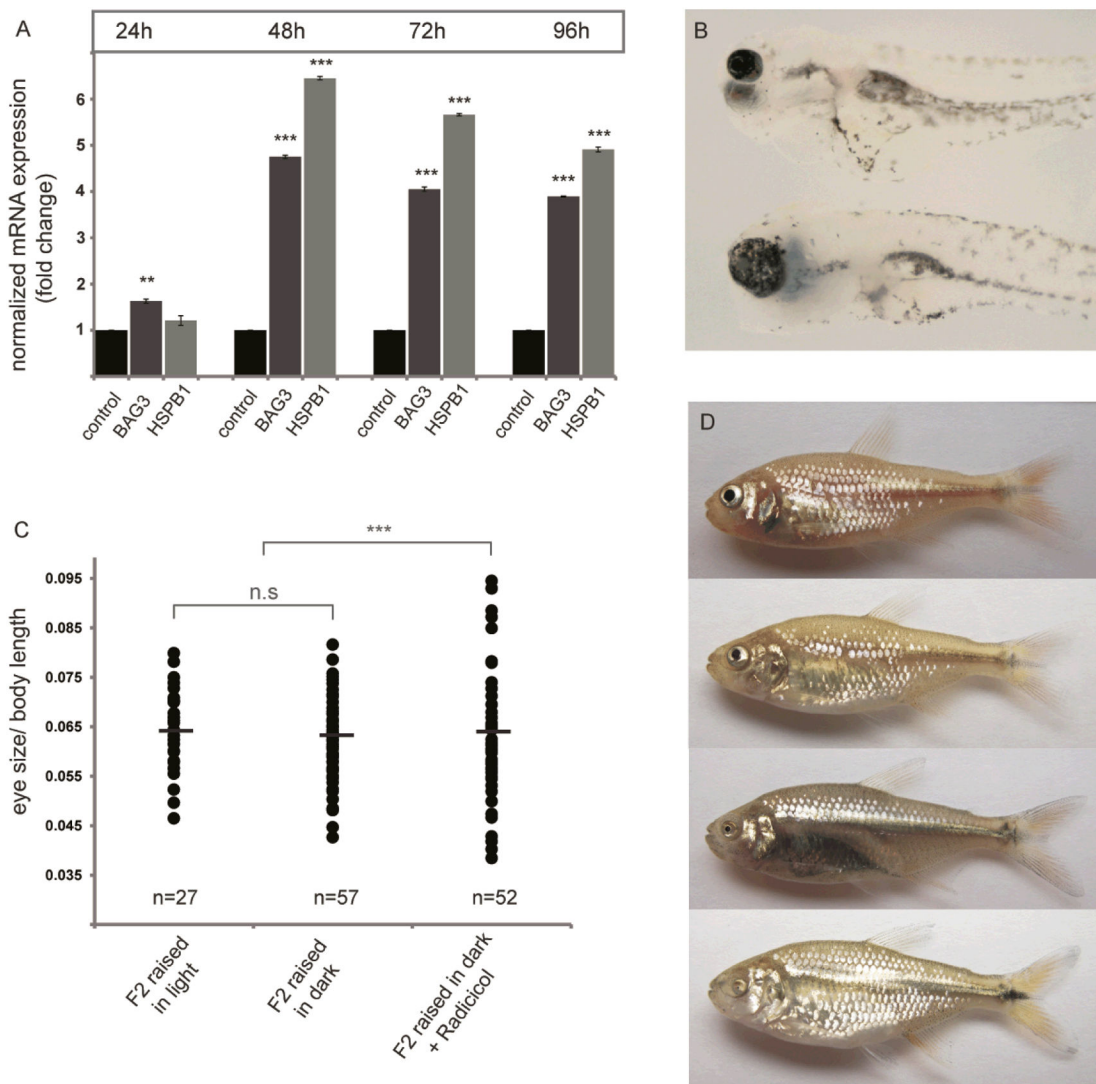
Fig. 1.
Reduction of HSP90 levels in *Astyanax mexicanus* using the chemical inhibitor Radicicol. (A) Inhibition of HSP90 using 500nM Radicicol leads to activation of BAG3 and HSPB1 (two-tailed t-test: **= $p<0.005$, ***= $p<0.0005$). Time scale refers to hours of treatment. (B) Variable eye sizes in surface *A. mexicanus* larvae after treatment. (C) Quantification of eye size in adult F2 hybrids after larval treatment of Radicicol reveals a significant increase in standard deviation of eye size while average eye size is not affected (two sided F-test: $p=0.0004$; Bartlett's test: $p=0.001$; Levene's test: $p=0.03$). Note that raising the fish in the dark alone does not affect eye size. Values were corrected for body size using standard length of the fish. (D) Examples of eye size variation in F2 population of hybrid *A. mexicanus*.
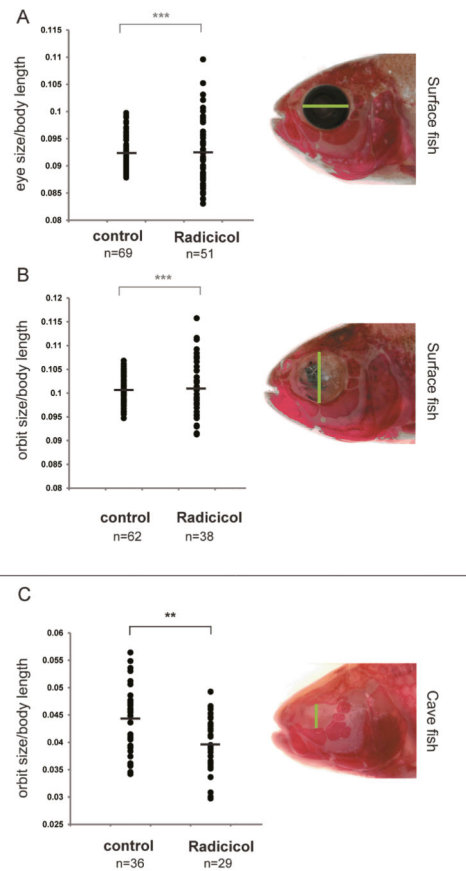
Fig. 2.

HSP90 inhibition in natural populations of *Astyanax mexicanus*. (A,B) HSP90 inhibition in natural populations of surface *Astyanax mexicanus* leads to an increase of variation in eye size (A) and orbit size (B). Eye size SD+ 83% - two sided F-test: p=8.1E-6; Bartlett's test: p<0.001, Levene's test: p<0.001; Orbit size SD + 108% - two sided F-test: p=3.4E-6; Bartlett's test: p<0.001, Levene's test p<0.001. (C) Orbit size decreases after Radicicol treatment in Tinaja cave populations (t-test: p=0.02), while change in variation is observed (SD -12%: two sided F-test: p=0.239). Control is DMSO. Asterisks in (A) and (B) compare standard deviations, while (C) compares averages.
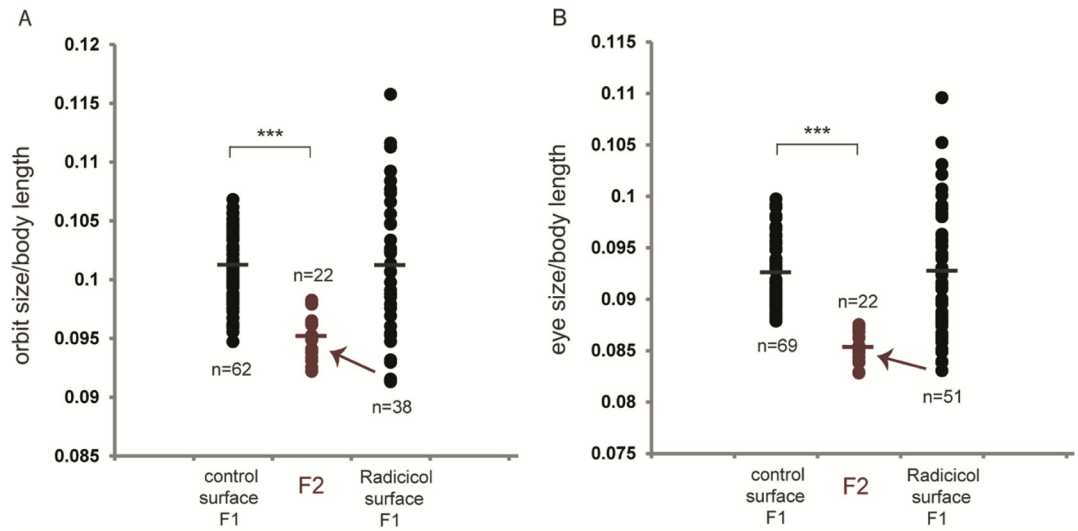
Fig. 3.
Genetic assimilation. (A–B) Selection for small eye size in surface fish generated by Radicicol treatment resulted in offspring with significantly smaller orbit size (A) and eye size (B) in the absence of treatment (two-tailed t-test: p=6.5E-13 for orbit size and p=7.2E-16 for eye size). The resultant range exceeded the range seen in any cross of untreated surface fish.
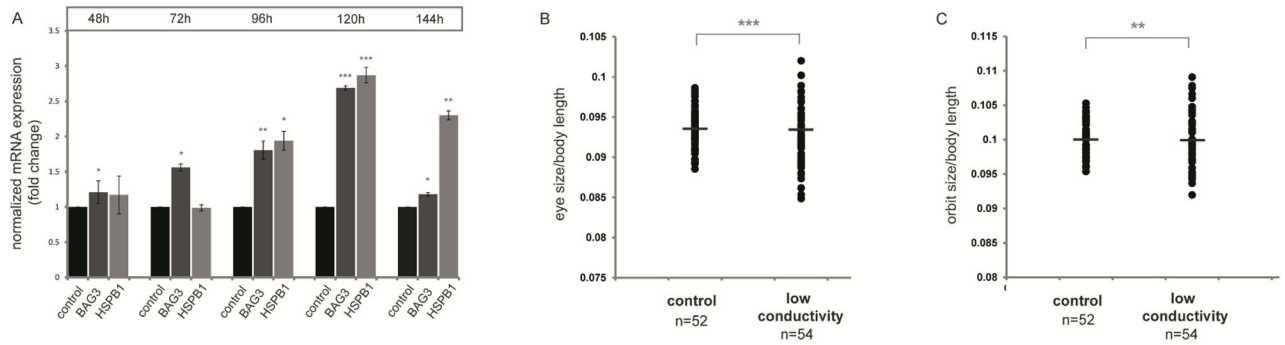
Fig. 4.

Low conductivity conditions in the cave natural habitat have a similar effect to Radicicol treatment on surface populations. (A) qRT-PCR of BAG3 and HSPB1 for surface fish reared under low conducitivity (230 µS) conditions compared to control conductivity conditions (two-tailed t-test: *= $p<0.05$, **= $p<0.005$, ***= $p<0.0005$). Time scale refers to hours of treatment. (B–C) Lower conductivity conditions reveal an increase in variation of orbit size (B) and eye size (C) (Eye size SD +50% - two sided F-test: $p=0.0018$; Bartlett's test: $p=0.006$, Levene's test: $p=0.005$; Orbit size SD +58% - two sided F-test: $p=5.9E-4$; Bartlett's test: $p=0.001$, Levene's test $p=0.01$).