

SHORT COMMUNICATION

Potential rapid evolution of foot morphology in Italian plethodontid salamanders (*Hydromantes strinatii*) following the colonization of an artificial cave

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Abstract

How organisms respond to environmental change is a long-standing question in evolutionary biology. Species invading novel habitats provide an opportunity to examine contemporary evolution in action and decipher the pace of evolutionary change over short timescales. Here, we characterized phenotypic evolution in the Italian plethodontid salamander, *Hydromantes strinatii*, following the recent colonization of an artificial cave by a forest floor population. When compared with a nearby and genetically related population in the natural forest floor and a nearby cave population, the artificial cave population displayed significant differences in overall foot shape, with more interdigital webbing relative to the other populations. Further, this population evolved significantly larger feet, which corresponded more closely to those found in other cave populations than to forest floor populations to which the cave population is closely related. Finally, we quantified the rate of evolution for both foot shape and foot area, and found that both traits displayed large and significant evolutionary rates, at levels corresponding to other classic cases of rapid evolution in vertebrates. Together, these findings reveal that the response to novel environmental pressures can be large and rapid and that the anatomical shifts observed in the artificial cave population of *H. strinatii* may represent a case of rapid evolution in response to novel environmental pressures.

Introduction

Abrupt environmental changes expose organisms to novel ecological challenges, which in turn can elicit rapid evolutionary responses in those phenotypic traits that enable organismal survival (Hoffman & Hercus, 2000; Hendry *et al.*, 2008). As such, examining the tempo of evolution is one way of identifying the processes responsible for phenotypic diversification. Furthermore, in some circumstances, it may be expected that evolutionary rates will differ across

clades, for instance, if taxa are exposed to distinct competitive or predation pressures across habitats (Dayan & Simberloff, 1998; Millien, 2006), or if species exploit new ecological regimes or display functional novelties (e.g. Mahler *et al.*, 2010; Martin & Wainwright, 2011). Thus, at the macroevolutionary level, understanding evolutionary rates provides a means of identifying the relationship between evolutionary trends in phenotypic diversification and the ecological and evolutionary processes that are responsible for them (Adams *et al.*, 2009; Thomas *et al.*, 2009; Harmon *et al.*, 2010; Rabosky & Adams, 2012).

At contemporary ecological timescales, it is often observed that extreme habitat perturbations caused by natural events or by anthropogenic activities, such as chemical pollution, harvesting or physical modification

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of the environment, have a profound effect on the pace of evolutionary change (Hoffman & Hercus, 2000; Hendry *et al.*, 2008). Indeed, numerous studies have shown differences in evolutionary rates between closely related populations that inhabit distinct habitats or that experience shifts in ecological conditions. For instance, species invading new habitats (McPeck, 1995; Revell *et al.*, 2007) or those introduced to new geographic localities (Klingenberg & Ekau, 1996; Clegg *et al.*, 2002; Herrel *et al.*, 2008) have been observed to display elevated rates of phenotypic evolution. Likewise, species introductions have caused rapid phenotypic responses in the native species with which they are now competitors (Stuart *et al.*, 2014), and recent habitat alterations, whether natural or human induced, have caused rapid phenotypic evolution in numerous taxa in both terrestrial and aquatic environments (e.g. Grant & Grant, 1995; Reznick *et al.*, 1997; Franssen *et al.*, 2013; Caruso *et al.*, 2014; Cureton & Broughton, 2014; for a review see Hendry *et al.*, 2008). Thus, current evidence suggests the hypothesis that shifts in habitats or ecological regime can result in rapid and adaptive changes in organismal phenotype.

In this study, we identify an instance of rapid changes in foot morphology in the Italian plethodontid salamander, *Hydromantes strinatii* (Aellen, 1958), following the colonization of an artificial cave. This medium-sized, terrestrial salamander is typically found in forest habitats, where it resides in the leaf litter, on talus slopes and on humid rock faces along streams (Lanza *et al.*, 1995, 2006; also Manenti, 2014). However, some populations of this species are also known to inhabit caves, where they can be found climbing the walls and ceilings due to the relatively well-developed interdigital webbing on their hands and feet (Lanza *et al.*, 2007; Adams & Nistri, 2010). One such population was discovered inhabiting an artificial cave, which was created approximately 70 years ago. This population therefore represents a contemporary colonization of a unique environment relative to that of the surrounding populations. Here, we exploit this natural evolutionary experiment and compare patterns of foot morphology in this population to what is observed in a nearby forest-dwelling population, using a third population inhabiting a natural cave as an additional comparison. Because the colonization of the artificial cave was from a forest-dwelling *H. strinatii* population, any phenotypic differences observed in this population may be inferred to be a response to selection pressures from this novel environment. Unfortunately, historical samples that predate the artificial cave colonization are unavailable. However, genetic analyses of several nearby populations indicated that that the present-day artificial cave population was more genetically similar to a neighbouring forest floor population than it was to a population inhabiting the natural cave (Forti *et al.*, 1997; Carranza *et al.*, 2008). Thus, comparisons of the artificial cave

population to that of the forest floor may provide insight into the short-term evolutionary responses to an environmental shift in this species over the past several decades (approximately ten generations: see below). Using this system, we addressed three main questions to enhance our understanding of patterns of phenotypic evolutionary change: (1) Are the forest floor (epigeal) and cave-dwelling (hypogean) populations distinct phenotypically? (2) How has the foot shape changed in the population that recently colonized the artificial underground habitat? and (3) if the observed changes are consistent with what is expected as an adaptive response to novel selection pressures, does the degree of phenotypic variation observed in the artificial cave habitat represent a case of rapid phenotypic evolution?

Materials and methods

Study species and collection localities

Hydromantes strinatii is a medium-sized terrestrial salamander endemic to Southern Europe (SE France and NW Italy: Fig. 1a). This long-lived species reaches sexual maturity in its fourth or fifth year (Salvidio, 1993), and in the field can exceed 12 years of age (Lindstrom *et al.*, 2010). We sampled a total of 170 adult specimens of *H. strinatii* from three geographic locations in November 2013 and May 2014 (Fig. 1a). Males were diagnosed by the presence of secondary sexual characteristics (i.e. a well-developed mental gland), whereas all animals lacking the gland and > 57 mm SVL were considered sexually mature females (Salvidio, 1993). The first salamander population inhabited a natural limestone cave (Grotta del Trabochetto, Pietra Ligure (Province of Savona), from which we obtained 51 adult specimens (30 females, 21 males). The second location represented a forest floor population found along a first-order Apennine stream, Rio Tonno, Val Brevenna (Province of Genova), where 60 adult specimens (36 females, 24 males) were obtained from the leaf litter, under logs and stones and in crevices in rocky outcrops along the stream. The third population was located in an artificial cave which was excavated during World War II and served as an air raid shelter, at San Bartolomeo, Savignone (Province of Genova). From this artificial cave, we obtained 59 adult specimens (23 females, 36 males). Salamanders were first reported at this site in 1972 (Pastorino & Pedemonte, 1972). This subterranean location displayed environmental conditions similar to those observed in natural cave habitats (Salvidio *et al.*, 1994). The two latter populations, inhabiting a similar nonkarstic area and separated by 10 km by air, belong to a genetically homogeneous *S. strinatii* phylogenetic cluster characterized by low genetic distance (mean $D_{Nei} = 0.03$; see Fig. 3 in Forti *et al.* 1997). Moreover, prior molecular evidence from these populations indicated that the forest floor and artificial cave

populations were more closely related to one another genetically than to the natural cave population (Carranza *et al.*, 2008). Based on the known age of the cave, the recent colonization of this subterranean habitat and the generation time of *H. strinatii*, this population has only inhabited the cave for approximately ten generations. For each locality, salamanders were transported from the field to the laboratory for processing. Afterwards, all animals were released to their site or origin. No mortality was observed during this study.

Morphometric and statistical analyses

Foot shape was characterized using landmark-based geometric morphometrics (Bookstein, 1991; Mitteroecker & Gunz, 2009; Adams *et al.*, 2013). These methods quantify the shape of anatomical objects from the coordinates of landmark locations, after the effects of nonshape variation (position, orientation and scale) have been mathematically held constant. For these analyses, we first obtained two-dimensional digital

images of the right foot of each specimen using a reflex Nikon D300 digital camera with a macro lens. From these images, we then digitized the locations of nine landmarks corresponding to the tips of the toes and the minimal extent of the webbing between them (Fig. 1b; see also Jaekel & Wake, 2007; Adams & Nistri, 2010). Next, a generalized Procrustes analysis (Rohlf & Slice, 1990) was used to align the specimens, and Procrustes tangent coordinates were obtained and treated as a set of shape variables for all subsequent analyses. Finally, we measured total foot area for each specimen.

We performed a number of statistical analyses to examine the patterns of foot morphology within and among populations. First, we used a Procrustes ANOVA (Goodall, 1991) with a factorial design to determine whether foot shape differed among populations and between the sexes. Pairwise comparisons between groups were then accomplished using the Euclidean (Procrustes) distances between group means, which were statistically evaluated using a permutation test. Differences in foot area among populations were also assessed using ANOVA, and patterns of shape variation were examined using principal component analysis. Shape differences were then visualized using thin-plate spline deformation grids. Finally, under the hypothesis that the observed changes in foot shape were adaptive, we estimated the rate of change in several foot traits to characterize the pace of evolutionary change that has occurred over the past seven decades. We used haldanes to estimate evolutionary rates in foot area and Mahalanobis distances to estimate rates of change in foot shape. Haldanes are a univariate measure of the evolutionary rate of change per generation in standard deviation units, whereas Mahalanobis distance is its multivariate generalization, and characterizes the mean change in a multivariate phenotype across generations relative to within-group variability (see Gingerich, 2009). Both estimates were based on the comparison between the artificial cave and the forest floor populations, because prior molecular evidence indicated that the two were genetically related to one another (Forti *et al.*, 1997; Carranza *et al.*, 2008). For both foot shape and foot area, we obtained estimates of evolutionary rates using the difference in mean trait values between the artificial cave and forest floor populations, relative to the pooled variation among populations. We then standardized both measures by ten generations, which represented a conservative estimate of the number of generations *H. strinatii* has occupied the artificial cave. The observed phenotypic evolutionary rates were then statistically evaluated using a randomization test, where specimens were shuffled among groups and a distribution of possible evolutionary rates was obtained (for details see Stuart *et al.*, 2014). All analyses were performed in R 3.1.2 (R Development Core Team, 2014) using version 2.1.2 of the *geomorph* library

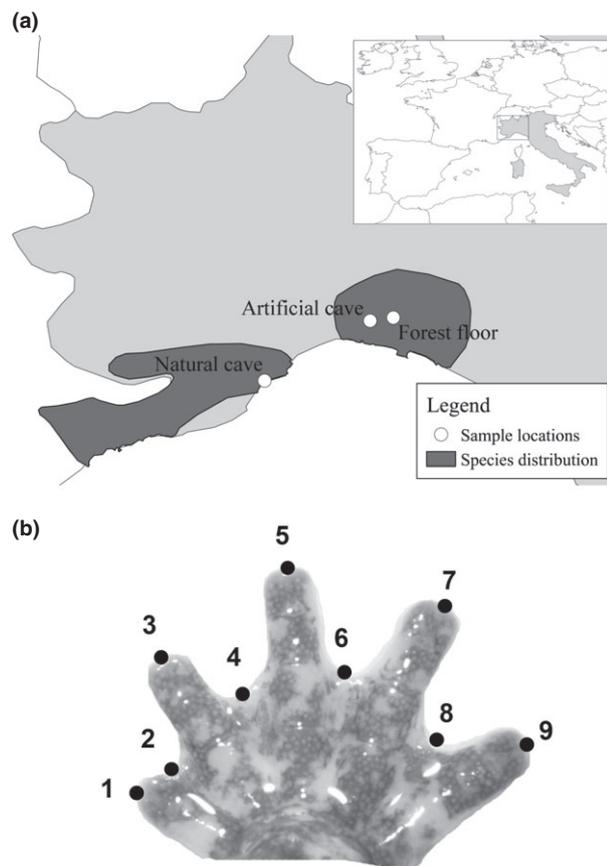


Fig. 1 (a) Geographic distribution of *Hydromantes strinatii*. Collection localities used in this study are shown. (b) Locations of landmark coordinates used to characterize foot shape (redrawn from Adams & Nistri, 2010).

(Adams & Otárola-Castillo, 2013; Adams *et al.*, 2014) and routines written by the authors.

Results

Using Procrustes ANOVA, we found significant differences in foot shape among populations (Table 1). Pairwise comparisons revealed that all populations differed significantly from one another (Table 1), a pattern visually confirmed using principal component analysis (Fig. 2a). Here, the 95% confidence intervals for all populations did not overlap in morphospace, implying that the three populations were phenotypically distinct (Fig. 2a). Furthermore, the most phenotypically distinct population was that found in the artificial cave. Thin-plate spline deformation grids of population means corroborated this finding and revealed that the shape differences among populations were primarily in the degree of interdigital webbing, with the artificial cave population displaying relatively more interdigital webbing as compared to the other two populations (Fig. 2a). When foot area was examined, we found significant differences among populations ($F = 14.3$, d.f. = 2,167, $P < 0.001$). Interestingly, here the natural and artificial cave populations did not differ from one another, but exhibited significantly larger foot areas as compared to individuals from the forest floor population (Fig. 2b). Thus, while the two cave populations were similar in their relative foot areas, the differences in foot shape between them implied that this was achieved in distinct anatomical ways (larger feet versus relatively more webbed feet).

Using Procrustes ANOVA, we found no differences in foot shape between males and females, but we did identify a significant population X sex interaction term (Table 1). This implied that the patterns of sexual dimorphism were not consistent among populations, although this result is difficult to explain from a biological point of view, and may simply reflect differences

Table 1 Results from factorial Procrustes ANOVA (with 999 permutations) and pairwise comparisons of populations (Euclidean distance below diagonal, significance level above diagonal, bold values indicate significant population differences).

Source	d.f.	SS	MS	<i>F</i>	<i>P</i>	<i>R</i> ²
Population	2	0.08880	0.04440	11.2902	0.001	0.1129
Sex	1	0.00357	0.00357	0.9087	0.568	0.0047
Pop × Sex	2	0.01629	0.00814	2.0710	0.035	0.0216
Residual	164	0.64497	0.00393			

	Nat. cave	Artif. cave	Forest floor
Nat. cave	–	0.001	0.001
Artif. cave	0.04709	–	0.001
Forest floor	0.03826	0.03337	–

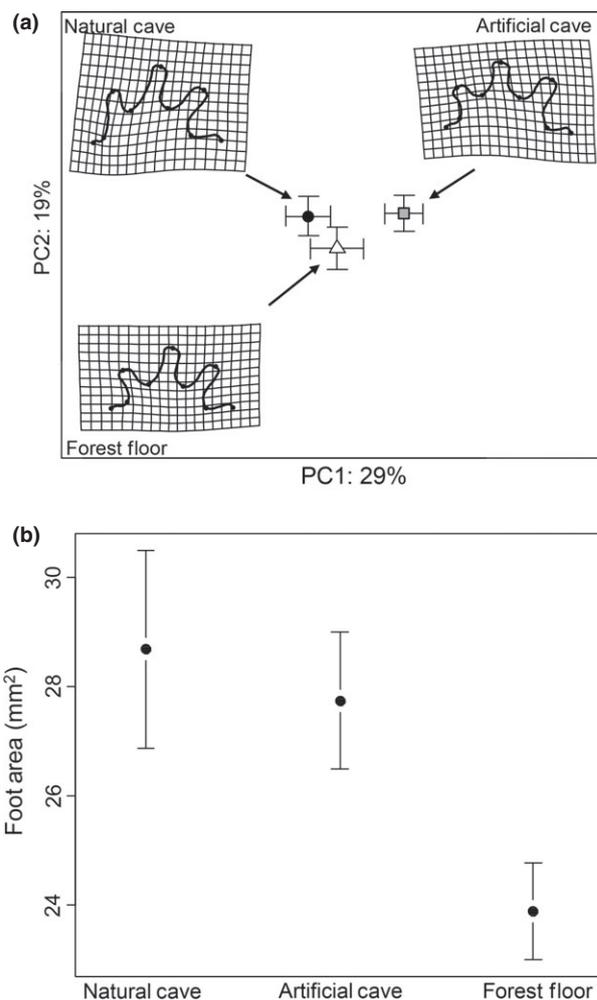


Fig. 2 (a) Principal component analysis displaying the means and 95% confidence intervals for three populations of *Hydromantes strinatii*: black circle (natural cave population), white triangle (forest floor population), grey square (artificial cave population). Thin-plate spline deformation grids of population means are shown (magnified by 3× to facilitate biological interpretation). (b) Mean foot area for each of the three populations sampled.

due to unbalanced sexual sampling ($\chi^2 = 6.49$ d.f. = 2, $P = 0.038$). Finally, we found large evolutionary rates for both foot area ($\text{rate}_{\text{haldane}} = 0.086$) and for foot shape ($\text{rate}_{\text{D-Mahal}} = 3.13$). Further, when we accounted for the fact that foot shape is highly multivariate, the net evolutionary rate for foot shape was still large ($\text{rate} = 0.174$ per trait dimension). Using a randomization test (*sensu* Stuart *et al.*, 2014), both evolutionary rates were significantly greater than expected ($P_{\text{rand}} = 0.001$). Therefore, under the hypothesis that these differences represent adaptive phenotypic change, we infer that both foot shape and foot area displayed significant evolutionary rates per generation in the artificial cave population.

Discussion

An important facet of evolutionary research is to elucidate the drivers of phenotypic diversity. Over the past several years, it has become increasingly apparent that evolutionary changes in phenotypes can occur rapidly (Hendry & Kinnison, 1999; Hendry *et al.*, 2008), particularly in response to shifts in selective pressures resulting from environmental perturbations (Franssen *et al.*, 2013; Caruso *et al.*, 2014; Cureton & Broughton, 2014; Stuart *et al.*, 2014). In this study, we characterized patterns of phenotypic evolution in the Italian plethodontid salamander, *Hydromantes strinatii*, following the recent colonization of an artificial cave by a genetically closely related forest floor population (Forti *et al.*, 1997; Carranza *et al.*, 2008). By comparing patterns of foot morphology in this population to those observed in a genetically related natural forest floor population and to a second population inhabiting a natural cave, we were able to determine the short-term evolutionary responses of this species to the environmental shifts associated with inhabiting this novel subterranean environment. Our findings provide several insights into the possible evolutionary processes associated with habitat shifts and how taxa respond to selection over contemporary timescales.

First, when comparing phenotypes between populations in similar environments, our study reveals that the phenotypic responses can display common components. This is evidenced by the fact that foot area in the artificial cave population was more similar to that found in the natural cave population than to what was observed in the forest floor population, this in spite of a high genetic similarity existing between the artificial cave and the forest floor populations. Indeed, given the natural history of this species, the evolution of a larger foot area in these populations is not surprising, as behaviourally the species displays a propensity to climb the rock walls and ceilings when found in subterranean habitats (Lanza, 1991; Lanza *et al.*, 2007). As such, the patterns observed here are consistent with the hypothesis that increased foot area is an adaptation to climbing in this group (Lanza, 1991; Lanza *et al.*, 2006; see Discussion in Adams & Nistri, 2010). Therefore, as is seen in other taxa (e.g. Losos, 1992; Jastrebski & Robinson, 2004; Berner *et al.*, 2008; Adams, 2010), patterns of phenotypic evolution in response to common selection pressures can display an adaptive signature, and can be repeatable in those populations of *H. strinatii* that occupy similar habitats.

Second, despite the common patterns of foot area between the two cave-dwelling populations, other anatomical shifts in the artificial cave population were more unique. Specifically, phenotypic changes in the artificial cave population were distinct in that this population displayed considerably greater interdigital webbing between their toes. Thus, in this system, the

artificial cave population has diverged from its ancestral form to one more consistent with that seen in hypogean populations in other species of Italian *Hydromantes*, where the degree of foot webbing is elaborated (see Adams & Nistri, 2010). When viewed in the light of the similarity in foot area between the two cave populations examined here, the differences in foot shape suggest that a similar biomechanical objective may be achieved through distinct anatomical pathways, where the foot area in one population is achieved through greater interdigital webbing while the same is accomplished in another population via relatively larger feet.

Together, these patterns are consistent with the hypothesis that novel ecological conditions can elicit strong selective pressures, resulting in both shared and unique phenotypic responses in just a few generations. Nevertheless, because of several limitations with the study system, alternative explanations of the observed patterns deserve consideration. For instance, because there is only one artificial cave, it is difficult to discern whether the observed patterns of phenotypic change are solely the result of a response to selection. Ideally, this could be addressed by comparing patterns of phenotypic change across replicate artificial caves to determine whether the patterns observed here are consistent, but unfortunately, no such replicate artificial caves are present in this system. Additionally, it is possible that the observed phenotypic differences have been caused, in part, by founder events, where individuals from the progenitor population displaying more extreme phenotypes were the founding individuals of the current artificial cave population. Unfortunately, while the genetic relationships among populations in this species have been previously studied (Forti *et al.*, 1997; Carranza *et al.*, 2008), no fine-scale molecular study within populations has been performed to inform on this alternative explanation.

Regardless of the underlying cause of the differentiation, what is clear is that the artificial cave population displays a unique phenotype that has evolved in only a few generations. As such, when the pace of this phenotypic change is considered, it is observed that the artificial cave population displayed both large and significant rates of evolution in foot shape and foot area. Notably, these evolutionary rates were comparable to other examples of rapid evolution in vertebrates (see Hendry & Kinnison, 1999), including the evolution of toepad area and lamellae number in anoles (Stuart *et al.*, 2014), the evolution of life history traits in guppies (Reznick *et al.*, 1997), the evolution of bill dimensions in Galapagos finches (Grant & Grant, 1995), evolutionary shifts in head morphology, feeding performance and digestive tract structure in wall lizards (Herrel *et al.*, 2008), and rates of body size evolution in plethodontid salamanders (Caruso *et al.*, 2014). From this, we can infer that the anatomical shifts observed in the artificial cave population of *H. strinatii* represent a case of rapid

evolution in a novel habitat with unique environmental pressures.

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