Why are not all chilies hot? A trade-off limits pungency

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Evolutionary biologists increasingly recognize that evolution can be constrained by trade-offs, yet our understanding of how and when such constraints are manifested and whether they restrict adaptive divergence in populations remains limited. Here, we show that spatial heterogeneity in moisture maintains a polymorphism for pungency (heat) among natural populations of wild chilies (Capsicum charuense) because traits influencing water-use efficiency are functionally integrated with traits controlling pungency (the production of capsaicinoids). Pungent and non-pungent chilies occur along a cline in moisture that spans their native range in Bolivia, and the proportion of pungent plants in populations increases with greater moisture availability. In high moisture environments, pungency is beneficial because capsaicinoids protect the fruit from pathogenic fungi, and is not costly because pungent and non-pungent chilies grown in well-watered conditions produce equal numbers of seeds. In low moisture environments, pungency is less beneficial as the risk of fungal infection is lower, and carries a significant cost because, under drought stress, seed production in pungent chilies is reduced by 50 per cent relative to non-pungent plants grown in identical conditions. This large difference in seed production under water-stressed (WS) conditions explains the existence of populations dominated by non-pungent plants, and appears to result from a genetic correlation between pungency and stomatal density: non-pungent plants, segregating from intra-population crosses, exhibit significantly lower stomatal density (p = 0.003), thereby reducing gas exchange under WS conditions. These results demonstrate the importance of trait integration in constraining adaptive divergence among populations.

**Keywords:** genetic correlation; secondary compounds; defence; capsaicinoids; capsaicin; natural selection

1. INTRODUCTION

Environmental heterogeneity drives spatial variation in selection and has long been assumed to play a key role in the maintenance of phenotypic variation by creating and enhancing adaptive trade-offs under divergent selection [1–5]. The basis for this assumption is that natural selection acts on whole organisms rather than isolated traits and, therefore, adaptation may often be constrained by trade-offs among traits that interact to determine individual phenotypes (i.e. functionally integrated traits [1,5]). Adaptive trade-offs are a cornerstone of evolutionary ecology [6–8], frequently explaining the maintenance of polymorphisms at range margins [9] and along environmental gradients [10,11]. Nonetheless, direct evidence of adaptive trade-offs is surprisingly rare [5] and most often represented by studies of experimental evolution in laboratory conditions [5,12,13]. Evidence of adaptive trade-offs in natural populations is lacking or surprisingly equivocal [6]; patterns attributed to fitness trade-offs resulting from environmental differences can often be attributed to alternative mechanisms [5]. Thus, despite many examples of trait polymorphisms, our understanding of the mechanism(s) underlying the maintenance of phenotypic variation among natural populations remains largely theoretical [14,15].

A central challenge in documenting adaptive trade-offs in natural populations is finding a species in which phenotypic variation is clearly tied to fitness consequences that change along a well-defined environmental gradient [16] and in which benefits and functionally linked costs of the phenotype are readily apparent [7]. Variation in the expression of constitutive chemical defences in plants has been suggested as a tractable area in which to examine these issues, as ecological benefits are easily measured and physiological costs appear unavoidable [17]. In practice, however, the benefits of chemical defence are often well documented, but the costs are often elusive [18,19]. One reason for the paucity of data on costs associated with a given plant defensive trait is that most defensive traits belong to an integrated suite of traits, which function concomitantly or even synergistically [20,21]. Such interactions make it difficult to ascribe fitness differences among phenotypes to a particular trait. Another limitation has been the focus on plant defensive traits related to herbivory. Although the measurement of herbivory itself is straightforward, the relationship between herbivory and fitness is complex and variable [22].

Here, we focus on a plant defensive trait that avoids these pitfalls: pungency in wild chili peppers (Capsicum spp.). Pungency in wild chilies has a simple genetic basis [23] and is expressed only in the fruit, where it acts to protect seeds.
[24], thereby providing the direct link to changes in fitness that is often missing from studies of chemical defence and herbivory [22]. Pungency is also polymorphic in some wild chili species, and multiple polymorphic populations have been identified along natural environmental gradients [24,25]. These attributes make the wild chili pepper an excellent system in which to investigate adaptive constraints.

Chili peppers get their ‘heat’, or pungency, from capsaicinoids, and two capsaicinoids, capsaicin and dihydrocapsaicin, account for more than 95 per cent of this pungency [26]. Pungency appears to be a synapomorphy, as basal chilies (Capsicum cilliatum) lack pungency and most derived chilies (e.g. Capsicum annuum) are all pungent [27]. Southeastern Bolivia is the putative centre of diversity for wild chilies; at least three Capsicum species co-occur and are polymorphic in pungency, with plants producing either pungent or non-pungent chilies growing side by side [25]. A pathogenic and pervasive fungus (Fusarium spp.) produces strong selection for pungency in at least one of these species, Capsicum chacoense [4]. In particular, capsaicin appears to protect chili fruits and seeds from Fusarium attack. It also protects chili fruits from consumption by granivorous rodents without reducing consumption by seed-dispersing birds [28,29]. Given these well-documented benefits of capsaicin, we ask: is the observed polymorphism in wild chilies maintained by a cost of pungency? In other words, ‘why are not all chilies hot?’ To answer this question, we focus on the evolutionary ecology of pungent and non-pungent forms of C. chacoense along a natural moisture gradient in southeastern Bolivia.

2. MATERIAL AND METHODS

(a) Pungency and populations

Natural populations of C. chacoense are either polymorphic for pungency (hot or not) or monomorphic (all hot). In addition, these populations vary in the degree of pungency among pungent plants [25]. We have examined variation in pungency and the polymorphism for pungency in 21 populations along a 300 km transect in southeastern Bolivia that co-occurs with a gradient in moisture (figure 1a). In the dry northeast, populations are 15–20% pungent and the percentage of pungent plants increases towards the wetter southwest, where populations are 100 per cent pungent (in 7 census years, we have not located a non-pungent chili in these populations; figure 1a and [25]). We have censused each of the focal populations over 5 years (between 2002 and 2009). To determine new recruits, we tagged plants each year and counted only new seedlings. We analysed changes in the proportion of pungent plants within locations through time by comparing model fit for a series of

Figure 1. (a) The natural rainfall gradient across southeastern Bolivia where polymorphic populations occur; pie charts indicate the proportions of pungent plants. (b) The proportion of pungent plants in each population increases as a function of precipitation ($r^2 = 0.83$, $F_{1,19} = 100.5$, $p < 0.001$). Triangles (black, 29%; grey, 45%; white, 88%) indicate the study populations.
Generalized Linear Mixed Models with binomial error distribution using R [30]. All models included location as a random effect. We tested the significance of pungency proportion with location, year and location by year as fixed effects. Model fits were estimated using maximum likelihood (ML) with a logit-link function. Pungency was determined by tasting in the field and verified by high-performance liquid chromatography as in Tewksbury et al. [25].

(b) Field sampling and growing conditions

We selected three polymorphic populations that span the range in rainfall, pungency (per cent of population pungent) and plant density found in polymorphic C. chacoense populations ([25] and figure 1b; electronic supplementary material, table S1). We collected fruits from pungent and non-pungent plants in each population, and seeds from these fruits were grown in the University of Washington glasshouse. We selected 11 maternal lineages from each pungency by population class for a total of 66 lineages, forming a population of 330 plants. These maternal plants were grown in the University of Washington glasshouse, and selfed fruits were selected to form a common garden population.

(c) Water stress experiment

All plants were grown under identical well-watered (WW) conditions in the University of Washington glasshouse, until after the first flower in each treatment group was observed, at which point, plants from within each lineage were assigned to one of two treatments and individually watered: a WW treatment (mimicking, as much as possible, the average rainfall during fruiting and flowering seasons in our WW site (electronic supplementary material) or a water-stressed treatment (WS; developed to mimic the average rainfall data from the driest location; electronic supplementary material). Under natural conditions, water is not limiting until fruit set, and fruit set has been described as the period in which chili peppers are most susceptible to drought stress [31]. We imposed the water stress treatment after the first flower in each treatment group was observed to replicate the natural water availability and ameliorate the effects of unnatural water stress on overall growth (electronic supplementary material). Data were analysed using a generalized linear model with lineage, treatment and pungency, and all interactions as fixed effects and a logit link transformation. Stepwise reduction [32] yielded a final model retaining the treatment by pungency interaction effect.

(d) Stomatal density

To isolate the relationship between pungency and stomatal density, we developed an artificial population segregating for pungency by reciprocally crossing two populations of pungent × non-pungent parents and analysing stomatal density from 30 plants in the F2 population. Stomatal density is influenced by plant and leaf age and by environmental conditions. To control for these effects, we standardized the watering regime (using the WW watering treatment, §2c), and age-matched all plants based on two age criteria: (i) date of germination and (ii) uniform height of 60 cm. Epidermal peels were obtained from sets of four leaves collected at 10, 25 and 50 cm. Stomata were visualized using a Zeiss microscope at 400×. Stomatal density was estimated by averaging the count of abaxial stomata in the visual field (using a reticle scale corrected for magnification) of four regions for each peel. There were no differences in the non-independent estimates of stomatal density along the height of the plant (a rough surrogate for leaf age). These averaged data were analysed using a t-test to detect mean differences in stomatal density between pungency classes. The pungency of each plant was verified by tasting and liquid chromatography.

3. RESULTS

Populations of wild chilies in Bolivia vary considerably in the proportion of pungent plants in the population (figure 1; electronic supplementary material, table S1), consistent with previous observations [25]. Measuring the frequency of pungent plants among new recruits over 7 years in these populations revealed no significant shifts in the proportion of pungent plants across the cline (electronic supplementary material, table S1). Variation in total annual rainfall predicts this stable cline in pungency (figure 1b, \( r^2 = 0.83, F_{1,19} = 100.5, \ p < 0.001 \)). Under restricted water conditions in the glasshouse, pungent plants produced 50 per cent fewer seeds per plant than non-pungent plants, but under WW conditions, fruit production was nearly identical (figure 2; GLM binomial error distribution, pungency × environment interaction \( F = 10.3, \ p = 0.005, n = 352 \)).

In these populations, pungent and non-pungent plants co-occur (within 1 m²) and are completely inter-fertile (within and between populations) [23]. Because water-use efficiency differences can result from differences in leaf morphology [33], we investigated morphological differences between pungent and non-pungent plants. For plants grown in the glasshouse, from field-collected seed, the only detected leaf morphological feature differing between types within population was stomatal density. To establish a mechanistic link between pungency and stomatal density, we constructed populations segregating for pungency and examined stomatal density. In these populations, pungent plants exhibited 40 per cent greater stomatal density than non-pungent plants; \( t\)-test, \( t = -3.7474, \ d.f. = 7.7, \ p\)-value = 0.006, \( n = 19 \) (figure 3).

4. DISCUSSION

In Southeast Bolivia, C. chacoense populations are distributed from high-rainfall regions in the southwest to the
level significantly impacts seed viability [24]. This suggests through all of the populations as even a moderate infection fungal pathogens, we would expect pungency to sweep this is unlikely, as Tewksbury Fusarium alone maintain the observed gradient, e.g. variation in that some frequency-dependent effect on pungency might portion of pungent plants within a population (figure 1 exhibited a 40 per cent lower abaxial stomatal density plants segregating for pungency, non-pungent plants ciency and stomatal density [35]. In this study, using negative functional relationship between water-use effi-

Additionally, empirical data from diverse taxa support a between stomatal density and water-use efficiency [34]. from cultivated chilies suggests a negative relationship trade-off in resource allocation is that non-pungent pressur
tions maintains this polymorphism in pungency. Natural selection is constrained by the relationship between popu-

Dry chaco regions north and east, and they are subject to multiple biotic and abiotic selective pressures that vary in importance across this space and among years. Physiological trade-offs appear to mediate the relative advantage of pungent versus non-pungent phenotypes across this cline. Non-pungent plants from our polymorphic C. chacoense populations show clear evidence of adaptation to water-limited conditions; non-pungent plants, which have significantly lower stomatal densities on their leaves than pungent plants, produce twice as many seeds under water stress (figure 2). These plants are disproportionately found in dryer areas (figure 1a), where water stress is more common and the fitness advantage of pungency is lower, owing to a reduction in the frequency of insect and fungal attack on chili fruit [4].

The positive correlation between rainfall and the proportion of pungent plants within a population (figure 1b) suggests a fitness trade-off between protection from fungal attack (chemical defence) and costs of producing capsicin in drier environments (figure 1c). It is possible that some frequency-dependent effect on pungency might alone maintain the observed gradient, e.g. variation in Fusarium abundance or infection (vector abundance), but this is unlikely, as Tewksbury et al. [24] found that more than 90 per cent of the fruit from all 12 populations were infected with Fusarium. Given the ubiquity of these fungal pathogens, we would expect pungency to sweep through all of the populations as even a moderate infection level significantly impacts seed viability [24]. This suggests that an integrated response to biotic and abiotic selective pressures maintains this polymorphism in pungency.

A potential mechanism underlying this proposed trade-off in resource allocation is that non-pungent plants use limited water resources more efficiently through fixed differences in stomatal density. Evidence from cultivated chilies suggests a negative relationship between stomatal density and water-use efficiency [34]. Additionally, empirical data from diverse taxa support a negative functional relationship between water-use efficiency and stomatal density [35]. In this study, using plants segregating for pungency, non-pungent plants exhibited a 40 per cent lower abaxial stomatal density than pungent plants (figure 3). This suggests that pungent plants have a lower water-use efficiency and consequently suffer a fitness cost under limited water conditions. This finding is consistent with other studies that have reported trade-offs in the production of defensive compounds under limited resource conditions [36].

The putative genetic correlation between stomatal density and pungency shows some degree of overlap in the full range of recombinants. Thus, while we failed to detect a recombinant pungent phenotype that overlapped the mean stomatal density of non-pungent plants, it is not clear from these data whether this genetic correlation is sufficient to constrain pungency in drier environments on its own. A plausible alternative is that pungency is constrained by the combination of the genetic correlation between pungency and stomatal density and relaxed selection on pungency [24].

The genetic correlation with stomatal density could be the result of tight linkage or pleiotropy. Although our data do not allow us to distinguish between these mechanisms, two lines of evidence suggest pleiotropy. First, our focal populations tend to be in Hardy–Weinberg equilibrium (D. C. Haak 2011, unpublished data) for presumably neutral microsatellite loci. Second, production of capsai-

Natural selection is constrained by the relationship between populations and their environments as well as by interactions among traits within organisms [40]. Although we do not know whether the genetic correlation between pungency and stomatal density is a result of linkage disequilibrium or pleiotropy, the result is ecological specialization, in which high performance in one environment carries a significant cost in other environments [7]. In contrasting environments, pungent and non-pungent chilies from the same population display fitness differences clearly attributable to changes at the pungency locus and perhaps its influence on stomatal density (a proxy for water-use efficiency). Thus, we propose that pungent C. chacoense plants are limited from adapting to the driest regions in this cline by a trade-off with water-use efficiency, thus explaining the gradient in proportion of pungent plants (figure 1a). More generally, stomatal density and pungency trade-off to limit the evolution of pungency, supporting a central tenet of evolutionary ecologie-
tory theory—that divergent natural selection can drive population differentiation [41].

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