Mechanisms maintaining species differentiation: predator-mediated selection in a *Bombina* hybrid zone

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SUMMARY

Mechanisms which prevent gene flow will maintain differentiation between species, and therefore contribute to biological diversity. We describe an experimental study of such mechanisms in a hybrid zone between the fire-bellied toad *Bombina bombina* and the yellow-bellied toad *B. variegata*. In this system, preference for different breeding habitats reduces the frequency of hybridization. A comparison of habitat ecology shows that the semi-permanent ponds in which *B. bombina* usually breeds have higher densities of aquatic predators than the temporary puddles typically used by *B. variegata*. We test for behavioural adaptations in tadpoles to these different levels of predation. *B. bombina* tadpoles are significantly less active than *B. variegata*, both before and after the introduction of a predator to an experimental arena; this reduces their vulnerability as many predators detect prey through movement. Behavioural differences translate into differential survival: *B. variegata* suffer higher predation rates in laboratory experiments with three main predator types (*Triturus* sp., *Dytiscus* larvae, *Aeshna* nymphs). This differential adaptation to predation will help maintain preference for alternative breeding habitats, and thus serve as a mechanism maintaining the distinctions between the two species.

1. INTRODUCTION

If two taxa interbreed naturally and produce viable, fertile hybrids, the persistence of numerous morphological, genetic and physiological differences between them requires explanation. Mechanisms which prevent the taxa from merging will contribute to the maintenance of biological diversity. These mechanisms may be genetic, environmental, or a combination of both; for example, adaptation of different genotypes to different ecological niches may play a significant role. We describe here an investigation of one such mechanism in two hybridizing taxa of fire-bellied toad (genus *Bombina* (Anura: Discoglossidae)).

The fire-bellied toad *Bombina bombina* is found in the lowlands of central and eastern Europe, whereas the yellow-bellied toad *Bombina variegata* occurs at higher altitudes of southern and western Europe; after common usage, we refer to the two as separate species. Divergence occurred during geographic isolation an estimated 2–7 million years ago. Following postglacial range expansion, their distributions now meet at altitudinal transitions, overlapping slightly (Arntzen 1978). Despite many morphological, life history and biochemical differences, the two species interbreed within the region of overlap and produce viable, fertile hybrids, resulting in a stable hybrid zone usually less than 10 km wide (Szymura 1993, and references therein).

The two species show a preference for different breeding habitats: *B. bombina* breeds mainly in semi-

permanent ponds, whereas *B. variegata* is a characteristic puddle breeder (Madej 1973; Arntzen 1978; Barandun 1995). Although interbreeding will continually break down original gene combinations, preference for alternative habitats is maintained within the hybrid zone, generating an association between habitat type and the genotype of individuals found there (MacCallum 1994; Bugter *et al.* 1995). For example, at the centre of our study site in Croatia, the frequency of individuals with more than half *B. variegata* alleles (at four diagnostic allozyme loci) was 9% in two large ponds, compared with a frequency of 61% in immediately adjacent puddles formed in wheel-ruts (MacCallum 1994). The preference appears most strong amongst *B. variegata* and *B. variegata*-like hybrids. Frequent desiccation of puddles forces migration in search of new sites, and yet the association remains consistent despite the availability of both habitat types within individual toads' dispersal ranges. To be maintained in areas of interbreeding, the habitat preference must have adaptive advantages. From an ecological perspective, these advantages would explain the occupation of different environmental niches; from a population genetics perspective, they reduce the potential for hybridization and so constitute a barrier to gene flow.

The different breeding habitats favoured by the two *Bombina* species can be classified by their permanence. The relative longevity of ponds creates a very different aquatic environment from ephemeral puddles, which will necessarily contain little vegetation and only the most opportunistic fauna. In particular, the abundance of potential aquatic predators, both vertebrate and

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invertebrate, on amphibian eggs and larvae should be higher in ponds. Previous studies have demonstrated the importance of interspecific interactions such as predation on larvae in determining species composition in anuran guilds (Morin 1983; Wilbur *et al.* 1983; Woodward 1983; Morin 1986; Cortwright & Nelson 1990). We therefore consider the potential role of predation in defining differential selection pressures within the *Bombina* system, and ask specifically whether predation could explain *B. variegata*'s avoidance of the pond habitat.

We test the hypothesis that predation pressure in ponds, *B. bombina*'s preferred habitat, is higher than in puddles, and that *B. variegata*'s avoidance of ponds is therefore associated with inferior adaptation of its tadpoles to a predator-rich environment. In such an environment, any trait which reduces the risk of mortality from predation should be strongly favoured. Movement, in addition to increasing encounter rate, is a cue to many aquatic predators which detect their prey using visual or mechanosensory reception (e.g. Richards & Bull 1990). A tadpole moving around foraging will therefore be at greater risk of predation than a less active one (Werner & Anholt 1993). Differences in microhabitat use, morphology and palatability will also determine the relative vulnerability of both species to predation. We consider three predictions from the above hypothesis:

- (1) the abundance of predators is higher in ponds than in puddles during the *Bombina* larval period;
- (2) the behaviour of *B. bombina* tadpoles makes them less vulnerable than *B. variegata* tadpoles to a predator: specifically, *B. bombina* tadpoles are less active;
- (3) *B. bombina* tadpoles suffer lower mortality rates from predators than *B. variegata*.

2. MATERIALS AND METHODS

(a) Study site

The study site is located around the village of Peščenica, 25 km south-east of Zagreb, Croatia, at an altitudinal transition between low, forested hills and arable flood plains of the River Sava. In this area, the distance across the hybrid zone between pure *B. bombina* and pure *B. variegata* populations is approximately 10 km (MacCallum 1994); altitude changes from *ca.* 100 m to 280 m. Both habitat types, ponds and puddles, are found within the hybrid zone; the majority of puddles are formed in tractor wheel-ruts.

(b) Ecological surveys

Five breeding sites of each habitat type, distributed across the hybrid zone, were surveyed. Predator abundance was compared using a catch-per-unit-effort approach: at two locations in each site, three sweeps with a metal sieve (25 cm diameter) were made. Sweeps were taken immediately below the water surface: preliminary samples with bottle traps indicated that, in deeper ponds, *Bombina* larvae were found in higher strata; depth was also constrained by the shallowness of wheel-ruts. All fauna caught were identified at least to the level of family; the abundance per sample of those known to be major tadpole predators (Fitter & Manuel 1995) and shown in pilot trials to attack *Bombina* larvae was then compared.

(c) Behavioural experiments

Bombina variegata eggs were collected from breeding sites on one side of the hybrid zone, *B. bombina* eggs from the other side. Within a site, egg batches were taken from as widespread an area as possible, in order to maximize the number of families sampled, and not all clusters were taken. Eggs were reared in laboratory aquaria; after hatching, larvae were provided with abundant food in the form of powdered nettle leaves. Eggs were collected between 24–28 May 1995, and experiments conducted between 9–16 June 1995, by which time tadpoles had reached Gosner developmental stages 26–28 (Gosner 1960). Within a trial, tadpoles were matched according to developmental stage and, as far as was possible, on size.

For predation trials we used the most common species of aquatic predators, as determined by the ecological surveys: final instar *Aeshna* (hawker dragonfly) nymphs, final and penultimate instar *Dytiscus* sp. (great diving beetle) larvae, *Triturus dobrogicus* (Danube crested newt) and *T. vulgaris* (smooth newt) adults. These were fed on tadpoles of other anuran species (*Rana* and *Hyla* sp.), until 24 h before an experiment.

Fresh non-chlorinated water at 20 °C was used for each trial, thus preventing possible carry-over of semiochemical cues (Petranka *et al.* 1987). Tadpoles were transferred to opaque plastic containers containing water and abundant food (nettle powder) 1 h prior to an experiment, during which acclimatization period container lids were left on.

(i) Experiment I: activity levels

Activity levels of the two species before and after the introduction of a predator were compared. For each trial, five tadpoles (at Gosner developmental stages 27–28) of the same species were placed together in a 2-litre plastic container. Behaviour was scored by an observer standing motionless approximately 1 m away; observations were dictated. We calculated an activity index of the mean number of tadpoles in the group of five which showed any movement during a series of 5 s observation periods taken at 1 min intervals; each series consisted of 12 observations. This gave a value on a continuous scale between 0 and 5; no assumption was required of independence of either individuals' behaviour or of behaviour across time points.

After the first 12 observations, a single predator was added to each container. *Triturus vulgaris* were used in 20 trials, and *Dytiscus* larvae in another 20, with the two predator types assigned equally to the two tadpole species. Tadpole behaviour was measured as a function of a perceived predation threat, and not of actual predation events. To facilitate this, *T. vulgaris* adults of a size too small to damage the tadpoles were used, and the mandibles of each *Dytiscus* larva were temporarily taped down. A further 12 observations were made immediately after the predator's introduction, and an activity index for the group calculated as before.

Trials were conducted at 0900–1100 and at 1700–1900 h. A total of 40 trials were run, 20 for each species; each tadpole was only used once.

(ii) Experiment II: survival rates

Vulnerability of the two species to predation was compared using predator preference trials. For each trial, ten *B. bombina* and ten *B. variegata* tadpoles were placed together in a 6-litre container, with a moderate density of aquatic plants. Containers were covered throughout to minimize external disturbance. After the acclimatization period, a single predator was introduced into each container. The number of

trials run with each predator type were: *Dytiscus* larvae, 16; *Aeshna* nymphs, 19; *Triturus dobrogicus*, 2; *Triturus vulgaris*, 5. Each predator was used in only one trial.

Predators were left to feed until approximately half the tadpoles present had been eaten; this was determined by brief scans of the containers at 2 h intervals, and so was not always exact. Average time taken was 13.4 h (± 9.9 s.d.). The number of surviving tadpoles of either species was then recorded; tadpoles of *B. bombina* and *B. variegata* are distinguishable by their stripe patterns. Predator feeding bias was quantified using Manly's preference index:

$$i = \frac{\ln(p_v)}{\ln(p_v p_b)},$$

where p_v is the proportion of *B. variegata* surviving out of the initial ten and p_b is the proportion of *B. bombina* (Manly 1974; Cheeson 1978). The index allows for the effect of prey depletion on availability. Values range from 0 to 1, with 0.5 representing random selection of prey and 1 representing only *B. variegata* being taken. Preference indices were analysed separately for two stage groups: tadpoles at Gosner stage 26 ('hatchlings'), and those at Gosner stages 27–28.

3. RESULTS

(a) Ecological surveys

The mean abundances of different predator categories are given in table 1. In all four categories a

null hypothesis of equal abundance in ponds and puddles can be rejected. The abundance of (i) newts (*Triturus* sp.), (ii) dragonfly (Suborder: Anisoptera) and damselfly (Suborder: Zygoptera) nymphs and (iii) diving beetle (Family: Dytiscidae) adults or larvae is significantly greater in ponds. Salamander (*Salamandra salamandra*) larvae were found exclusively but only occasionally in puddles. The density of predators in pond samples was therefore substantially higher than in puddle samples.

(b) Experiment 1: activity levels

There were no tadpole groups for which zero activity was recorded, either before or after the introduction of a predator. Movement was strongly associated with feeding. Mean activity indices for each species in the absence and then presence of a predator are presented in figure 1. Table 2 contains an analysis of variance of the data.

The difference in the overall activity levels of the two species was highly significant: *B. variegata* tadpoles were consistently more active than *B. bombina*, with the parameter estimate indicating an average increase of 2.85 units. After the introduction of a predator, activity was significantly lower in both species: the overall effect was to lower the activity index by 0.58. There

Table 1. *Abundance of predator categories in pond and puddle samples*

(Means and standard errors of abundance of each category in sieve-sweep samples. Categories are as follows: 1. Newt adults: *Triturus dobrogicus*; *T. alpestris*; *T. vulgaris*; 2. Dragonfly (Anisoptera) and damselfly (Zygoptera) nymphs; 3. Diving beetle (Dytiscidae) adults and larvae; and 4. Fire salamander (*Salamandra salamandra*) larvae.)

predator category	ponds (<i>n</i> = 5)	puddles (<i>n</i> = 5)	<i>G</i> -test of equal distribution; d.f. = 1
1. newts	4.83 $\pm$ 2.41	1.33 $\pm$ 0.76	12.6; <i>p</i> < 0.01
2. dragonfly/damselfly nymphs	16.5 $\pm$ 7.07	0	137.0; <i>p</i> < 0.01
3. diving beetles	12.0 $\pm$ 3.50	0.17 $\pm$ 0.17	90.6; <i>p</i> < 0.01
4. salamander larvae	0	0.50 $\pm$ 0.22	4.16; <i>p</i> < 0.01

Table 2. *ANOVA and parameter estimates of tadpole activity levels*

(Species term compares *B. variegata* with *B. bombina*; presence of predator term includes both predator types. Time of day effect (morning or afternoon) is nested within species, to give a parameter estimate for either species. Repeated measures on each tadpole group (one before the introduction of a predator, one after) allow comparison between groups, nested within time and species.)

source	d.f.	sequential SS	<i>F</i> ratio	<i>p</i> value
species	1	17.500	91.047	< 0.001 ***
presence of predator	1	11.438	59.509	< 0.001 ***
species * predator presence	1	0.657	3.418	0.0723
time[species]	2	5.030	13.085	< 0.001 ***
group[time (species)]	36	15.593	2.253	0.0075 **
predator * time[species]	2	0.107	0.2682	0.766
error	36	7.197		
total	79	57.522		

term	parameter estimate ($\pm$ s.e.)
intercept	1.024 ($\pm$ 0.329)
species	2.848 ($\pm$ 0.450)
presence of predator	−0.575 ($\pm$ 0.139)
species * predator presence	−0.363 ($\pm$ 0.196)
<i>B. bombina</i> : time effect	0.125 ($\pm$ 0.438)
<i>B. variegata</i> : time effect	−1.958 ($\pm$ 0.438)

Figure 1. Mean activity indices (and s.e.) for *B. bombina* and *B. variegata* tadpoles before and after the introduction of a predator (see text for derivation of indices). Table 2 contains ANOVA of activity level.

was no evidence of a difference between species in response to predator introduction.

Time of day did not affect the activity level for *B. bombina*, but reduced that of *B. variegata* by nearly two units, despite controlled water temperatures. There was also significant variation between groups of tadpoles within species and time of day.

Activity level in the presence of a predator can be expressed as a proportion of the activity level before the introduction. *B. bombina*'s activity was reduced to an average of 55.12% (± 6.77 s.e.) of the original level; *B. variegata*'s to 61.04% (± 8.36). An analysis of variance of log-transformed percentages showed no difference between species (F ratio = 0.23; d.f. = 1,36; $p = 0.64$),

nor between predator types (F ratio = 1.38; d.f. = 1,36; $p = 0.25$). There was therefore no evidence that *B. bombina* reduce their activity more than *B. variegata* or vice versa, nor that the effect of a *Dytiscus* larvae was different from that of a *Triturus vulgaris* adult.

(c) Experiment II: survival rates

The Manly preference indices for the two developmental stage groups are presented in figure 2. Amongst the hatchling size class (Gosner stage 26), prey depletion was apparently random with respect to species (mean index = 0.496 ± 0.057 s.e.; t -statistic = -0.078 ; $n = 19$; $p = 0.93$). However, at the later stages (Gosner stages 27–28) *B. variegata* were more vulnerable than *B. bombina*: the mean preference index was significantly greater than 0.5 (mean index = 0.612 ± 0.045 ; t -statistic = 2.49; $n = 23$; $p = 0.02$). ANOVA of the preference index (*Triturus* sp., *Dytiscus* larvae and *Aeshna* nymphs) showed no evidence of a difference between the three predator types (*Triturus* sp., *Dytiscus* larvae and *Aeshna* nymphs: F ratio = 2.03; d.f. = 2,36; $p = 0.146$) nor of an interaction between predator type and developmental class (F ratio = 0.065; d.f. = 2,36; $p = 0.937$).

4. DISCUSSION

The results confirm the prediction that, during the *Bombina* larval period, predator density is higher in the semi-permanent ponds in which the fire-bellied toad *B. bombina* breeds than in the temporary puddles which are the typical habitat of the yellow-bellied toad, *B. variegata*. In experimental trials, *B. bombina* tadpoles differed from *B. variegata* in their activity levels: *B. bombina* were consistently less active and spent less time moving around feeding than *B. variegata*. Both species responded to the disturbance induced by introduction of a predator by reducing activity, but *B. variegata* still remained more than twice as active. Once past the hatchling stage, *B. variegata* tadpoles suffered higher levels of mortality than *B. bombina* in predator choice experiments. We were restricted by availability of hatchlings at Gosner stage 26, using them for Experiment II only. Whilst a similar comparison of their activity levels would provide a more complete picture, we know that at this stage tadpoles are largely inactive (Lawler 1989; personal observations), so it is unlikely that their inclusion would affect our conclusions.

Do the behavioural differences demonstrated imply adaptation to different breeding habitats? Development under the risk of predation creates a trade-off between resource-acquisition and predator avoidance (Wilbur & Fauth 1990; Werner & Anholt 1993; Skelly 1995). Low activity rate will reduce vulnerability, but will also decrease foraging rate and, therefore, growth and development rate. Conversely, higher activity levels facilitate faster development rates and so a shorter time to reaching either a size refugia (e.g. Richards & Bull 1990) or metamorphosis: the average larval period for *B. variegata* is 87% of that for *B. bombina* (Nürnberg *et al.* 1995). This implies that *B. variegata* tadpoles are at risk for less time (a frequently

Figure 2. Distribution of Manly preference index (see text for derivation) from predator preference trials for two categories of larval developmental stage.

overlooked point). The benefit of higher activity might therefore outweigh the cost of increased vulnerability in a predator-rich environment. However, physiological constraints should impose diminishing returns on increases in development rate with time active, whereas the probability of an encounter with a predator will increase linearly with time active. The incremental benefit to development rate of increased activity would be lower than the incremental cost in terms of predation rate (Werner & Anholt 1993). A less active species will therefore be better adapted to an environment in which predation is a regulating factor, in spite of the longer larval period incurred. Our results imply that the response to the growth–mortality trade-off differs between *B. bombina* and *B. variegata* tadpoles as predicted by the ecology of their usual breeding habitats; the genetically-determined behavioural patterns demonstrated here will serve to increase *B. bombina*'s fitness relative to *B. variegata* in a semi-permanent pond.

Differences in tadpole behaviour and susceptibility to predation have been found in other taxa and, similarly, have been invoked to explain species' distributions along environmental gradients or occupation of alternative ecological niches (Morin 1983; Woodward 1983; Lawler 1989; Chovanec 1992; Werner & McPeck 1994; Skelly 1995). The data are consistent with the hypothesis that predator-rich environments should contain less active species. We do not, however, know of any other study where the contrast has been drawn between two regularly interbreeding species. Traits characteristic of either *Bombina* species are in strong statistical association (linkage disequilibrium) within hybrid populations (MacCallum 1994); selection on differences at the larval stage will therefore cause a correlated advantage of habitat preference in adults. Simulation models confirm that a selectively-advantageous habitat preference, by forcing assortative mating, will play an important role in maintaining the integrity of the genomes of two hybridizing taxa (L. Kruuk, in preparation).

Further modes of natural selection act to maintain the *Bombina* hybrid zone. There may be differential adaptation to the puddle habitat: *B. bombina*'s slower development rate (Nürnberg *et al.* 1995) will reduce its fitness relative to *B. variegata* in temporary water bodies where desiccation is often the main source of mortality (e.g. Skelly 1995). Hybrid unfitness will also create a barrier to gene flow (Barton 1983, 1986) between the two species. It is known that morphological abnormalities and embryonic mortality are higher in hybrids than in either parental form (Madej 1965; Czaja 1980; Koteja 1984; all cited in Szymura & Barton 1986; L. Kruuk & J. Gilchrist, unpublished data). Finally, environmental factors such as the effect of terrestrial habitat on adult survival require further investigation, as does the role of competition between adults for mates and territories.

A combination of environmental and genetic factors therefore interact to maintain the genetic integrity of two populations despite their lack of reproductive isolation. Differential adaptation of larvae to predation

pressures is one of a suite of traits that prevent the merging of *B. bombina* and *B. variegata*. We have shown that experimentation can give insight into the relevant interactions, and so clarify the mechanisms maintaining differentiation between species.

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