Mechanisms for partial reproductive isolation in a *Bombina* hybrid zone in Romania

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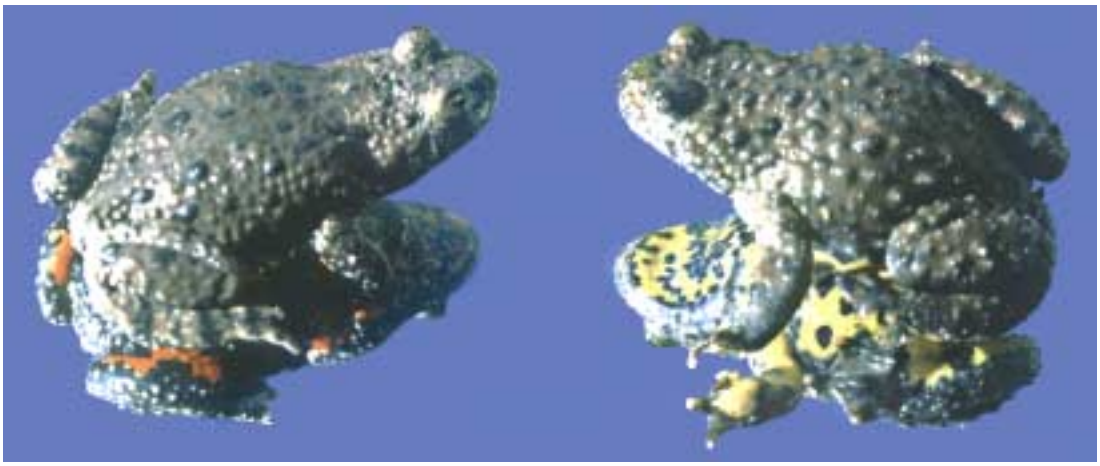

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Sonja Köhler

aus Bocholt

1. Gutachter: PD Dr. Beate Nürnberger
2. Gutachter: Prof. Dr. Wolfgang Stephan

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Abstract

Differences between taxa which have developed in allopatry can contribute to reproductive isolation in the case of secondary contact. Hybrid zones are ideal study systems in which to investigate the role of pre- and postzygotic mechanisms for the reduction or inhibition of gene flow. This thesis describes a hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata* in Romania.

The spatial arrangement of populations in this hybrid zone resembles a broad mosaic, with *B bombina* restricted to scattered big ponds and *B. variegata*-like hybrids occupying the surrounding less permanent water bodies. This structure is in striking contrast with the steep clinal transitions found in hybrid zones in Croatia, Poland and the Ukraine. A detailed comparison between the transects in Romania and Croatia revealed that the underlying distribution of habitat is the most likely factor determining the structure of a *Bombina* hybrid zone. Furthermore, habitat preference is stronger in Romania than in Croatia. Despite habitat preference, *B. bombina* adults occasionally migrate out of ponds and reproduce in intermediate habitat, thus causing introgression at neutral markers in the *B. variegata*-like populations there. In Vines et al. (in press), we used the genetic structure to quantify this migration and then assessed how much selection is required to counteract the breakdown of adaptive differences. The necessary level of selection is plausible but neutral divergence is probably collapsing.

Breeding site preference in adults and natural selection in embryos and tadpoles may be important forces against immigrant *B. bombina* alleles in *B. variegata*-like populations. I found a consistent shift in breeding habitat preference towards *B. variegata* in intermediate habitat. I also quantified natural selection in tadpoles as this should constitute a similarly important but postzygotic mechanism for partial reproductive isolation. There was significant intrinsic selection against *B. bombina* alleles in *B. variegata*-like families. This fits the prediction that selection should be against immigrant *B. bombina* alleles rather than heterozygotes. There was no direct evidence for extrinsic selection in tadpoles, although it is strongly suggested by breeding habitat preference in adults. This issue is worth further investigation. I also investigated tadpoles after selection at the phenotypic level. *B. variegata*-like tadpoles grow and develop faster than *B. bombina*-like ones in intermediate habitat, which affords them an adaptive advantage in the face of desiccation. Considering phenotypic plasticity, *B. bombina*-like tadpoles show the same high level and continuous range as *B. variegata*. This finding is probably related to the high rate of introgression in the Romanian hybrid zone.

I showed that habitat preference and selection are important mechanisms for the maintenance of reproductive isolation in this *Bombina* mosaic hybrid zone and may play an important role for reproductive isolation in incipient species.

Abbreviations

cm	centimeter
dNTP	Desoxyribonucleosidtriphosphate
EDTA	Ethylenediaminetetraacetate
km	kilometer
m	meter
M	molar
mM	millimolar
min	minutes
rpm	rotations per minute
SDS	Sodium Dodecyl Sulfate
TA	Tris-acetate
TBE	Tris/Borate/EDTA
TNES	Tris/NaCl/EDTA/SDS
V	volt

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1 INTRODUCTION

1.1 Overview

Speciation is the process of reproductive isolation and differentiation by which new species are formed from a single ancestral population. This very process is one central subject of evolutionary biology. How did the current diversity of organic life develop from a few simple forms? Theories concerning ”The origin of species” have been debated since the publication of Darwin’s (1859) landmark text. However, the speciation process has rarely been observed due to the time scales involved. This renders the direct testing of hypotheses difficult. Rather, one draws indirect conclusions about historical speciation events from recent degrees of relationship across clades. Even more fruitful for insights into the speciation process itself is the investigation of reproductive isolation mechanisms in incipient or hybridizing species. The two foci of this thesis are two reproductive isolation mechanisms in the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata* in Romania. First, the environment is an important determinant of the dynamics and the pattern of genotypes. Second, I determine the mode and strength of natural selection on hybrid tadpoles in this system. Data on the importance of these issues in nature are essential for progress on any general theory concerning the process of speciation.

The study of speciation is complicated by a multitude of opinions on how to define a species. Since speciation is a continuous process but species are discrete categories, the decision of ”when” to attribute the species status to a population is difficult. Therefore, the definition of a species remains subject of different conceptual approaches with diverse theoretical priorities (for a review see Berlocher 1998, Hull 1997, Otte & Endler 1989). Usually, species definitions are devised from a conceptual basis with specific taxonomic, phylogenetic, ecological or genetic questions in mind. The biological species concept has been most widely used by geneticists and ecologists. It was coined by Mayr (1942, 1963) who defined a species as ”groups of naturally or potentially interbreeding natural populations which are reproductively isolated from other such groups”. However, this concept suffers a lack of relevance to asexually reproducing organisms and to cases of hybridization between two species (Hull 1997). It has become

apparent that the maintenance of distinct gene pools is (at least on some limited time scale) no contradiction to the formation of fertile hybrids, for example in *Bombina*. I will therefore use the uncontroversial term “taxa” throughout this thesis when referring to *Bombina bombina* and *B. variegata*.

The species concept of common gene pools is immediately linked to the two issues that characterize the speciation process. How does phenotypic divergence proceed at the genetic level? Which mechanisms prevent gene flow between diverging gene pools? How are the two issues related? I will use the term ”reproductive isolation” to refer to any heritable trait that prevents gene flow despite migration between populations thus keeping alleles between them separate. Reproductive isolation may be apparent either before or after the formation of a hybrid zygote, i.e. either pre- or postzygotic. Traits involved in reproductive isolation may either affect hybrid fitness directly or be of an ecological or behavioral nature and cause hybrid unfitness indirectly. Little is known about the temporal order in which reproductive isolation mechanisms appear – if there is any general pattern at all (Coyne & Orr 1998). The authors recommend comparative studies of allopatric species pairs in different stages of divergence to get a more detailed idea of how reproductive isolation evolves in allopatry. However, such studies are tedious because when investigating taxa that have already diverged considerably, it is difficult to infer from current patterns which and how many traits were involved in initial reproductive isolation and which ones diverged only after the original divergence occurred. The study system of choice are therefore populations that have diverged significantly but can still interbreed. This is the reason why hybrid zones are regarded as ideal ”evolutionary laboratories” for the study of reproductive isolation (Hewitt 1988).

In the next section (1.2) I first characterize different modes of reproductive isolation and then illustrate in which geographic context these may evolve. In doing so, I give an overview of the major issues that are discussed in the context of speciation theory, including the biogeography of speciation, the frequency of reinforcement, the roles of sexual and natural selection, and the evolution of prezygotic and postzygotic isolation. In the remainder of this Chapter, I discuss the insights into reproductive isolation that one may obtain from the study of hybrid zones (1.3). Finally, I introduce the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata* and summarize previous work on these taxa (1.4).

1.2 Reproductive isolation

1.2.1 Modes of reproductive isolation

Prezygotic isolation

The speciation process is complete when full reproductive isolation is attained. This may be manifest in pre- or postzygotic reproductive isolation between two species, i.e. before or after the formation of a hybrid zygote. Prezygotic isolation mechanisms prevent either heterospecific mating or fertilization events and hence, hybridization. Heterospecific mating may be avoided if two species differ in their use of habitats or resources in or on which mating occurs, if they differ in their timing of reproduction or, if they exhibit different male traits and the corresponding female choice for mating partners. Prezygotic reproductive isolation may still be attained after heterospecific mating by the inhibition of heterospecific fertilization through biochemical or mechanical mechanisms. These can arise from an ongoing conflict between males and females. In particular, polygynous males have a selective advantage if they carry mutations that increase their mating frequency and decrease the likelihood that females remate subsequently with other males. This in turn decreases the females' fitness, and any mutation that counteracts this effect will be favored. Such conflicts or "arms races" may lead to perpetual antagonistic co-evolution between males and females and may thus generate rapid evolutionary divergence of traits involved in reproduction. Males from other populations lacking the ability to compete with sperm or to defend themselves against female counter-measures will be outcompeted for fertilizations. Evidence for postmating, prezygotic isolation has come recently from comparative studies of speciation rates in insect species with polygamous mating versus species where females mate only once (Arnqvist et al. 2000). Preferential use of conspecific sperm when a female is sequentially inseminated by heterospecific and conspecific males has been shown in many insect groups such as grasshoppers (Bella et al. 1992), crickets (Gregory & Howard 1994), flour beetles (Wade et al. 1994) and *Drosophila* (Price 1997).

Postzygotic isolation

Postzygotic reproductive isolation mechanisms comprise the dysfunction (inviability or infertility) of hybrids after a zygote has been formed. A distinction is made between intrinsic and extrinsic postzygotic isolation. The former applies to hybrid dysfunction

that occurs irrespective of the environment due to genetic incompatibilities in hybrids if the parental species have either different ploidy levels, or different alleles that do not function properly when brought together in hybrids. Polyploidization is common in plants (Masterson 1994), but occasionally found in animals, e.g. tree frogs (Gerhardt 1994). Polyploidization results in the immediate interruption of gene flow, and the new type may then be maintained through selfing or asexual reproduction. In animals, allelic differences between species play a more important role in hybrid sterility and inviability (Coyne & Orr 1998). Extrinsic postzygotic isolation on the other hand depends on the environment in which the hybrid occurs. If the parental populations are adapted to different environmental conditions, hybrid phenotypes are intermediate and thus inferior in both parental habitats.

Since it is costly to produce inviable or infertile hybrid offspring, postzygotic reproductive isolation should theoretically entail the evolution of prezygotic isolation mechanisms in areas of hybridization. This process has been termed reinforcement (Dobzhansky 1937). However, this is to this day one of the most controversial issues in speciation (Coyne & Orr 1998). An important requirement for reinforcement is the persistent association of male traits and female choice with fitness determining loci through very strong selection against hybrids, so that strong linkage disequilibrium between mate choice traits and fitness is maintained. As will be shown below, *Bombina*, has escaped from confinements connecting these three traits.

1.2.2 The evolution of reproductive isolation

Allopatry

In which geographical context do the above mechanisms of reproductive isolation evolve? The rate of evolution of reproductive isolation is determined by the counteracting forces of migration, recombination and selection. Traditionally, the evolution of reproductive isolation in allopatry is regarded as the most important scenario of speciation (Mayr 1963). In an allopatric distribution, a physical barrier separates two populations. For example, it is generally assumed that glaciers acted as such barriers during ice ages in the northern hemisphere, forcing separate populations of a species into different refugia for many generations. If two populations become geographically isolated, gene flow between them is inhibited. Genetic divergence in allopatry requires only geographic isolation and time without other forces such as

selection or reinforcement acting (Turelli et al. 2001). This is because in isolated populations new alleles accumulate with time (via genetic drift), but cannot spread from one population to the other. Therefore, they may be incompatible with alleles fixed in the other population. However, understanding the evolution of this intrinsic isolation had posed a serious problem to evolutionary biologists for a long time. Darwin (1859) had been puzzled how something as maladaptive as hybrid nonviability or sterility could arise by natural selection. In other words, it was unclear how two genotypes descended from a common ancestor could become separated by an adaptive valley unless one of the lineages passed through the valley. The solution was finally found by Dobzhansky (1937) and Muller (1942) who proposed that with the interaction of two or more loci, hybrid nonviability or sterility can evolve without inhibition by natural selection. The model is the following: an ancestral species has the alleles *aa* and *bb*. As the two subpopulations become geographically isolated, gene flow is impeded. In one population the *A* allele arises and becomes fixed so that all genotypes are *AA bb*. Similarly, in the other population, the *B* allele is fixed at the other locus, and all genotypes in this population are *aa BB*. If both populations interbreed upon secondary contact the *A* and *B* alleles may not be compatible in *AaBb* hybrids since they have never been tested in the same genetic background. This pattern of epistasis has been termed Dobzhansky-Muller (DM) incompatibilities. It is important to note that DM incompatibilities may arise due to any out of several evolutionary forces, i.e. drift or natural selection. DM incompatibilities need not have a big effect on hybrid fitness. Rather, the simultaneous action of many DM incompatibilities, also involving interactions among three or more loci, will cause problems in hybrids. A model by Orr (1995) has shown that the strength of postzygotic isolation as well as the number of DM incompatibilities between taxa increases much faster than linearly with time once two populations are diverging, because later mutations have a higher impact on hybrid unfitness.

The evolution of genetic divergence in allopatry can be accelerated by divergent selection (Schluter 2001). Populations in allopatry will probably encounter slightly different environmental conditions where different phenotypes will be respectively favored. Therefore, at selected loci different alleles will be advantageous in different populations, and genetic divergence will accrue. Divergent natural selection is a more potent force than drift to cause postzygotic reproductive isolation in the case of secondary contact (Rice & Hostert 1993). Reproductive isolation will then be either

extrinsic (if it is functionally related to adaptive divergence) or intrinsic (as a result from allelic incompatibilities in hybrids). Sexual selection may also enhance reproductive isolation, either in direct or indirect connection with natural selection. For example, natural selection may drive adaptation to alternative habitat types or resources that are also used as mating sites. On the other hand, natural selection may favor differently adapted traits in different places. These traits may be used as species recognition mechanisms during mate choice. If such sexual selection is involved in genetic divergence, secondary contact may reveal prezygotic isolation.

Parapatry

The empirical basis for the viewpoint that allopatry is the most important geographic scenario for speciation is the observation that closely related sister species tend to be geographically separated with either allopatric or parapatric ranges and that the probability of sympatry increases with the distance of relationship (reviewed in Barraclough et al. 1998). However, given a sufficiently broad geographical range, any mechanism that can produce divergence among allopatric populations can also cause divergence in parapatry where populations' geographic ranges adjoin along a transition zone. Indeed, even if most genetic divergence arises in allopatry, the diverging populations are likely to get temporarily and spatially into contact. For divergence in parapatry, selection becomes a more important force than drift in relation to the mixing effects of migration and recombination (but see Gavrilets et al. 2000). In a single locus model, if local selection overpowers dispersal, differentiation will result in a stable cline of genotypes with prezygotic or postzygotic isolation (Slatkin 1973, Endler 1977). However, a cline might as well be the consequence of secondary contact along an ecotone between two species diverged in allopatry. Therefore, observing a cline does not reveal its causing agent, and the frequency of parapatric divergence is debatable.

Sympatry

Finally, speciation in sympatry implies that there is initially only one population that splits sympatrically into two. It requires an abrupt, considerable constraint of gene flow. As purely genetic mechanisms, polyploidization or chromosomal rearrangements can cause an instant inhibition of gene flow. Sympatric speciation through these genetic mechanisms is therefore restricted to organisms that can reproduce asexually or by selfing. Sympatric speciation in sexually reproducing organism on the other hand has received much attention over the last decade, and many empirical and theoretical studies

have supported its plausibility (reviewed in Rice & Hostert 1993, Via 2001). In this context, the primary evolution of prezygotic isolation is a necessary component of sympatric divergence. Most models of sympatric speciation assume that reproductive isolation is driven by adaptation to alternative discrete resources or habitats via strong competition. Assortative mating may arise either through resource choice and mating on the resource or through sexual selection of alternative phenotypes (e.g. in cichlid fish: Seehausen et al. 1999). Additionally, disruptive natural selection against intermediate phenotypes may drive divergence in habitat preference (Hatfield & Schluter 1999, Via 1999). If more than one gene is involved in the adaptation to one of the alternative habitats, recombination between the genes must be reduced for the set to be established (Felsenstein 1981). Many well documented cases of sympatric speciation have been found in herbivorous insects because of a highly specialized relationship with their host plants which serve as habitat and resource as well as as mating site (e.g. Bush 1969, Via 1999). A common feature of early sympatric divergence is that despite adaptive differences at selected loci, neutral loci may be introgressed from other populations as long as some gene flow occurs (Via 2001).

To summarize, genetic divergence requires only time if gene flow is restricted while uninhibited gene flow counteracts divergence between populations because alleles can spread rapidly even in the face of limited migration. On the other hand, ecological adaptation to alternative habitat types can drive the accumulation of differences between populations even at high migration rates if there is a selective disadvantage to moving into the wrong habitat.

1.3 Hybrid zones

Hybrid zones offer unique opportunities to study the nature of speciation. In the most rewarding case, the wide range of genotypes produced allows us to quantify the selective mechanisms that counteract gene flow and keep the populations distinct despite hybridization. Hybrid zones are ”regions in which individuals from two populations which are distinguishable on the basis of one or more heritable characters meet, mate and produce hybrids” (Harrison 1990). Arnold (1997) adds that this process occurs naturally and that the hybrids produced are viable and at least in part fertile. Barton & Hewitt (1985) emphasize that hybrid zones are local phenomena: in general, they are very narrow relative to the total distribution and dispersal ranges of the parental

populations. Most hybrid zones involve changes in a range of morphological and genetic characters and are therefore characterized by a cluster of parallel gradients in gene frequencies, termed clines (Haldane 1948, Slatkin 1973, Barton & Hewitt 1985, 1989). In this section, I give an overview of the origin and fate of hybrid zones, portray different types of hybrid zones and illustrate inferences that are drawn from genetic patterns in hybrid zones for the maintenance of reproductive isolation.

1.3.1 The origin of hybrid zones

There are two possible ways in which hybrid zones may originate. First is the intuitive case in which previously allopatric populations come into secondary contact. If reproductive isolation is not fully established, hybridization will occur. On the other hand, hybrid zones may arise in situ, that is between populations whose distribution ranges adjoin in parapatry and who differentiate along an ecological gradient without an initial split between them. These two scenarios of origin cannot be distinguished given some time after the onset of hybridization, because the pattern of a hybrid zone will not be determined by the way in which it evolved, but by dispersal and the mode and strength of selection (Endler 1977). Additionally, distribution ranges of species vary over evolutionary periods as they may follow climatic zones and expand into or retreat from local regions. Thus populations may evolve in allopatry and still experience occasional gene flow along narrow transition zones. Therefore, the recent structure of a hybrid zone may not shed light on its origin, but it does allow inferences about the mechanisms maintaining differentiation between the involved taxa. Estimates of the strength of habitat preference and/or assortative mating, selection and migration rates again allow predictions about the fate of the hybrid zone.

1.3.2 The fate of hybrid zones

The fate of a hybrid zone is determined by migration, the rate at which the parental genomes are broken down by recombination and by the strength of selection against hybrids. There are several possible outcomes of hybridization, ranging from hybrid speciation and the strengthening of reproductive isolation to the merging of the taxa involved (reviewed in Hewitt 1988). One scenario is the case in which reproductive isolation is insufficient to counter the merging effects of migration and recombination. Single advantageous alleles will spread as a wave front through the species' distribution

range and the clines for these traits will flatten quickly (e.g. in sunflowers: Carney et al. 2000). Similarly, neutral alleles for which the taxa differ will diffuse from one into the other, producing progressively shallower clines. Considering a single locus, the rate of diffusion depends on the balance of selection on that locus and other adjoining loci on the same chromosome and the local recombination rate dissociating them (Barton & Bengtsson 1986). Selection based on only a few loci causes a weak barrier effect for neutral variants elsewhere in the genome. Even if there are some selected differences that are maintained, the two taxa may merge into one and would not be considered distinct by any species definition.

If the barrier to gene flow is weak and hybrids fall into several distinct genotype classes whose fitness may be lower, equal or higher relative to the parental taxa, then some hybrid genotypes may persist (Arnold & Hodges 1995). The fitness of hybrid genotypes often varies with the environment (e.g. Campbell & Waser 2001). Indeed, extrinsic selection is thought to play a central role in the establishment of relatively fit hybrids. In the absence of niche differentiation, new hybrid genotypes are likely to be overwhelmed by competition and gene flow from the parental taxa (Buerkle et al. 2000). For rare hybrid genotypes to prevail, the abrupt interception of gene flow is essential. The maintenance of stable hybrid genotypes is more common in plants than in animals since the former may rely on asexual reproduction or selfing for the maintenance of the fit hybrid genotype class while in animals, recombination is likely to break up favorable combinations of alleles. If a group of hybrids is temporarily and/or spatially fitter than either parental taxon, it will predominate in these patches in space and time. This model of bounded hybrid superiority has been postulated in the big sagebrush (*Artemisia tridentata*) hybrid zone where the fitness of a genotype varies with the habitat it occupies (Wang et al. 1997, 1999). The extreme outcome of bounded hybrid superiority would be the formation of a new hybrid species. There are a number of well documented cases of homoploid hybrid speciation in plants (reviewed in Rieseberg 1997), suggesting that in plants, natural hybridization may play an important role in evolutionary diversification (e.g. in *Iris* species: Arnold et al. 1990, Emms & Arnold 1997).

A different potential fate of hybrid zones may be observed if postzygotic reproductive isolation is nearly complete. The total barrier to gene flow is strong when selection affects many loci and when these are spread evenly across the genome because then the

unit of selection tends to be the entire genome (Barton 1983). In this case, prezygotic mechanisms may quickly strengthen and reinforce postzygotic isolation provided that selection against the production of unfit offspring is more effective than recombination which tends to dissociate mate preference and fitness traits. Selection against hybrids does not, however, prevent the introgression of alleles from one population into the other as long as any fertile hybrid occurs. Intensive introgression has been reported in some studies despite considerable fitness reduction of the F1 generation (review in Arnold et al. 1999).

Most fruitful for evolutionary studies is the intermediate outcome of hybridization, when considerable divergence has developed between two taxa but fertile hybrids are still formed as is the case in *Bombina*. In contrast to the scenarios depicted above, this type of hybrid zone may remain at a stable equilibrium for many generations. Depending on the counterbalancing forces of dispersal and selection the long term outcome may be either full reproductive isolation or the merging of the two taxa.

1.3.3 Patterns of hybrid zones

Models of hybrid zones fall into two classes: dispersal-independent and dispersal-dependent hybrid zones (Barton & Hewitt 1985, 1989). In population genetics, the dispersal rate σ^2 is defined as the variance in distance between parent and offspring across a continuous landscape, while the migration rate *m* is the fraction of individuals per population within a patchy environment that were born elsewhere. In a continuous landscape and a shallow cline, the local allele frequencies are mostly determined by the local selection forces with dispersal playing a subordinate role. In dispersal-independent hybrid zones, local allele frequencies will directly reflect the local environmental conditions. For example, bounded hybrid superiority may be essentially dispersal-independent since hybrids are favored in the intermediate habitat between the two parental types (see above). In dispersal-dependent hybrid zones or step environments, dispersal and selection interact at comparable strength producing linkage disequilibrium and positive feedback among selected genes. In environmental pockets, the threshold width l_c of the pocket beyond which the locally adapted allele can persist is $l_c = \sigma / \sqrt{s}$, where σ is the mean distance between parent and offspring and *s* is the selection coefficient (Slatkin 1973). Depending on the mode of selection, there are two types of dispersal-dependent hybrid zone models. The first one is that of a tension zone, where

selection is intrinsic, against hybrid individuals (Barton & Hewitt 1985, 1989). The second one – which I will refer to as the ecotone model – is characterized by extrinsic selection along environmental gradients or discontinuities (Endler 1977).

Tension zones and ecotones

A tension zone (Key 1968) forms when the distribution ranges of two differentiated taxa adjoin in parapatry and reproductive isolation is principally in the form of postzygotic allelic incompatibilities. Assuming a diffusion model of dispersal in a continuous environment, gradients in allele frequencies follow clines, which can be described by sigmoidal curves along the transition from one taxon into the other. These can be moved from place to place by a variety of fitness and density factors (Barton & Hewitt 1985). Due to the pushing forces of alleles from both sides, tension zones tend to minimize their length, hence their name. For example, a tension zone will move in favor of a fitter allele and, if the density or dispersal rates are not equal, in the preferred direction of dispersal (e.g. downwind) and towards regions of low density (May et al. 1975, Nagylaki 1976). Tension zones are likely to get trapped at physical barriers or areas of low density such as mountain ranges, rivers or inhospitable habitat, where dispersal is hampered. This feature leads to tension zones being easily confused with ecotones, which are similarly found along ecological gradients. Another feature of tension zones is the concordance (identical shape and width) and coincidence (consistent place) of clines at a wide range of traits. This is due to the fact that dispersal and recombination are constantly counteracted by selection against hybrids so that alleles at different loci only recombine away from each other with considerable delay. Significant linkage disequilibria are generated by the dispersal of parental allele combinations into the center of the hybrid zone (Slatkin 1975). However, since allelic incompatibilities through drift alone accumulate rather slowly and only in allopatry or with the help of drift in small populations, it is rather likely that ecological divergence plays an additional role in many hybrid zones.

An ecotonal hybrid zone results if two ecologically differentiated taxa interbreed in a zone of secondary contact which coincides with an environmental selective gradient. Adaptive differences will be maintained even when they are only weakly selected (Haldane 1948), while differences in neutral traits may again break down via recombination. So, despite the fact that the nature of selection differs, the observable outcomes of hybridization in ecotones and in tension zones resemble each other: we

find a set of clines and significant linkage disequilibrium at environmental barriers (Kruuk et al. 1999b).

After reviewing a range of hybrid zones, Barton & Hewitt (1985) concluded that most hybrid zones are in fact tension zones and thus maintained entirely by hybrid dysfunction. This conclusion elicited considerable debate. Examples of hybrid zones where the tension zone model is applicable include the atlantic mussels *Mytilus edulis* and *M. galloprovincialis* (Bierne et al. 2002), the hybrid zone between two karyotypic races of the common shrew *Sorex araneus* in Britain (Hatfield et al. 1992) and hybridization between the two land snail species *Albinaria hippolyti aphrodite* and *A. h. harmonia* in Greece (Schilthuizen & Lombaerts 1995) just to name a few. A less clear case is the hybrid zone between two subspecies of the grasshopper *Chorthippus parallelus* in the Pyrenees. Laboratory crosses between the two pure taxa have revealed hybrid male sterility, though less hybrid dysfunction than expected has been observed in the field as well as non-coincident clines for hybrid dysfunction (Virdee & Hewitt 1994), meaning that hybrids are not universally unfit as assumed in the tension zone model. Similarly, the hybrid zone between the two European subspecies of the house mouse (*Mus musculus domesticus*, *M.m. musculus*) seems to fit the tension zone model: the hybrid zone is very narrow, patterns of introgression are similar in all transects, and hybrids have a genetically determined higher susceptibility to intestinal worms (Sage et al. 1986). However, the clines are not in concordance and even an increased developmental stability in mixed populations has been found (Alibert et al. 1994). The hybrid zone between the water strider species *Limnoporus dissortis* and *L. notabilis* in Canada also resembles a tension zone in some features, e.g. a steep cline at a sex-linked locus and intrinsic genetic incompatibilities in hybrids (Sperling & Spence 1991). However, there is differential habitat association between the two species. Another example is the hybrid zone between the hard clam species *Mercenaria mercenaria* and *M. campechiensis* in Florida lagoons. In this case, reduced hybrid fitness supports the tension zone model while differential, environmentally mediated selection affecting both the parental and hybrid genotype classes suggests that the ecotone model may also be applicable (Bert & Arnold 1995). A purely ecotonal hybrid zone exists for example between the plant species *Prunella grandiflora* and *P. vulgaris* in Canada. The parental taxa exhibit different clonal growth strategies in adaptation to specific soil types while hybrid plants show intermediate performance in the parental habitat and better performance in intermediate patches (Fritsche & Kaltz 2000).

These examples illustrate that inferences about the predominant mode of selection in a hybrid zone from either the geographical pattern in genotype distribution or from hybrid performance in the laboratory are not unambiguous. Evidence for which mode of selection predominates in a hybrid zone can only come from studies investigating the fitness of different hybrid and parental genotype classes directly in alternative habitat types. What matters more for the fate of a hybrid zone is the relationship between recombination and total selection, because only a strong barrier to gene flow can hold clines in different traits together and significantly slow introgression at neutral loci. If the area covered by both populations is very large relative to their dispersal range, the movement of animals can be approximated by diffusion (Haldane 1948, Nagylaki 1975). This simplifies the analysis of a hybrid zone. The following holds both for tension zones and for environmental steps.

Historically, the theory describing clines has been based on single locus models (Haldane 1948, Slatkin 1973, May et al. 1975, Endler 1977, Barton 1979). Later Barton (1983) developed a multilocus model, allowing for the strong linkage disequilibria between loci found in many hybrid zones (e.g. *Heliconius*: Mallet & Barton 1989, *Bombina*: Szymura & Barton 1986, 1991). The techniques for the analysis of hybrid zones comprise three steps which I will explain in this order below: 1) to estimate the strength of selection from the cline width; 2) to estimate the dispersal rate from the observed linkage disequilibrium between alleles in neutral loci and 3) to calculate the barrier to gene flow of a locus from the ratio of the step in the center of the cline and the gradient at the edges.

1) When there is spatially varying selection in the form of an environmental step such that alternate alleles are favored at a given locus, a sigmoidal allele frequency cline results. The width of the cline is defined as the inverse of the maximum slope (Barton 1983). The width of a cline is again equal to the characteristic length scale of the variation of gene frequencies l_c if the cline is maintained by a balance between dispersal and selection (Slatkin 1973, May et al. 1975). Once the dispersal rate has been estimated from the linkage disequilibrium (see below) and the cline width has been measured directly, one may obtain estimates of the selection strength. Although it is possible to test for the magnitude of selection by examining zone width, it is problematic to use these estimates to discern the form of selection, i.e. whether it is mainly extrinsic or intrinsic (Emms & Arnold 1997), which has

generally little effect on the shape of clines (Barton & Gale 1993, Kruuk et al. 1999b).

2) If the two parental taxa are fixed for alternate alleles at a sample of marker loci, any migrant into the hybrid zone is more likely to carry sets of alleles characteristic of its source population than of the destination. Alleles typical for one population will occur together in the same individual, and alleles at physically unlinked loci will be associated non-randomly within populations. This association has been termed linkage disequilibrium (*D*). In the neutral case *D* is halved with every generation of random mating through recombination between physically unlinked loci. This process may be counteracted by selection against hybrids. High levels of *D* can only be maintained by ongoing influx of pure individuals into the hybrid zone, whereas selection is mainly responsible for the maintenance of the clines. Therefore, linkage disequilibrium may be used to estimate dispersal, given the rate of recombination between the loci (Barton & Gale 1993).

3) Instead of a smooth sigmoidal curve many hybrid zones exhibit a sharp step at the center of the cline at any one locus. This sharp step indicates a barrier to gene flow between two hybridizing taxa. The net effective selection over many linked loci can result in a sharp step at the center of the cline (Barton 1983, Szymura & Barton 1986). Despite this sharp step, foreign alleles may still penetrate far into either side of the hybrid zone. The barrier to gene flow, *B*, is measured as the ratio of the step in allele frequency at the center of the cline and the gradient at the edges (Nagylaki 1976). *B* has the units of distance and corresponds to the length of unimpeded habitat that would prevent gene flow in neutral loci in an equal way (Szymura & Barton 1986, 1991). *B* may be used to estimate the expected time an allele at a neutral locus may need to pass the barrier given an estimate of dispersal, recombination and selection at the locus in question.

To summarize, the outcome of hybridization is determined by the counterbalancing forces of dispersal, recombination and selection. The analysis of steep clines allows inferences about how genomes of diverged populations interact and predictions about the fate of a hybrid zone. These are invaluable for the interpretation of data on the impact of single isolating mechanisms which can only be obtained directly from the hybrid zone.

Mosaic hybrid zones

When two ecologically differentiated taxa interbreed upon secondary contact, the outcome of hybridization will depend on the distribution of habitat. The ecotone model described above will only apply in a hybrid zone that occurs along a steep ecological gradient. But if the two habitat types are intermingled in a patchy distribution, a mosaic of different genotypes with an associated increase in hybridization will be the result (Harrison & Rand 1989). If there is no habitat preference, local associations between genotype and habitat type can only be maintained if the patches exceed a critical size of l_c (Slatkin 1973). But if there is differential habitat preference and/or assortative mating or mating on the resource, then divergence of loci under selection or those determining habitat choice can be preserved even in habitat patches that are smaller than the dispersal range. However, since parental differences that do not provide any adaptive advantage in either habitat type are eroded by recombination, these traits will introgress from one parental taxon into the other. The distribution of genotypes in a mosaic hybrid zone may then resemble the situation in early sympatric speciation by ecological differentiation (Via 2001). There are some well-studied cases of mosaic hybrid zones. For example, the two cricket species *Gryllus pennsylvanicus* and *G. firmus* are adapted to different soil types and form a mosaic hybrid zone in Connecticut where the two habitat types are found closely adjacent (Rand & Harrison 1989). At patch boundaries however, steep coincident and concordant clines are found at selected loci (Ross & Harrison 2002). Therefore, a mosaic hybrid zone may appear clinal at one scale and mosaic at another. Furthermore, mosaic hybrid zones need not necessarily be maintained by extrinsic selection alone. Additional intrinsic selection against hybrids may play an important role in maintaining reproductive isolation, as assumed for the mosaic hybrid zone between the ground crickets *Allonemobius fasciatus* and *A. socius* in New Jersey (Howard et al. 1993), the two genetic types of the grass shrimp *Palaemonetes kadiakensis* in Texas (Garcia & Davis 1994) and the fire ant species *Solenopsis invicta* and *S. richteri* in the southern USA (Shoemaker et al. 1996).

Mosaic patterns can also arise in homogeneous metapopulations due to drift if there are few migrants between demes or if the neighborhood is small (Wright 1943). In this case however, one would not expect an association between allele frequencies and habitat. Considerable drift occurs when long distance migrants move into the space between two differentiated populations, but as soon as secondary contact has been fully established, the pattern of the hybrid zone is dominated by dispersal and selection. However, if the

space recurrently invaded by the pure long distance migrants consists of unoccupied habitat patches, the mosaic pattern may be preserved over a long time scale (Nichols & Hewitt 1994). This mechanism has been postulated for the hybrid zone between the grasshoppers *Chorthippus brunneus* and *C. jacobsi* (Bridle et al. 2001).

1.4 *Bombina* hybrid zones

This thesis focuses on a hybrid zone between the fire-bellied toads *Bombina bombina* and *Bombina variegata*. Despite their considerable divergence, the two taxa interbreed regularly and produce fully fertile hybrids. This renders them a unique study system in which to detect mechanisms that generate partial reproductive isolation. A thorough literature review about *Bombina* and hybridization in this system is given by Szymura (1993). In this section I give a brief description of the two taxa, followed by a compilation of studies conducted so far on *Bombina* hybrid zones.

1.4.1 The genus *Bombina*

The genus *Bombina* is one of only four members in the family Discoglossidae although some authors define a separate family Bombinatoridae (arguments summarized in Günther 1996). Discoglossids are characterised by a disc shaped tongue, a mostly aquatic life style and a rather small body compared to real toads. Other typical Discoglossid characteristics are a medioventral spiracle in the tadpole and an inguinal amplexus. The family predominantly occurs in Europe and is one of the oldest amphibian families (Duellmann & Trueb 1994). *Bombina* adults reach a maximal snout-vent length of 5 cm, are brown to gray and have a warty, glandular skin. When attacked, they display a bright ventral warning color in an aposematic arching of their body – the so-called Unkenreflex – to advertise that they are unpalatable (Bajger 1980).

1.4.2 Biogeography

The origin of the two *Bombina* taxa is thought to be the result of the splitting of an ancestral Laurasian population during a Pliocene glaciation period, between 2 to 6 million years ago. The current view is that during that time in allopatry, the group that retreated into eastern lowlands developed into *B. bombina* while in the western refuges the mountainous form *B. variegata* originated. Fossils of *B. bombina*-like animals found

in Poland and of both *B. bombina* and *B. variegata*-like animals in Slovakia that probably date back to the Upper Pliocene suggest that at that time, the two taxa had already split (reviewed in Szymura 1993). This view is supported by the application of a molecular clock to albumin data which proposes that the split occurred about 2 million years ago (Maxson & Szymura 1984). A different analysis of allozymes suggests an even earlier split of the two taxa, about 6.8 million years ago (Szymura 1983). Although the accuracy of both biochemical and paleontological evidence may be doubted, Szymura (1993) argued that the two taxa are probably older than previously assumed by Mertens (1928) and Arntzen (1978) who suggested the split having occurred in the Pleistocene. The ranges of the toads must have contracted and expanded periodically following ice-sheet movements during Pleistocene glaciations (Arntzen 1978). During periods of strong glaciation, subpopulations were presumably restricted to various refugia: the plains bordering the Black Sea for *B. bombina*, and southern Italy and two patches on the Balkan peninsula for *B. variegata*, which lead to the formation of the three main subgroups of the latter taxon (see Szymura 1993). When the climate finally ameliorated, the two taxa spread over Europe into their current distribution ranges. Arntzen (1978) proposed that *B. bombina* migrated up the Danube valley and thus entered the Hungarian Plain from the south-east, while *B. variegata* mainly spread into a western direction and north to the Carpathians (Figure 1.1).

Today *B. bombina* occupies lowland plains in eastern and northern Europe whereas *B. variegata* occupies mountainous regions throughout western, southern and central Europe. It is divided into three subgroups: *B. v. pachypus* in Italy, *B. v. scabra* in Greece and *B. v. variegata* in western and central Europe (Figure 1.1). There are isolated populations of *B. variegata* on hills scattered over the Danubian plain today. These suggest that *B. variegata* once had a wider distribution in this region but has since been displaced by *B. bombina* as the latter invaded the lowlands of the Hungarian Plain. Arntzen (1978) proposed that this displacement was a continuous process of hybridization and outcompeting. However, *B. variegata* might as well have retreated as the climate changed gradually, leaving no suitable habitat in the lowlands. The current distributions of the two taxa are parapatric, overlapping slightly at the altitudinal transitions at which they meet. Hybridization occurs where the distribution ranges adjoin or overlap. The resulting hybrid zone extends from Austria for about 3000-4000 km along the southern edge of the Danube valley to the Black Sea and completely surrounds the Carpathian mountains along their foothills (Szymura 1988).

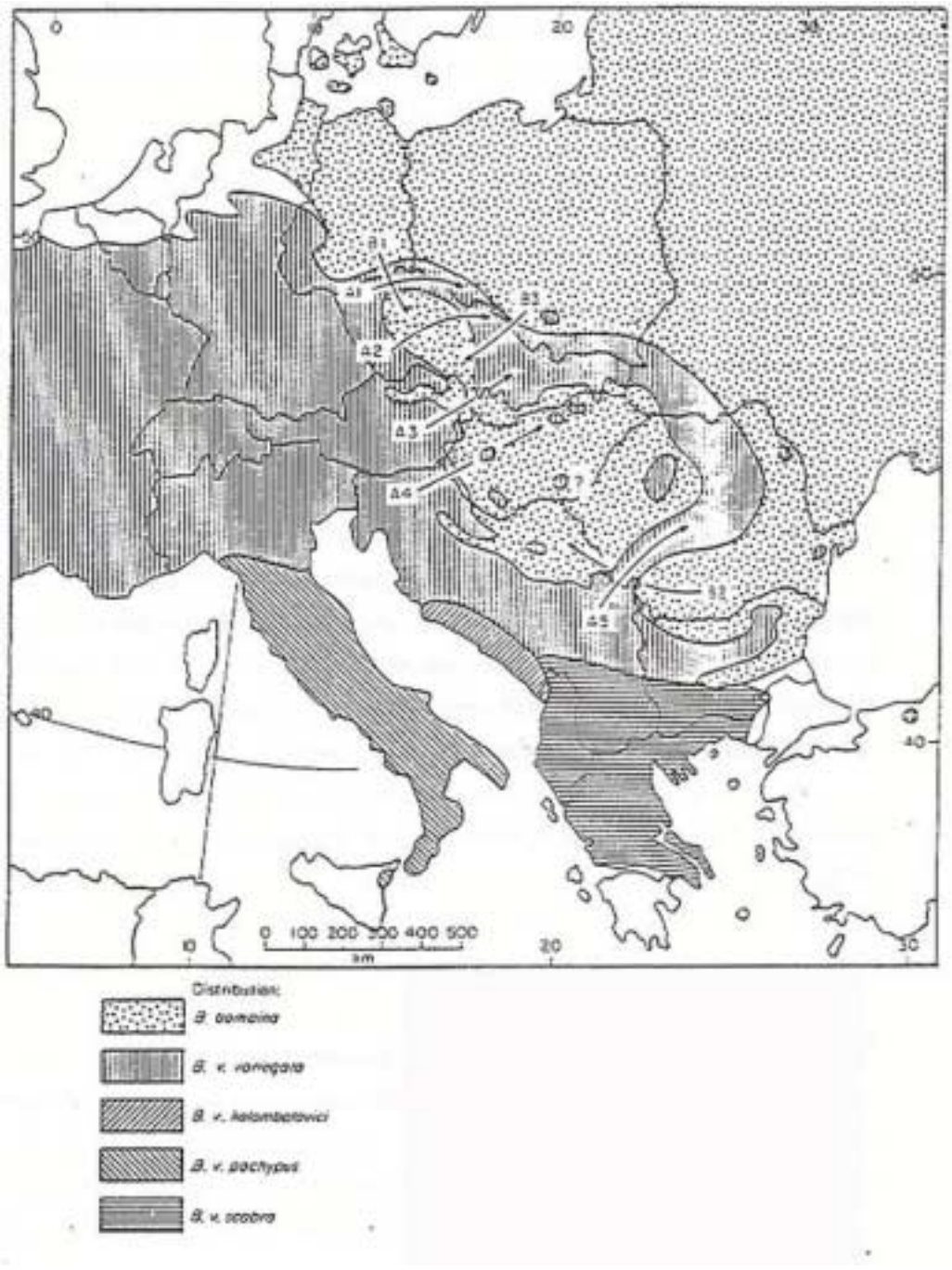

Figure 1.1: The distribution of *Bombina* in central Europe. The arrows represent some hypotheses relating to their post glacial migrations (from Arntzen 1978).

1.4.3 Differences between *Bombina bombina* and *Bombina variegata*

Morphological differences

B. bombina and *B. variegata* (Figure 1.2) not only differ in their distribution ranges, but also in various morphological characteristics with the ventral pattern being the most obvious: *B. variegata* (the yellow-bellied toad) has bright yellow, interconnected spots

on a pale gray background, whereas *B. bombina* (the fire-bellied toad) has a predominantly black ventral surface with numerous small white and distinct red to orange spots. The belly pattern of hybrids is intermediate between these two, and the extent to which the ventral patches are connected has long been used to classify hybrids (Michalowski & Madej 1969, Gollmann 1984). This spot score is highly concordant with clines in diagnostic enzymes (in Poland: Szymura & Barton 1986, 1991, in Croatia: Nürnberger et al. 1995) and with a range of other morphological traits (Nürnberger et al. 1995) and is therefore a good indicator for a toad's genotypic status in the field. Furthermore, each belly pattern is unique and may be used for recapture identification. The dorsal pattern also differs between the two taxa. *B. bombina* adults have a smooth dorsal skin with dark spots on the gray background, whereas *B. variegata* adults have a completely gray back but exhibit a rough, warty surface.

a) **b)**

Figure 1.2: a) *Bombina variegata* and **b)** *Bombina bombina* (from www. whose-tadpole.de).

Other traits that differ between the two taxa are thought to reflect alternative life styles and adaptation to different ecological niches:

1. *B. bombina* prefers open, permanent habitat in lowland plains, from lakes to semi-permanent ponds, drainage ditches and marshy areas, whereas *B. variegata* only occurs in ephemeral habitat like wheel ruts, water-filled hoof prints, puddles and drainage ditches and is mainly restricted to forested upland areas (Lörcher 1969, Madej 1973, Barandun 1995).

2. The temporary nature of the *B. variegata* habitat implies that sites dry out quickly over the season. This necessitates a more terrestrial lifestyle and frequent migrations of the adults in search of new sites. It is generally thought that as adaptations to this, *B. variegata* adults have a thicker skin, a larger body, relatively longer legs and a more robust skeleton than *B. bombina* adults (Nürnberger et al. 1995).

3. The two taxa have different territorial mating calls: *B. bombina* males have longer, louder calls of lower frequency with a longer duration between call pulses than *B. variegata* males (Lörcher 1969). Since *B. bombina* breeds in larger water bodies, the males form big choruses that can be heard from several hundred meters distance (pers. obs.). Furthermore, the males defend bigger territories than *B. variegata* males to which they try to attract females (*B. bombina*: 2 to 3 m and *B. variegata*: 1 to 1.4 m in diameter: Lörcher 1969). Only *B. bombina* males posses internal vocal sacs, in this taxon the calls are generated when air is pushed from these sacs into the lungs (Günther 1996). It has not yet been shown that the different calling patterns confer adaptive advantages to the respective breeding habitats.

4. Ephemeral puddles with a high risk of desiccation impose strong selection on the breeding system in *B. variegata*. Presumably to increase the chance that at least some eggs survive until metamorphosis, *B. variegata* females lay bigger, but fewer eggs in small batches (Rafinska 1991, Nürnberger et al. 1995) scattered over time and space (Barandun & Reyer 1997), whereas *B. bombina* females lay more but smaller eggs (Rafinska 1991).

5. The tadpoles of the two taxa differ in development time and in morphological and behavioral features. From the relatively bigger *B. variegata* eggs, bigger tadpoles hatch. The larval period until metamorphosis in *B. variegata* is reduced approximately ten days compared to *B. bombina* tadpoles (Nürnberger et al. 1995). Furthermore, *B. bombina* tadpoles are more quiescent and have higher tail fins, presumably as adaptations to the presence of predators which are more abundant in permanent habitat (Kruuk & Gilchrist 1997, Vorndran et al. 2002).

Genetic differences

Szymura developed six diagnostic allozyme loci that differ between the two taxa and may be analyzed by electrophoretic techniques (Szymura 1976, Szymura & Barton 1986, 1991). Since then, Nürnberger et al. (in press) have provided a linkage map

comprising a battery of neutral DNA-markers (Microsatellites and SSCPs) that exhibit between two and 30 alleles and therefore allow more detailed analysis, e.g. family assignments in cohort studies. The genetic divergence between *B. bombina* and the different subgroups of *B. variegata* expressed as Nei's genetic distance (Nei 1972) was estimated from 29 allozyme loci, and ranges between 0.37 and 0.59 (Szymura 1993).

1.4.4 Previous work on *Bombina* hybrid zones

Méhely (1892) was the first who mentioned naturally occurring hybrids between *B. bombina* and *B. variegata* (Figure 1.3). It was not until decades that the detection of allozyme markers, and even later diagnostic DNA-markers, enabled intensive studies of hybridization in *Bombina*.

Figure 1.3: Hybrid *Bombina* individuals from one site in Apahida.

Two *Bombina* hybrid zones in Poland (Szymura & Barton 1986, 1991) and one in Croatia (MacCallum 1994, MacCallum et al. 1998) have been investigated thoroughly using diagnostic allozyme markers. They showed narrow clinal transitions in diagnostic traits from one taxon to the other. However, different patterns have been found in other transects. Below I briefly summarize the main results and inferences from *Bombina* hybrid zones to date.

The two hybrid zones in southern Poland were situated near Cracow and 200 km away, near Przemysl. In both transects, there was a coincident, smooth, clinal transition in six diagnostic allozymes and in morphological traits between *B. bombina* and *B. variegata*. In both transects, the clines were very narrow (maximum likelihood estimates: 6.15 km in Cracow, 6.05 km in Przemysl). Furthermore, the clines in morphological traits seemed to be stable in width and position, since these matched samples taken 33 and 55 years earlier (Szymura & Barton 1991). Populations were in Hardy-Weinberg equilibrium, suggesting that there was random mating within both hybrid zones. But there was considerable linkage disequilibrium among the physically unlinked allozyme loci, the standardized parameters reaching maximum values of 0.22 at Przemysl and 0.17 at Cracow at the center of the cline. Linkage disequilibrium is mainly generated by dispersal, which was estimated to be 0.89 km/generation in Cracow and 0.99 km/generation in Przemysl. The set of sharp clines reflected a strong barrier to gene flow from one taxon to the other, in Przemysl equivalent to 51 km (22 km – 81 km) of unimpeded habitat. Hybrid fitness was estimated to be 58% (54% - 68%) that of pure populations. There was also direct evidence of reduced fitness in hybrids: embryonic mortality was higher (Szymura & Barton 1986) and increased developmental and morphological abnormalities were observed in tadpoles and adults in the hybrid zone (reviewed in Szymura 1993).

This and the similarity of the estimates of linkage disequilibrium from two different transects were interpreted as evidence that *Bombina* hybrid zones are maintained by dispersal and selection against hybrids, i.e. that they fit the tension zone model. However, this model does not take into account the ecological differentiation of the two *Bombina* taxa. Indeed, some transects of hybridization in *Bombina* that have been investigated for the pattern of morphological features did not fit the tension zone model. Szymura proposed the existence of three different types of hybridization patterns in *Bombina*, depending on the relief and ecological structure of a zone (Szymura & Barton 1991, Szymura 1993). The first was the clinal pattern described above, found in Przemysl and Cracow, and later also in Pescenica in Croatia (see below). The second type was characterized by an overall bimodal hybrid index distribution where the hybrid index is the number of *B. variegata* alleles summed over all loci investigated. This pattern was found in a transect near Kostajnica in Croatia. There, the pure parental types occured in close proximity in a mosaic pattern, hence facilitating the formation of F1 hybrids. Since neutral markers had introgressed from one taxon into the other,

Szymura excluded the possibility that the Kostajnica transect was of relatively recent origin. Based on the finding of a strong association between habitat type and phenotype, he proposed that the situation could be stable with habitat preference (which entails non-random mating), operating in the context of a heterogeneous environment. Indeed, the transect in Kostajnica featured a patchwork of hills, meadows and forests (Szymura 1993). A similar mosaic distribution of morphological characteristics was found in a transect in eastern Slovakia, also coinciding with a patchy environmental structure (Gollmann 1986). Szymura depicted the third type of *Bombina* hybridization as relics of previous hybrid zones (Szymura & Barton 1986). These transects lack central populations, probably through the destruction of habitat by agricultural activities. Marginal populations show traces of introgression but lack heterozygote deficit, in contrast to populations in mosaic hybrid zones. Putative relic hybrid zones were found at Waldviertel in Austria (Gollmann 1984) and near Zagreb (reviewed in Szymura & Barton 1986). To summarize, Szymura suggested that the outcome of hybridization in *Bombina* is not homogeneous in space and time since it depends on several factors – especially environmental ones – that differ from place to place. Szymura's discussion of the relative roles of selection against hybrids and selection by the environment was followed by the thorough analysis of another transect in Pescenica, Croatia (MacCallum 1994, MacCallum et al. 1998).

Superficially, the transect in Pescenica resembled the two hybrid zones in Przemysl and Cracow in that they featured concordant transitions in allozyme and in morphological traits in adults (Nürnberger et al. 1995) over similar scales with a sharp central step and shallow tails. But there were most interesting differences, corroborating the importance of Szymura's discussion summarized above. The populations in Pescenica were not in Hardy-Weinberg-equilibrium; heterozygote deficit reached a maximum value of $F_{IS} = 0.26$ at the center of the cline. The linkage disequilibrium was also significantly higher – even when the heterozygote deficit was taken into account: the maximum value for D was 0.139, compared to 0.055 and 0.043 at Przemysl and Cracow respectively. Furthermore, in the center of the hybrid zone, breeding habitats showed a clear mosaic distribution, together with a significant association between a population's mean genotype and the habitat type. A similar tendency was observed in the Ukraine (Yanchukov, pers. comm). MacCallum et al. (1998) found that this association occurred on a scale much smaller than the annual dispersal range, which indicated that there was active habitat preference. The genotype-habitat association caused non-random mating

which in combination with influx of pure genotypes from the periphery produced the observed linkage disequilibrium. Heterozygote deficit, estimated in adults at the beginning of the season, may come about in three different ways: i) if some migrating adults choose the habitat of the opposing taxon by mistake (this is the same process that generates *D*), ii) if there is random mating but selection against hybrid individuals or selection by the habitat against the wrong alleles and iii) if there is assortative mating. These three factors are not mutually exclusive. For example, MacCallum (1994, MacCallum et al. 1998) showed that active habitat preference in adults accounted for the association between marker alleles and habitat. As a consequence, there was incomplete mixing of the two gene pools or non-random mating by habitat, which might explain the high level of F_{IS} and *D*. Nürnberger et al. (1995) carried out a large scale breeding experiment with toads from Pescenica. A wide range of adults was scored for belly pattern, skin thickness, skeletal properties and mating call before crosses were made within population across the cline and also between putatively pure populations. The offspring were scored for egg size, development time, larval and metamorph survival. While the clines in adult traits and allozyme allele frequencies were concordant, the clines in egg size and development time were shifted in different directions, presumably due to strong directional selection. Selection by habitat was suggested by the maintenance of the habitat preference as narrowing the range of acceptable breeding habitat must be compensated by a fitness gain of breeding in the preferred habitat. When mating occurs within the habitat substantial linkage disequilibrium is generated between habitat preference genes and those conferring fitness in the habitat (Diehl & Bush 1989). Moreover, Kruuk et al. (1999a) demonstrated that there was also increased mortality in some hybrid tadpole families that had been taken from the center of the hybrid zone.

1.5 Aims of thesis and Chapter outline

Analysis of hybridization in *Bombina* provides a rare opportunity to study both the genetic dynamics and the balance of roles between selection against hybrid individuals and selection by the habitat in maintaining reproductive isolation between the two taxa. This thesis extends the investigation of hybridization in *Bombina* to a new *Bombina* hybrid zone near Apahida in Romania. My research on this hybrid zone was carried out in collaboration with Tim Vines (University of Edinburgh). It focuses on two main

issues. First, genotypes are distributed in a more patchy way in Apahida than in Poland or Pescenica. In Chapter 2, I describe the topography of the study site, the distribution of habitat types and genotypes and I point out how the Apahida study area differs in these issues from the one in Pescenica. A direct comparison of equivalent data from both hybrid zones allows to test whether the distribution of habitat or rather the overall cline primarily determines the spatial arrangement of genotypes in a *Bombina* hybrid zone. This test and a detailed analysis of the pattern of genotypes and inferences about habitat preference, migration and selection strength are part of Tim Vines' thesis (Vines 2002, Vines et al. in press). I summarize the findings briefly in Chapter 3. They lead to the conclusion that strong natural selection is needed to maintain this particular transect.

Insight into the form of selection (i.e. whether it is intrinsic and/or extrinsic) has to come from measurements of survival in the field, which is the second issue and main focus of my thesis research. As noted above, the two forms of selection are not mutually exclusive. Hybrid dysfunction has been observed in tadpoles in the Pescenica hybrid zone (Kruuk et al. 1999a) and is a possible explanation for heterozygote deficit among adults in Apahida. On the other hand, habitat preference in adults must have an adaptive advantage since it means a restriction of resource use which can only be outweighed by better performance in the preferred habitat. Therefore, selection against alleles in the wrong habitat is also likely. I investigate natural selection on hybrid *Bombina* tadpole cohorts in the field concentrating on naturally deposited egg batches in 14 sites. I take a sample of around ten eggs per batch for genotyping and allow the remainder of the egg batches to develop in the sites. In Chapter 4, I test for intrinsic selection, apparent as segregation bias or as heterozygote deficit within the collected egg families. In Chapter 5, I look at breeding habitat preference in adults and extrinsic selection in tadpoles, which may be apparent as shifts in mean allele frequencies between the egg and late tadpole stages. I concentrate on the tadpoles since they are primarily affected by the adults' preference of different breeding habitats. In Chapter 6, I explore whether there are genetically determined differences in the tadpoles' growth, development and phenotypic plasticity and if they are associated with the habitat preference in the adults. Finally, the wider implications of this thesis in the context of research of speciation and hybrid zones are discussed in Chapter 7.

2 THE APAHIDA HYBRID ZONE

2.1 Overview

This Chapter describes the topography and the distribution of habitat and adult genotypes in the study area around Apahida in Romania. Extensive sampling in the Transylvanian plain in 1998/99 (by B. Nürnberger, T. Vines, A. Hofmann, R. Sieglstetter) had revealed a distribution of genotypes that did not follow our expectation of clinal transitions at altitudinal gradients. Unexpectedly, pure *B. bombina* populations were found in the Transylvanian highland, i.e. a long way from the Hungarian heartland. *B. variegata* was not restricted to forested hills, and the transition between the taxa, in particular the location of a possible steep cline was entirely unknown. Small scale analysis was needed to clarify on what spatial scale the two taxa meet and hybridize. The analysis from Pescenica had suggested that habitat preference plays an important role as a reproductive isolation mechanism in *Bombina* hybrid zones. With habitat preference, the distribution of habitat types in a *Bombina* hybrid zone should be a major determinant of the pattern of recombinants found in the zone (see 1.4.4). We concentrated on a subset of the Transylvanian plain around Apahida and analyzed the distribution pattern of habitat and genotypes in detail. The results are presented in this Chapter. Inferences for habitat preference, migration and selection are summarized in Chapter 3. The study of selection within tadpole cohorts (Chapters 4 to 6) was conducted in the same region of the Apahida hybrid zone, and this Chapter outlines field and molecular methods relevant for the entire thesis. I also look at the concordance of allele frequencies across marker loci to test whether they may be treated as equivalent indicators for the state of an individual's genome.

2.2 The study site

The study area lies near the village of Apahida, about 20 km to the north-east of Cluj-Napoca on the Transylvanian plain in Romania (Figure 2.1). The south west corner of this 400 km² area is located at 46°50' N and 23°47' E.

Figure 2.1: Map of Romania (http://www.grida.no/db/maps/prod/level3/id_1277.htm). The arrow indicates the study area.

The Transylvanian plain is almost entirely enclosed by mountain ranges. To the north, east and south, the Carpathian mountains form an arc with peaks of up to 2550 m. Adjoining to the west are the Bihor- or Apuseni mountains which are up to 1850 m high. The only connections to the Hungarian plain further beyond are the Somes and the Mures valleys which drain the Transylvanian plain to the north and south of this isolated mountain range. The landscape in the Transylvanian plain is characterized by rolling hills between 200 m and 500 m above sea level. The bedrock is formed by tertiary marl and in the river valleys quaternary sandy loam has been deposited.

The study area itself (Figure 2.2) is dominated by the two main river valleys Apahida and Gadalin which dissect the landscape in an east-west direction and finally meet the Somes which flanks the study area in the west. Side-arms of these valleys have a north-south orientation. Around Cluj-Napoca, all valleys and slopes have been logged entirely, beginning from the 14th century (Pounds 1979). In the valleys there are small arable strip fields where farmers grow crops for subsistence while the slopes and hills are used as pastures and fruit orchards. Only on some hilltops have small beech woodlands (dominated by beech, oak and hornbeam) remained, and these are used as

pastures and for wood. The landscape has suffered intensely from erosion, and water bodies are rare. In Cluj-Napoca, the mean temperatures reach –3.4°C in January and 18.2°C in July; the mean annual temperature is 8.2°C. The mean precipitation is 24 mm in January and 81 mm in July; the mean annual precipitation is 548 mm (http://www.klimadiagramme.de/Europa/cluj.html). In both field seasons the Transylvanian plain was affected by severe drought and temperatures up to 40°C which caused most water bodies including large ponds to dry out in early June.

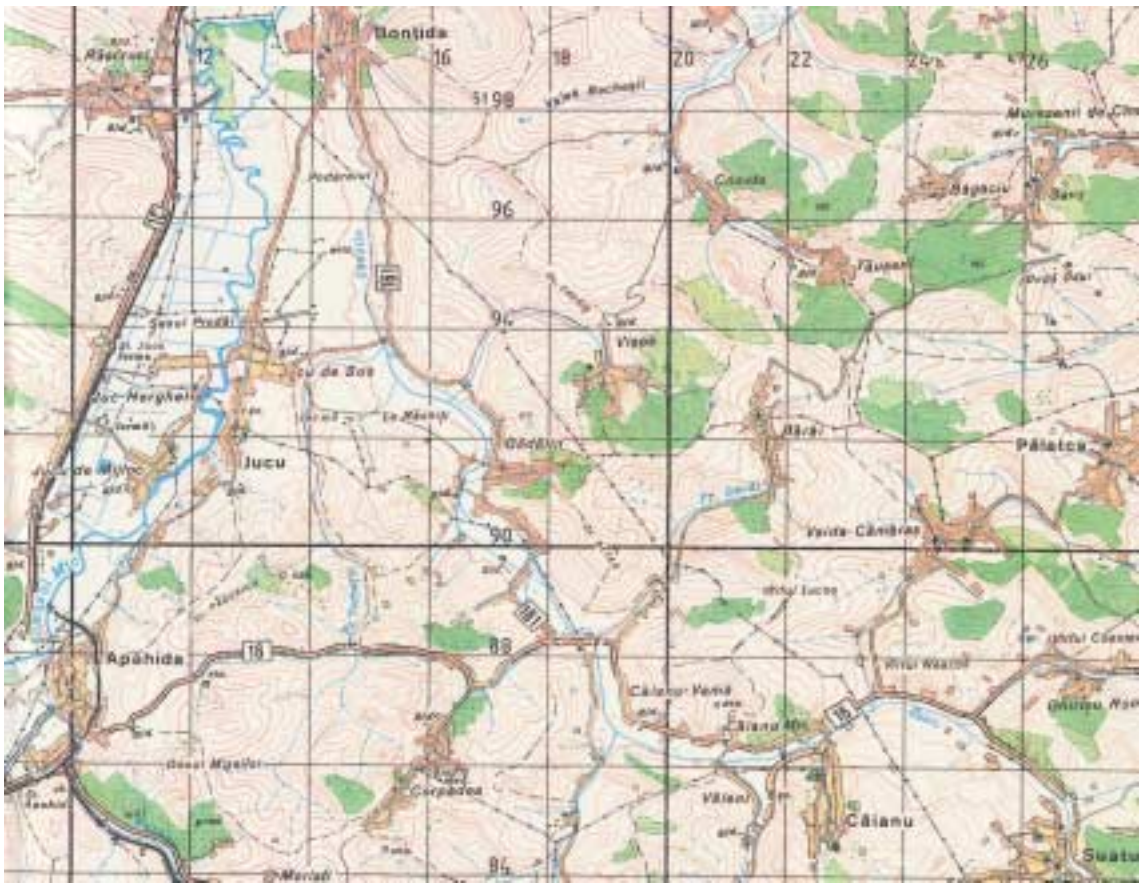

Figure 2.2: The study area to the north-east of Cluj-Napoca. It is situated between the villages Apahida, Suatu, Coasta and Bontida. One grid length equals 2 km.

2.3 Material and Methods

2.3.1 Collection and processing of animals

The field season extended from mid April to mid July both in 2000 and 2001. Collections were made by T. Vines, myself, B. Nürnberger and M. Thiel in 2000 and by

myself and T. Sands in 2001. The area was searched intensively for water bodies with the aid of topographic maps (1:25,000) which were obtained from the Institute of Geography at the University of Cluj-Napoca. Since water bodies were rare, every water body that could be found was visited and searched for toads. Sites were labeled beginning from number 145 continuously over both years up to number 411. However, due to limited capacity for genotyping many sites were dropped from the analysis. The criterion for excluding a site was its redundancy, i.e. close proximity to and a similar composition of toad phenotypes as in adjoining sites. Due to the abundance of puddles containing *B. variegata*-like animals most sites that were dropped were rather of this type because we placed greater value in finding *B. bombina*-like intermediate and pure *B. bombina* sites. In total 991 toads were collected from 94 sites: 750 individuals from 75 sites in 2000 and an additional 241 toads from 4 old and 19 new sites in 2001. Toads were collected by hand and with nets from the aquatic habitat and sampled immediately. They were anaesthetized in 0.2% MS222 (3-amino benzoic acid ethyl ester, Sigma). The anaesthetic lasted for approximately ten minutes; during this time the toads' individual belly pattern was photographed before a small portion of a single toe was removed as a tissue sample from either the right (in 2000) or left (in 2001) foot. The photographs served for recapture identification during subsequent visits. The toes were labeled and stored in Eppendorf cups with 99.9% ethanol. Morphometric measurements of the snout-vent and tibiofibula lengths were also taken, along with recording the presence of nuptial pads (by which breeding males may be identified), warts (typical for *B. variegata*) and dorsal spots (typical for *B. bombina*). This information, however, will not be discussed in this study. The belly pattern was used to approximate an individual's genotype state in the field (see 1.4.3). To obtain the so called spot score one records whether the yellow to red spots are connected (1) or not (0) over ten critical points along a toad's ventral side. Thus, the spot score varies from 0 (pure *B. bombina*) to 10 (pure *B. variegata*). After this procedure, when again fully awake, the toads were immediately released into the habitat where they had been caught. No negative effect of the anaesthetic or toe amputations has been reported yet.

2.3.2 Collection of ecological data

B. bombina and *B. variegata* breed in permanent and ephemeral habitat respectively (see 1.4.3). The permanence of an aquatic habitat correlates with a number of physical measures and with the type of vegetation found in and around the water body. For

example, in deep, large ponds water is most likely to persist throughout the season. These sites will therefore contain specialized aquatic vegetation. Table 2.1 lists the variables that were measured for each site to quantify its degree of permanence. Data were recorded once between 20. April and 17. May in the respective year whenever a site was visited for the first time. Measurement of other variables such as flora, fauna and pH would have been worthwhile as well, but given the limited time available for this aspect of our field work, we concentrated on those parameters that had been identified as particularly informative in a previous study (MacCallum 1994, MacCallum et al. 1998). We also aimed at obtaining a data set that would enable direct comparison of the two hybrid zones (see Chapter 3).

Table 2.1: List of habitat variables recorded for each site.

Grid reference (GPS)
Habitat type (pond, puddle, etc.)
Length of water body
Width of water body
Depth of water body
Percentage of surface area with emerged vegetation
Percentage of surface area with submerged vegetation
Percentage of bank vegetation < 15 cm high
Percentage of bank vegetation 15 to 50 cm high
Percentage of bank vegetation > 50 cm high

2.3.3 Multivariate statistics for the analysis of the ecological data

The ecological variables chosen were assumed to be a measure of a site's permanence. Therefore, they were expected to be correlated with each other. For example, small, shallow sites are most prone to quick desiccation and are therefore not suitable habitat for aquatic vegetation. For an investigation of the correlation between habitat and genotype one needs an independent, objective measure of habitat differences. For this the large number of inter-correlated variables describing the habitat had to be reduced. The multivariate method most appropriate for this is a discriminant function analysis (see MacCallum 1994). A discriminant function requires at least two known groups prior to the analysis. A linear combination of the variables is produced which

maximizes the difference between these groups relative to the variance within them. The importance of each variable to the overall function is based on the minimization of Wilk's λ, which is the ratio of the within groups sum of squares to the total sum of squares when considering variables individually. At each step of the analysis the variable with the smallest Wilk's λ is added to the function. If the effect that the addition of the new variable has on the function is significant (measured as an F statistics) then this variable is retained, meaning that the variable makes a significant contribution to discriminating between the group means, reducing Wilk's λ. Variables that do not improve the discrimination significantly are eliminated. This model can then be used to position the intermediate cases along a single axis between the two extremes.

All variables were transformed to improve their normality (log for continuous variables, arcsine for percentages: Sokal & Rohlf 1995). To enable a direct comparison between the Apahida and the Pescenica sites, a discriminant function axis was computed by T. Vines (2002) following MacCallum (1994) based on a joint sample of 152 sites using a stepwise routine with forward and backward selection. The subjective categorization of typical ponds and puddles was used as the basis for the discriminant function analysis, and the linear combination of habitat variables that best separated them calculated. The overall function was applied to all sites, and the discriminant score was rescaled to run from 0 (ponds) to 1 (puddles), using the most pond-like and the most puddle-like sites in the dataset as a whole as endpoints. The habitat index was denoted as *H*.

2.3.4 Solutions for the molecular analysis

10 x TA	1 M Tris-Acetate/HCl, pH 7.5 in H_2O
10 x TBE	1 M Tris, 0.8 M Boric acid, 0.01 M EDTA, pH 8.0 in H_2O
TNES	0.05 M Tris, 0.4 M NaCl, 0.1 M EDTA, 0.015 M SDS, pH 7.5 in H_2O

2.3.5 Genotyping

The adults sampled in 2000 were genotyped by T. Vines (507 individuals) and M. Thiel (243 individuals), while all adults sampled in 2001 (241 individuals) were genotyped by myself. The molecular analysis of the tissue samples comprised four physically unlinked, presumably neutral DNA markers, two microsatellites (*Bv12.19*, *Bv24.12*) and

two single strand conformation polymorphisms (SSCPs) (*Bb7.4, Bv24.11*). There were two *B. variegata* alleles and one *B. bombina* allele at *Bb7.4*, *Bv12.19* and *Bv24.11* respectively, whereas locus *Bv24.12* had five alleles characteristic of *B. variegata*, one characteristic of *B. bombina* and one of exactly intermediate length which could not be assigned to either taxon and was left unscored since it was only observed in one population (13 cases). For every animal, the sum of *B. variegata* alleles across all loci was computed. Through division by the number of loci times two this hybrid index was rescaled to vary from 0 (pure *B. bombina*) to 1 (pure *B. variegata*). A site's mean allele frequency, $\bar{p}$, is the mean over all genotyped individuals.

DNA preparation

The ethanol-preserved tissue samples were dried before DNA was extracted following a standard protocol. Briefly, the tissue was digested overnight at 56°C with 0.2 mg/ml proteinase K in 0.25 ml TNES. The solution was then mixed with 0.25 ml 2.6 M NaCl and centrifuged to pellet the cell debris (room temperature, 10 min, 13,000 rpm). The supernatant was transferred to a fresh vial and DNA was extracted once with 0.5 ml chloroform. After incubation on ice (15 min), the aqueous phase was transferred to a new vial. DNA was precipitated in 1 ml 99.9% ethanol at –60°C for 30 min. After pelleting the DNA (4°C, 15 min, 13,000 rpm), it was washed twice with 0.25 ml 70% ethanol and then air-dried at room temperature. Finally, the DNA was resuspended in 20 µl ultrapure water (Roth) and stored at –20°C. The DNA concentration and purity was measured in a GeneQuant photometer (Pharmacia) as absorptions at 260 nm and 280 nm using a 1:20 dilution. For subsequent PCR the DNA was diluted to a final concentration of 10 ng/µl.

Polymerase chain reaction (PCR)

PCR were set up in a final volume of 30 µl with 50 ng (5 µl) template DNA, 50 mM KCl, 10 mM Tris (pH 9.0 at room temperature), dNTPs (0.2 mM per nucleotide), 10 pm of each primer (Table 2.2) and 0.5 units Taq polymerase (rTaq, Amersham Pharmacia Biotech). For the microsatellite loci, flourescent-labelled primers were used. $MgCl_2$ concentrations varied among loci between 1.5 mM and 2.5 mM (Table 2.2). Amplification was carried out on a Hybaid Touchdown thermocycler with oil overlay. After the initial denaturing step of 3 min at 95°C, the cycle profile was the following: 15 sec denaturing at 94°C, 30 sec annealing at x°C and 60 sec elongation at 72°C,

where x°C is the primer specific annealing temperature (Table 2.2). Depending on the locus, 33 or 35 cycles were conducted.

Table 2.2: PCR and electrophoresis parameters for the four DNA markers used in this study. The primer sequences (Nürnberger et al. in press) can be found in the GenBank records. The first two loci are SSCPs for which conditions for electrophoresis through native polyacrylamide gels are given, whereas the latter two loci were resolved on denaturing polyacrylamide gels under conditions given in the text.

Primer	GenBank Acc #	PCR $MgCl_2$ conc. (mM)	PCR anneal. temp. (x°C)	PCR cycle #	Gel conc. (%)	Gel runtime (h)	Gel temp. (°C)
Bb7.4	AF472441	2.5	57	33	8	5	2
Bv24.11	AF472425	1.5	56	33	8	2	4
Bv12.19	AF472423	1.5	52	35	-	-	-
Bv24.12	AF472426	2.5	51	33	-	-	-

To verify successful amplification, ¼ volume of every PCR product was electrophoresed for 30 min in a 2% agarose gel and visualized with ethidium bromide staining. The products were further analyzed using polyacrylamide gels.

Native polyacrylamide gels

The SSCP amplification products were mixed 1:1 with an electrophoresis buffer (100% formamide, 0.05 µM xylene cyanole), denatured for 3 min at 95°C, shock-chilled on wet ice and immediately electrophoresed on native horizontal polyacrylamide gels. These gels had a size of 12 cm x 25 cm x 0.5 cm and contained 8% polyacrylamide (Roth) and a gel buffer of 1 x TA (pH 7.5 at room temperature). Electrophoresis was conducted at constant low temperatures (Table 2.2) and a constant voltage of 40 V/cm for 2 or 5 hours in MultiPhor gel rigs (Pharmacia) with an electrode buffer of 2 x TBE. DNA was fixed on the gel for 30 min in 10% acetic acid before it was stained for 30 min in 60 mM $AgNO_3$. The staining was visualized in a strongly reducing solution of 0.375 M NaOH, 2.2 mM Boro-Na-hydride and 0.12% formaldehyde. Gels were softened for 15 min in 10% glycerol and air-dried overnight at room temperature. Finally, they were fixed on both sides with a plastic matrix.

Denaturing polyacrylamide gels

Amplification products of microsatellites were mixed 1:1 with an electrophoresis buffer (95% formamide, 10 mM EDTA, 0.025% bromphenole blue, 0.05 µM xylene cyanole), denatured for 3 min at 95°C, shock-chilled on wet ice and size-separated by electrophoresis through 0.5 cm thick polyacrylamide gels on an automatic sequencer (ALF expressTM, Amersham Pharmacia Biotech). The gels had a concentration of 6% polyacrylamide (Long Ranger, BMA), 6 M urea (Roth) and a gel buffer of 1 x TBE and pH 7.5 at room temperature. Electrophoresis was performed at 55°C and 55.5 V/cm for 8 hours with an electrode buffer of 0.5 x TBE. The length polymorphisms were analyzed with the software Fragment Manager 1.2 (Amersham Pharmacia Biotech).

2.3.6 Statistical techniques for the analysis of genetic data

The concordance between the four DNA markers can be demonstrated by plotting the mean *B. variegata* allele frequency at each locus (p_i) against the overall mean *B. variegata* allele frequency ($\bar{p}$). If all these allele frequency ranges coincided perfectly, all points would fall onto the diagonal. Scatter around this diagonal means that there is some variation among loci in the transition from one taxon into the other. There are two possible explanations for this variation: there may be consistent differences in both the width and the position of allele frequency ranges at different loci and there may be random fluctuations due to genetic drift (Szymura & Barton 1986, 1991). Consistent differences in cline position and width can be estimated using a cubic polynomial model that describes the allele frequency at a single locus as a function of the overall allele frequencies of *B. bombina* alleles ($\bar{q}$) and *B. variegata* alleles ($\bar{p}$):

$p_i = \bar{p} + 2\,\bar{p}\,\bar{q}\,[\alpha + \beta(\bar{p} - \bar{q})]$ (Szymura & Barton 1986, 1991).

α reflects an overall change in position of an allele frequency range in favor of *B. variegata* while β denotes a narrowing or widening of a particular allele frequency cline relative to the overall one. Random deviations from complete concordance due to genetic drift are quantified by the standardized variance in allele frequency F_{ST} which is here a measure of the variance between loci within sites which equals concordance (see MacCallum et al. 1998).

2.4 Results

2.4.1 Habitat types

Sites varied considerably in size and depth, age, water regime and vegetation type. There were only four ponds (Figure 2.3a) which had a surface of more than 2000 m^2 and were more than 3 m deep. Although they must have existed for many years, two of these ponds dried out during the extremely dry 2001 season. Two lakes where *B. bombina* could be heard are situated in the study area, however, they were not sampled due to prohibited access. A collection of 13 smaller ponds had a mean surface of 120 m^2 and did not exceed 1 m in depth (Figure 2.3b). They were characterized by abundant aquatic vegetation but all dried during the season. A special group of sites consisted of eight excavated holes around 3-4 m deep and 3-5 m in diameter which had been dug for fish along a stretch of 200 m between 1950 and 1990 (Figure 2.3c). These holes had no shallow water zones but exhibited a wide range of successive stages depending on their age. Older holes contained dense aquatic vegetation and also other anuran species. Another group of sites comprised six man made watering holes dug for horses (Figure 2.3d). They had an average size of 0.5 m x 0.3 m x 0.5 m. These sites only lasted for a few weeks and were barren of any aquatic vegetation. Another group of sites comprised 15 drainage ditches which occurred along roads and on the bottom of major valleys (Figure 2.3e). They were densely covered by emergent aquatic vegetation. The majority of sites (48) comprised small puddles in tractor wheel ruts and hoof prints, often around concrete wells (Figure 2.3f). These puddles were prone to high turbidity.

a) b)

Figure 2.3: Typical habitats in the Apahida hybrid zone. a) big pond (site Nr. 293); b) small pond (290).

c) d)

e) f)

Figure 2.3 continued: c) artificial pond (200.4); d) dug hole (373); e) ditch (321) and f) puddles around a well (385).

The computation of the habitat permanence index, *H*, revealed that the four retained variables were width, % emerged vegetation, depth and % submerged vegetation (Table 2.3). The variables are the same as those in the axis calculated by MacCallum (1994) for the Pescenica sites alone. The distinction between the two habitat types was highly significant ($F_{4,146} = 91.3$; $p < 10^{-6}$).

Table 2.3: Discriminant function coefficients (standardized) and associated Wilk's λ, a stepwise measure of the additive effect of all entered terms on the function. Variables are ordered by their contribution to the function. All Wilks λ gave $p < 0.001$. From: T. Vines (2002).

	Overall	Ponds	Puddles	Wilk's λ	Correlation
Width	0.5	2.48	0.79	0.41	0.75
% em. veg.	0.79	-7.21	-0.22	0.34	0.58
Depth	2.19	-2.24	4.93	0.29	0.57
% subm. veg.	0.66	0.67	-1.58	0.28	0.34
Constant	0.49	-5.60	-5.11		
Group mean		2.33	-1.05		

The minimum and maximum habitat index values in Apahida are 0.15 and 0.91 respectively (Figure 2.4, Appendix 2.1). Most sites exhibit an intermediate habitat index between 0.5 and 0.8. In contrast to this, in the majority of the sites in Pescenica the habitat index ranges between 0.7 and 0.9. At the same time there is more habitat available at the permanent end of the habitat index range and less intermediate habitat than in Apahida.

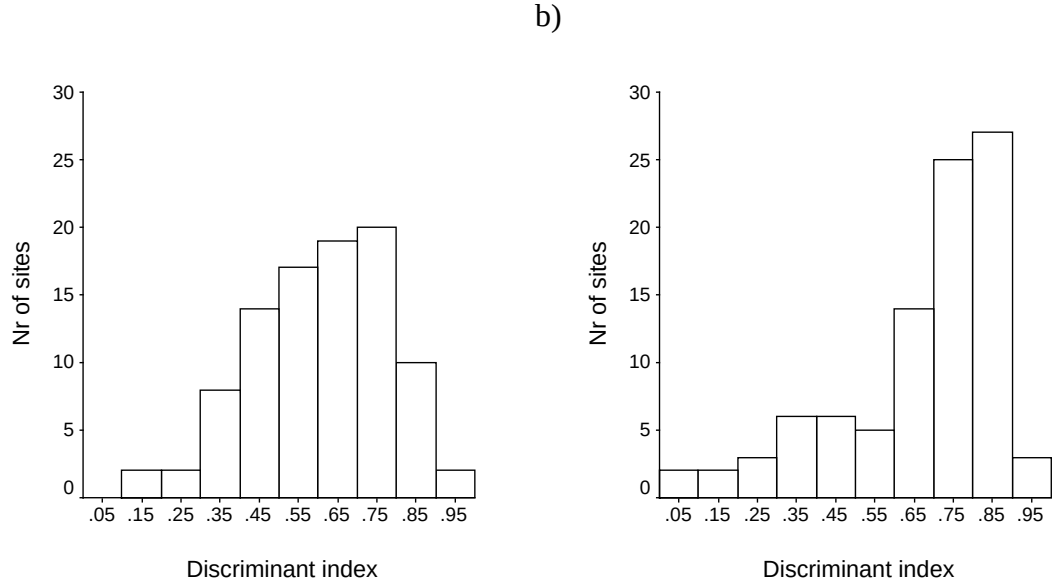

Figure 2.4: Frequency of sites across the range of the habitat index *H* in the Apahida (a) and Pescenica (b) hybrid zones. Apahida: overall $\overline{H}$ = 0.61; S.D. = 0.18; N = 94; Pescenica: $\overline{H}$ = 0.67; S.D. = 0.21; N = 93. Data from Pescenica are from MacCallum (1994).

No correlation between the log-transformed altitude and arsine-transformed *H* could be found in Apahida (r_{SP} = 0.004, p = 0.967, Figure 2.5). This means that in contrast to the study sites in Poland and Pescenica where permanent ponds occur only in lowland plains, there is no obvious environmental gradient in the Apahida study area. For example, large ponds may be found just below hilltops, and puddles are not restricted to high elevations.

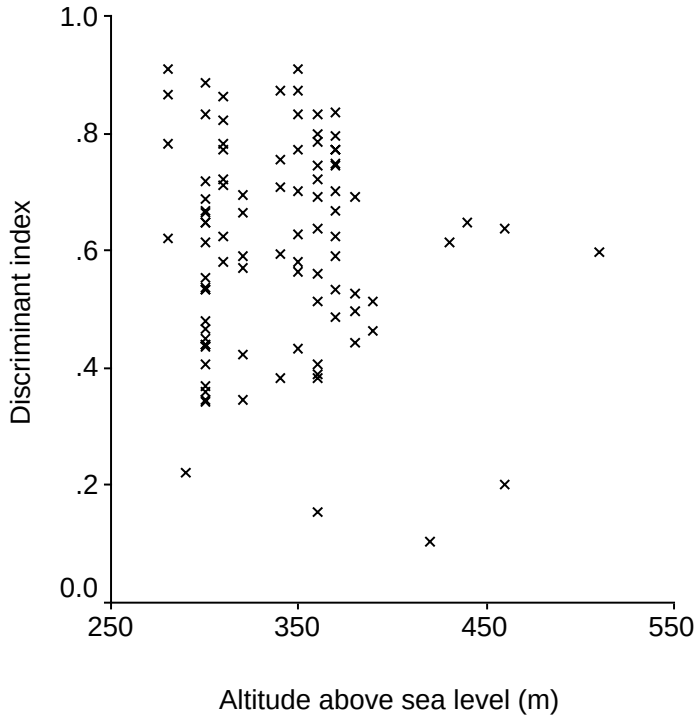

Figure 2.5: Relationship between altitude and the habitat index *H* in the Apahida hybrid zone. There is no altitudinal gradient in the distribution of aquatic habitat types (r_{SP} = 0.004, p = 0.967).

2.4.2 Concordance between loci

All four neutral DNA markers change approximately in concordance across the range of $\bar{p}$ (Figure 2.6) which justifies treating them as equal and as approximate indicators for the status of a toad's genome. The cubic polynomial model described above was fitted by T. Vines (2002) to the data which allows to determine the overall deviation of the allele frequency as well as the steepness of divergence at every locus relative to the average (Table 2.4). Three out of eight estimates were significant. Locus *Bv24.11* had the largest excess of *B. bombina* alleles (4%) and the smallest difference in allele frequency between either end of the genotype spectrum. The largest difference in allele

frequency over the genotype spectrum was found at locus *Bv24.12*. However, the estimates do not exceed those found for the Pescenica data for allozymes (MacCallum et al. 1998), indicating that there is roughly the same level of concordance in Apahida. Around the regressions there is still variation unaccounted for. The overall estimate of this F_{ST} = 0.033 which indicates that allele frequencies in Apahida are more affected by genetic drift than those in Pescenica where F_{ST} = 0.0068 (MacCallum et al. 1998).

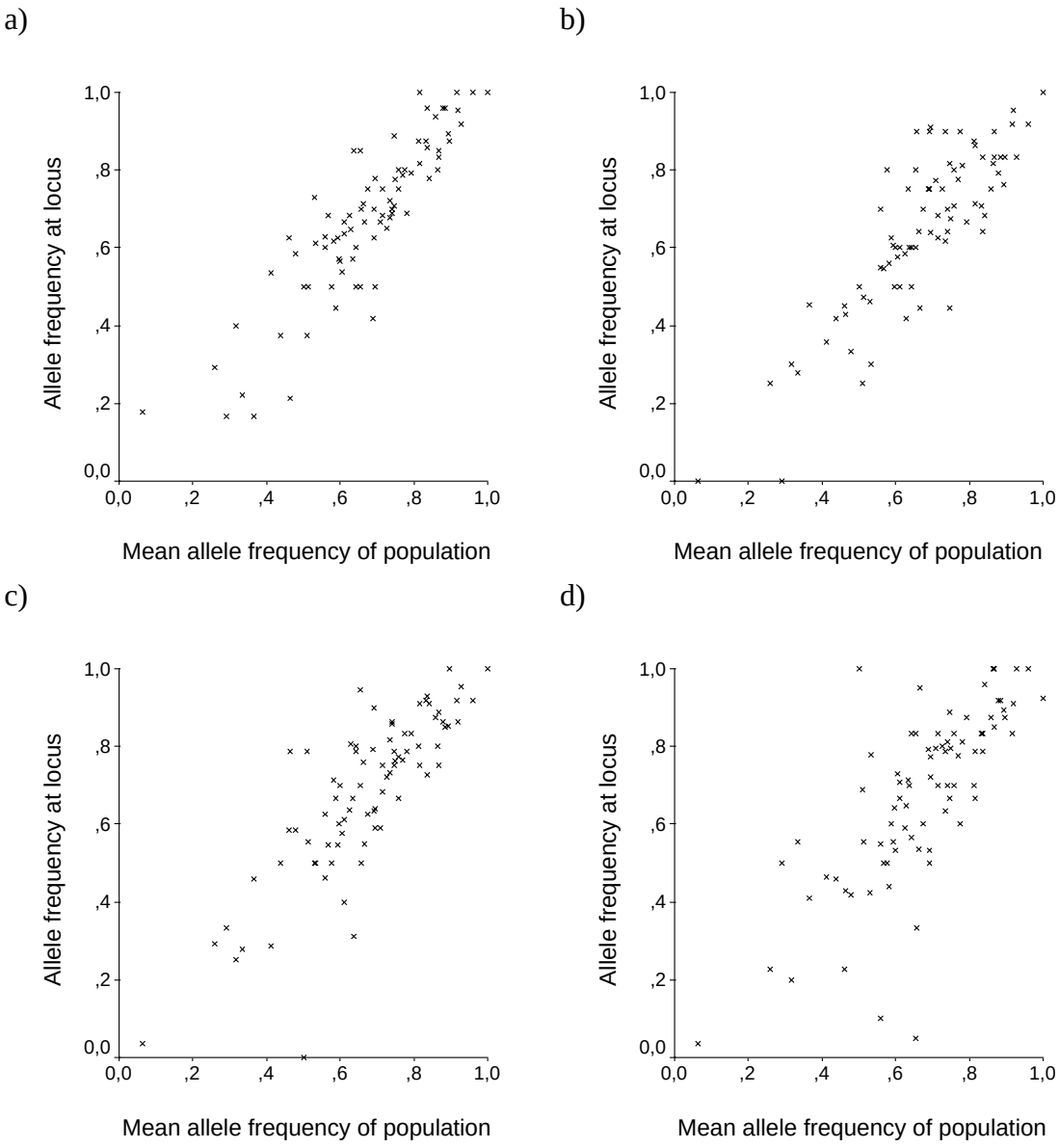

Figure 2.6: Concordance of allele frequency across loci. Each graph shows the allele frequency at each of the four loci (p_i) plotted against the mean across all loci ($\bar{p}$). a) locus *Bb7.4*; b) locus *Bv24.11*; c) locus *Bv12.19* and d) locus *Bv24.12*.

Table 2.4: Variation in the position (α) and width (β) of the allele frequency ranges at the different loci relative to the overall average. F_{ST} denotes the variance of each locus around the overall allele frequency range. Limits are given in brackets. Significant values are printed in bold. From: T. Vines (2002).

Locus	α	β	F_{ST}
Bb7.4	0.028 (-0.01; 0.08)	0.041 (-0.08; 0.16)	0.000
Bv24.11	**-0.08** (-0.12; -0.04)	**-0.16** (-0.28; -0.03)	**0.022**
Bv12.19	0.004 (-0.03; 0.03)	-0.025 (-0.15; 0.09)	**0.048**
Bv24.12	0.052 (0.00; 0.09)	**0.15** (0.02; 0.26)	-0.001

Total F_{ST} = **0.033**

2.4.3 The distribution of adult genotypes

The vast majority of the populations are of intermediate allele frequencies (Figure 2.7a, Appendix 2.2). Most of these hybrid populations exhibit a mean *B. variegata* allele frequency $\bar{p}$ around 0.7, so that $\bar{p}$ over all populations tends towards *B. variegata*. Out of the total 98 populations, 76 populations are hybrid ($0.2 < \bar{p} < 0.8$), $\bar{p}$ exceeds 0.8 in 21 populations and only one population has a $\bar{p}$ below 0.2. It is important to note that when looking for sites, we placed particular effort in finding sites with *B. bombina*-like toads which were less abundant in the area. In reality, the imbalance between *B. variegata*-like and *B. bombina*-like populations in Apahida is even more drastic than these data suggest, because *B. variegata*-like populations are underrepresented in the genetic analysis relative to the situation in the field. Hybrid populations dominate the Apahida hybrid zone, and there are only few pure populations of either taxon. This finding stands in striking contrast to the clearly bimodal distribution in populations' allele frequencies in the Pescenica hybrid zone (MacCallum 1994). There, 30 out of 85 populations have a $\bar{p}$ between 0.2 and 0.8 while 32 populations have a $\bar{p}$ below 0.2 and $\bar{p}$ exceeds 0.8 in 23 populations (Figure 2.7b).

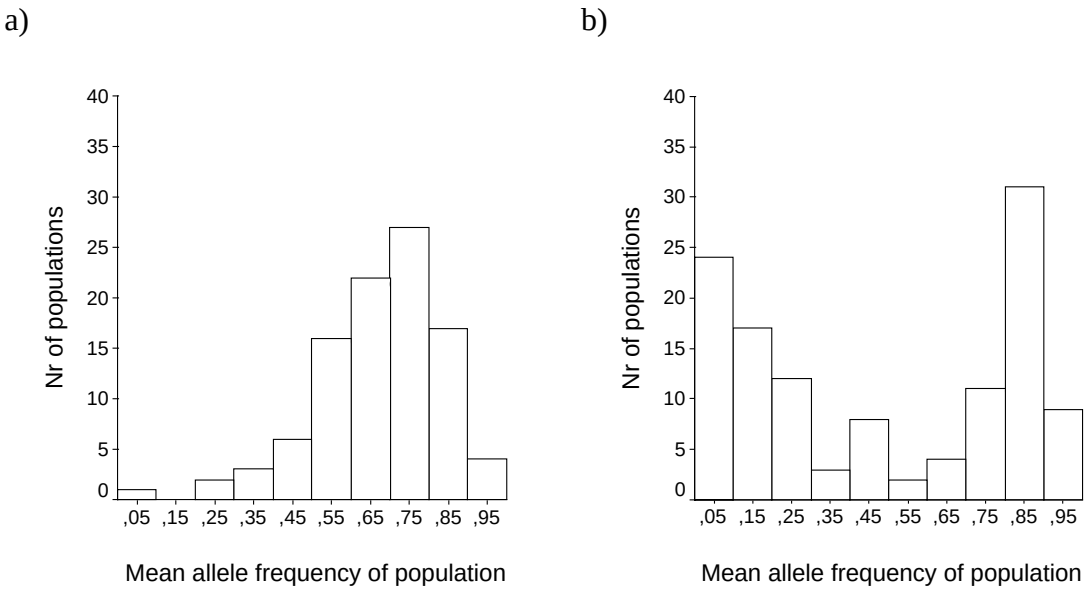

Figure 2.7: Number of populations across the range of mean *B. variegata* allele frequencies ($\bar{p}$) in steps of 0.05 in the Apahida (a) and the Pescenica (b) hybrid zones. Apahida: overall $\bar{p}$ = 0.68 (S.D. = 0.16); N = 98; Pescenica: overall $\bar{p}$ = 0.49 (S.D. = 0.36); N = 121. Data from Pescenica are from MacCallum (1994).

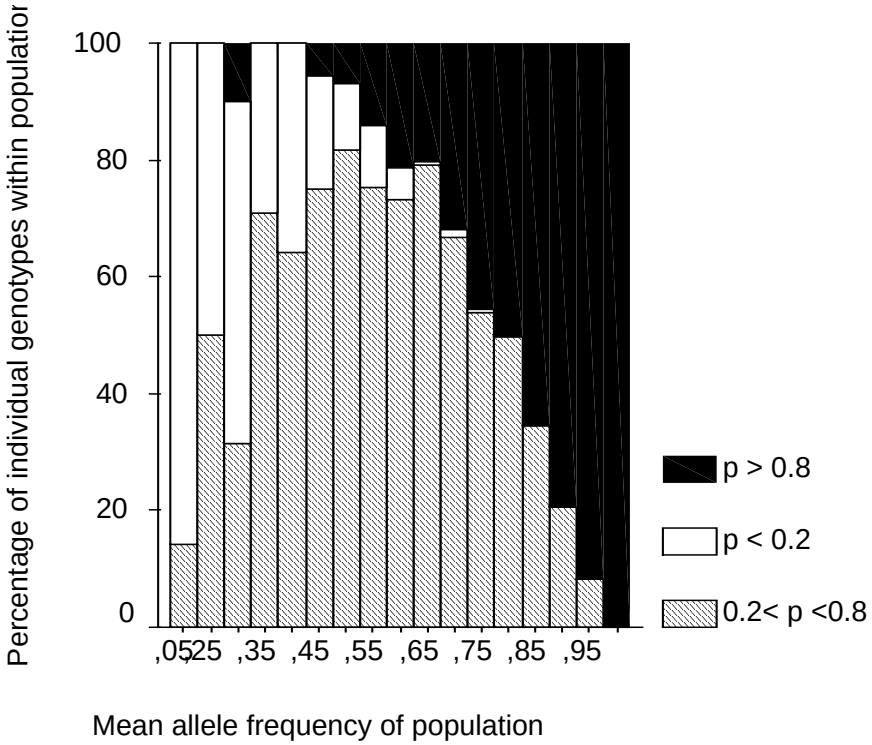

Figure 2.8: Distribution of individual genotypes across the spectrum of populations' mean *B. variegata* allele frequencies. Bars represent the mean percentage of individuals whose genotype is below 0.2 (white), between 0.2 and 0.8 (striped) or above 0.8 (black). Genotype classes are in steps of 0.05.

Considering the allele frequencies of individuals within populations, a similar picture emerges. In Apahida there is a large number of hybrid adults ($0.2 < p < 0.8$), and these constitute the majority in populations with $\bar{p}$ between 0.35 and 0.75 (Figure 2.8). On the other hand, the distribution of allele frequencies among adults in the Pescenica hybrid zone is clearly bimodal with only few hybrid individuals which were found in the less frequent hybrid populations (MacCallum 1994).

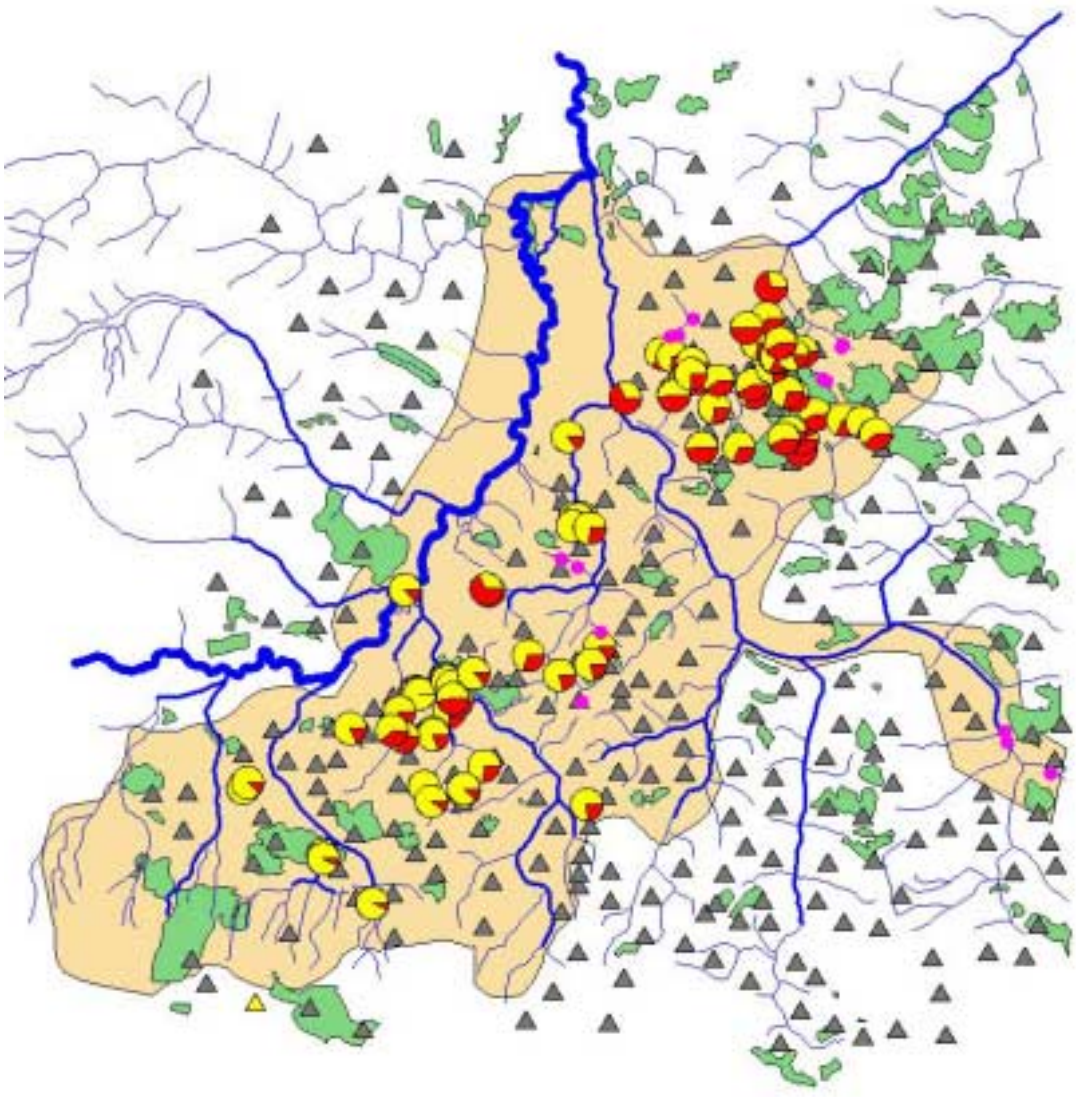

Figure 2.9: All populations and their mean genotype across the Apahida study area (see Figure 2.2). Given is the ratio of *B. variegata* (yellow) and *B. bombina* (red) alleles per population. The intensively searched area is highlighted in brown. Pink spots represent sampled sites without genotype data. Forests are marked green, hilltops as gray triangles and rivers are blue. The main river in the west is the Somes.

Considering the structure of the hybrid zone, the observed distribution of mean allele frequencies could mean that we have sampled the *B. variegata*-like section of a very broad cline. But instead, no clinal pattern is present: the few *B. bombina* populations are scattered over the map and occur in proximity to relatively pure *B. variegata* populations (Figure 2.9). Pure *B. variegata* populations seem to be more frequent in, but are not restricted to, the southern part of the study area. No significant gradient emerges. There is a broad mosaic around Apahida. The similarity between the distribution of mean allele frequencies and habitat indices suggests that the spatial pattern of habitat distribution may determine the composition of genotypes within populations in Apahida.

2.5 Summary and conclusions

The Apahida study area differs from the one in Pescenica in its topography, in the distribution of aquatic and terrestrial habitat and in the pattern of allele frequencies within and among populations. The terrestrial habitat is more uniform in Apahida. All sites are situated in arable fields or pasture, because forests have completely vanished from around Apahida, while they cover much of the Pescenica transect, and especially the hills there where *B. variegata* dwells. A discriminant function as a measure of the aquatic habitat's permanence was computed based on ecological data jointly for Pescenica and Apahida. There was no correlation between this habitat index *H* and the altitude in Apahida, in contrast to Pescenica, where an altitudinal gradient was observed, with ponds restricted to the lowland plain. The four neutral DNA marker loci change in concordance along the overall mean allele frequency which justifies treating them as equal, and the degree of concordance is very similar in the two transects. The Apahida study area is dominated by hybrid populations, while mean population allele frequencies in the Pescenica study site have bimodal distributions and the numerous pure populations are clearly situated at opposite ends of the cline. Similarly, there are far more hybrid individuals in Apahida than in Pescenica. A preliminary inspection revealed the lack of any clinal structure in the distribution of allele frequencies in Apahida. Instead, the hybrid zone exhibits a mosaic pattern, and the similarity of histograms showing habitat and genotype distributions suggests the strong impact of habitat on the pattern of recombinants found in a *Bombina* hybrid zone. The most tempting explanation for the difference between the allele frequency cline in Pescenica

and the mosaic in Apahida is that the former exhibits a spatial gradient in habitat distribution while the latter does not. The strength of habitat preference may differ as well. In Chapter 3, the major determinants of hybrid zone structure will be identified and habitat preference quantified.

Noting the differences in the number of hybrid populations and in the amount of hybrid individuals, one may ask to which extend heterozygote deficit and linkage disequilibrium differ between Pescenica and Apahida and under what circumstances the situation in Apahida may be stable. These questions will likewise be addressed in Chapter 3. For the hybrid zone to be stable one would have to assume either strong assortative mating or strong selection counteracting the breakdown of linkage disequilibrium by recombination. As no evidence for assortative mating within sites exists (Vines 2002), selection must be the critical issue. This is what the remainder of this thesis will address in Chapters 4 to 6.

3 HABITAT PREFERENCE, MIGRATION AND SELECTION

3.1 Introduction

In this Chapter I summarize the analysis of the *Bombina* hybrid zone in Apahida, which has been presented in the paper "The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*" by T.H. Vines, S.C. Köhler, M. Thiel, I. Ghira, T.R. Sands, C.J. MacCallum, N.H. Barton and B. Nürnberger. Since detailed information can be obtained from the paper, I restrict this Chapter to a summary of the main issues treated there. In Chapter 2, I showed that the *Bombina* hybrid zone in Apahida exhibits a mosaic pattern where *B. bombina* occurs in a few single ponds surrounded by an extensive population of *B. variegata*-like hybrids. In contrast to the smooth clinal transitions found near Cracow and Przemysl (Poland), Pescenica (Croatia) and the Ukraine (Szymura & Barton 1991, MacCallum et al. 1998, A. Yanchukov, pers. comm), the *Bombina* hybrid zone in Apahida exhibits no obvious gradient in allele frequencies. Why do *Bombina* hybrid zones differ from place to place? In this study we compared the *Bombina* hybrid zones in Apahida and Pescenica (Croatia: McCallum 1994, MacCallum et al. 1998), we quantified the proportion of the variation in genotype distribution that may be explained by i) a clinal component and ii) habitat type and we used the association between habitat and genotype to estimate the strength of habitat preference.

How do heterozygote deficit and linkage disequilibrium differ between Apahida and Pescenica? Under which circumstances can divergence persist despite intensive hybridization in the mosaic in Apahida? To explore these issues we inferred estimates of migration, selection strength and the strength of the barrier to gene flow from the heterozygote deficit and from the standardized linkage disequilibrium in the Apahida hybrid zone.

3.2 Methods

Chapter 2 gave a detailed description of toad sampling and genotyping methods and illustrated the resulting data set comprising 991 adult toads from 94 populations.

Genotyping was based on six allozyme loci in Pescenica whereas four molecular markers were used for Apahida, but they were treated as equivalent because of their very similar clines in the Ukrainian transect (Yanchukov, unpublished data). For each individual, the number of *B. variegata* alleles was summed over all loci and then divided by the twofold number of loci so that it ranges from 0 for pure *B. bombina* to 1 for pure *B. variegata* (see Chapter 2). A population's mean *B. variegata* allele frequency is denoted as $\bar{p}$. The habitat permanence index H was similarly scaled to vary between 0 for pure ponds and 1 for pure puddles (see Chapter 2).

To estimate heterozygote deficit (F_{IS}) and standardized linkage disequilibrium (R), maximum likelihood techniques were applied (see references in Vines et al. in press for details). Heterogeneity between sites and between loci in F_{IS} can be assessed by comparing log$L$ when F_{IS} is held constant or allowed to vary between them. Pairwise linkage disequilibrium (D) was standardized by the allele frequencies: $R_{ij} = D_{ij} / \sqrt{p_i q_i p_j q_j}$ to facilitate comparisons between sites with different allele frequencies. However, the full range of $-1 < R < 1$ is only possible when $p = q = 0.5$. In the computations of R, F_{IS} was accounted for in order to remove any undue inflation of disequilibrium through correlations of alleles within loci.

3.3 Results

The spatial pattern of hybridization in the *Bombina* hybrid zone around Apahida differs strikingly from the *Bombina* hybrid zones described so far in genetic detail. In Cracow, Przemysl, Pescenica and the Ukraine, transition zones from pure *B. bombina* into pure *B. variegata* allele frequency pools form a cline of 6 – 9 km width and are located at environmental gradients between forested hills and open plains (Szymura & Barton 1986, 1991, MacCallum et al. 1998, A. Yanchukov unpublished data). Around Apahida, a mosaic structure emerges with a large extent of hybrid populations and no obvious environmental transition (see Chapter 2). The nearest pure population for *B. variegata* lies about 20 km to the SW in the Apuseni mountains, and 100 km to the north in the Hungarian plains for *B. bombina*.

Spatial pattern of genotype and habitat distribution in Apahida and Pescenica

What is the major determinant of a population's allele frequency in the Apahida and Pescenica hybrid zones? Is it rather the overall cline or the distribution of habitat types?

We quantified this by fitting a multiple regression model to explain the mean *B. variegata* allele frequency where the geographic x-, y-coordinates and the discriminant habitat index *H* for each site were entered simultaneously as independent variables. Neither the north-south nor the east-west spatial axis were significant and the habitat was of overwhelming importance in the Apahida study area (Figure 3.1). The same analysis applied to the Pescenica data revealed that both the spatial location and habitat were highly significant there. The importance of spatial coordinates is expected since the overall gradient in allele frequencies runs SW to NE across the transect.

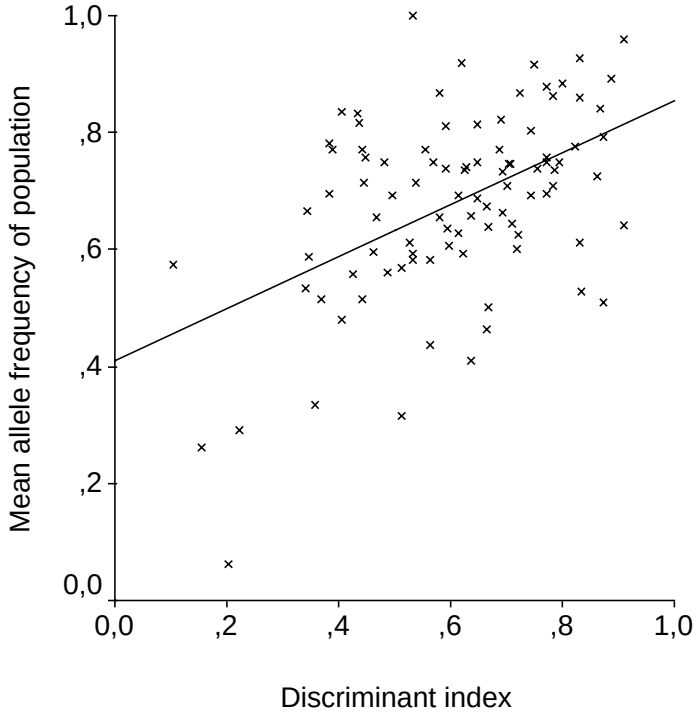

Figure 3.1: Mean *B. variegata* allele frequency p_i per population as a function of the habitat index *H* in the Apahida transect. $p_i = 0.46 + 0.39\ H$ ($F = 30.2$; $p < 0.001$).

In Chapter 2, I suggested that the distribution of habitat is the major determinant of the structure of a *Bombina* hybrid zone and that the difference between the cline in Pescenica and the mosaic in Apahida may be explained by the gradient in habitat distribution in the former and the interspersed pattern in the latter. We tested for a gradient in occupied habitat with a least squares regression of the habitat index *H* against the x-, y-coordinates. There is a significant correlation between the E-W axis and habitat type in Pescenica, but not in Apahida (see Chapter 2). However, proving that a gradient in habitat types will result in a cline is complicated in that the pattern in

occupied habitat need not necessarily be equivalent to the distribution of available habitat. For example, there are puddles in the lowlands in Pescenica which are not occupied by *B. variegata* for some unknown reason. I discuss this issue further below.

In addition to the difference in habitat distribution, the strength of the toads' habitat preference may differ between Apahida and Pescenica as different subgroups of *B. variegata* are involved in the two hybrid zones. To estimate the strength of habitat preference, the overall difference in $\bar{p}$ between pure ponds ($H = 0$) and puddles ($H = 1$) was estimated by a regression of $\bar{p}$ on H which gave $\Delta\bar{p} = 0.39\ \Delta H$. However, a more reliable estimate of the genotype-habitat association should be made over local scales that do not exceed the estimated lifetime dispersal range of 1 km (which is based though on limited recapture data in a Polish transect where habitat distribution differs from Apahida: Szymura & Barton 1991). Therefore the regression was repeated on pairs of ponds and puddles that were less than 1 km apart. This revealed a slightly lower difference in $\bar{p}$ between the extreme habitat types: $\Delta\bar{p} = 0.30\ \Delta H$.

With the Pescenica data an overall regression of $\bar{p}$ on H is difficult because the strong cline predominates the change in allele frequencies over large scales. A regression of $\bar{p}$ on H for pairs of ponds and puddles gave an estimate of $\Delta\bar{p} = 0.16\ \Delta H$. These estimates indicate a stronger genotype-habitat association in Apahida, and this is probably due to a stronger habitat preference.

Heterozygote deficit and linkage disequilibria

There are far more hybrid individuals in Apahida (see Chapter 2) despite stronger habitat preference. This observation can only be explained by high migration between habitat types which needs to be counterbalanced by pre- or postzygotic isolation mechanisms if the situation is to be stable. Rates of migration and selection strength can be explored through heterozygote deficit and linkage disequilibrium (see section 1.3.3). First, all sites were divided into seven groups by their mean allele frequencies $\bar{p}$. F_{IS} was estimated across all loci and peaks in populations at the *B. bombina* side of the genotype spectrum ($F_{IS} = 0.22$ at $\bar{p} = 0.21$); a similar asymmetry had been detected in Pescenica ($F_{IS} = 0.23$ at $\bar{p} = 0.32$). Standardized linkage disequilibrium $R = 0.090$ across all sites for each pair of loci (support limits: 0.083, 0.097). This means that there is an overall excess of parental allele combinations, despite their constant break-up by recombination. Maximum R is shifted considerably towards the *B. bombina* side of the

genotype spectrum as well (R = 0.38 at $\bar{p}$ = 0.28), again in striking similarity to the Pescenica estimate (R = 0.39 at $\bar{p}$ = 0.39). Second, all Apahida sites were divided into seven groups by the habitat index H and the same process was repeated. This gave a less clear pattern due to the low number of sites at the lower end of the H spectrum, although values of F_{IS} and R again peak at the *B. bombina* side of the habitat axis. The implication of linkage disequilibria in intermediate Apahida populations for the migration rate of *B. bombina* out of ponds and the minimal selection strength necessary to maintain the mosaic are discussed below.

3.4 Discussion

Our survey revealed that the hybrid zone in Apahida fits a mosaic pattern without any clinal gradient and thus stands in contrast to the clinal structures that have been found in the *Bombina* hybrid zones in Cracow, Przemysl and Pescenica. Interestingly, Gollmann (1986) found another mosaic pattern in a *Bombina* hybrid zone in Slovakia (see 1.4.4). The distribution of genotypes in Apahida is asymmetric: scattered ponds are the only stronghold for *B. bombina* and are surrounded by *B. variegata*-like hybrid populations in temporary habitat (see Chapter 2). Compared to Pescenica, the association between habitat and genotype is slightly stronger in Apahida, suggesting that habitat preference is stronger there. Despite the difference in the spatial pattern, heterozygote deficit and standardized linkage disequilibria are very similar between the two hybrid zones and peak in sites with *B. bombina*-like toads in both regions. With these findings we raised the following questions: Why do hybrid zones differ from place to place? How is strong linkage disequilibrium maintained within a broadly sympatric distribution? And, under what circumstances will either selected or neutral divergence persist despite migration and hybridization?

We discussed three hypotheses that could account for the difference between the *Bombina* hybrid zones in Apahida and Pescenica. The contribution of *B. variegata* subgroups with different habitat preference, different ages or alternative stable equilibria and a different distribution of the alternative habitat types might lead to different patterns of genotype distribution. Our data suggest a stronger habitat preference in Apahida. However, it is difficult to determine whether this is the cause or the consequence of the different hybrid zone structures. Stronger habitat preference

could have allowed the two taxa to move past each other into sympatry. With weaker habitat preference, establishing pure populations in the range of the other taxon would be impossible in the face of recombination, and hybridization would result in a parapatric distribution. On the other hand, stronger habitat preference could have evolved in response to selection against toads migrating to the wrong habitat type and producing unfit hybrids. This reinforcement may occur more easily in a mosaic than in a cline (Sanderson 1989, Cain et al. 1999), which might explain why habitat preference is weaker in Pescenica.

Apahida and Pescenica might also differ because they are of different ages or have arrived at alternative stable states. We cannot be precise about this since we do not know for how many generations hybridization has been going on. Finally, a potential cause for the difference between Apahida and Pescenica is the spatial distribution of habitat. The regressions of *H* onto x-, y-coordinates have clearly shown that there is a gradient in occupied habitat in Pescenica while no such pattern emerged in Apahida. However, we cannot be sure that the patterns in occupied habitat reflects the patterns in available habitat. For example, while ponds are restricted to lowland areas in Pescenica, there are puddles in the floodplain which are not utilized by *B. variegata* for unknown reasons. Similarly, without mark-recapture data from Apahida we cannot prove that all habitat types are equally available there, although the distribution of occupied habitat plausibly reflects the distribution of available habitat. The above possibilities are not mutually exclusive, and more ecological data need to be collected to understand habitat preference and the availability of appropriate habitat more fully. We tentatively concluded that the spatial arrangement of habitat contributes most strongly to the differences between the two hybrid zones. In this case, stronger introgression should ensue and preserving divergence may be harder. We obtained an estimate of migration of *B. bombina* into intermediate habitat in Apahida and discussed which selective force would be needed to maintain divergence.

It is possible to derive migration rates from linkage disequilibria (see 1.3.3). Migration is by far the more important factor generating linkage disequilibria relative to epistatic selection at least as far as associations between neutral markers are concerned (Barton & Gale 1993, Kruuk et al. 1999b). However, it is necessary to discern between the clinal component (i.e. migration of pure individuals from the periphery) and the mosaic component (i.e. migration from nearby populations of different habitat) of migration.

While both need to be considered in Pescenica, migration from the periphery may be neglected in the broad mosaic in Apahida. In Apahida, linkage disequilibrium peaks in *B. bombina*-like hybrid populations, which is likely caused by the immigration of pure *B. bombina* from ponds. This is suggested by the gap in $\bar{p}$ between pure *B. bombina* and *B. bombina*-like hybrid populations and by the observation of pure *B. bombina* adults in intermediate habitat. Even in hybrid populations with $\bar{p}$ = 0.2, these are unlikely to have been generated locally by recombination and so may be assumed to be immigrants from ponds. We assumed that a site with allele frequency $\bar{p}_i$ receives immigrants from ponds with $\bar{p} \cong 0$, and so $\Delta\bar{p}_i \cong \bar{p}_i$ for a computation of *m*. We estimated the immigration rate for each site from $D_i = (m_i \Delta \bar{p}_i^2)/r$. For populations with a *B. variegata* allele frequency between $0.2 < \bar{p} < 0.6$ we obtained $\bar{m} = 0.19$ (S.D. = 0.19). Although it is clearly a simplification to compute a single migration rate between two subgroups of populations, this estimate can in the same time explain the observed heterozygote deficit in these populations (see Appendix in Vines et al. in press) and was used in the following analysis.

Under what circumstances may divergence be maintained in Apahida despite high rates of migration between habitat types and intensive hybridization? We considered neutral loci and those traits mediating differential adaptation (including habitat preference) separately. First, linkage disequilibria between selected and neutral loci will eventually disappear, even in a clinal hybrid zone. In general, hybrid zones are regarded as barriers to gene flow. They delay the introgression of neutral traits from one taxon into the other but cannot prevent this process as long as there is any recombination (Barton & Bengtsson 1986). However, introgression might be too slow to be detected in hybrid zones with a post-glacial origin.

Second, under what circumstances will the divergence at selected loci persist? At single loci, this will be the case if $s > m$ (Haldane 1932). If linkage disequilibria build up between several selected loci, the total selection *S* ($S = ns$, where *n* is the number of loci and *s* is the per locus selection) will determine the structure of a hybrid zone (Barton 1983). The stability of divergence at selected loci depends on precisely how selection acts: mainly against hybrids or against alleles in the wrong habitat. If selection acts against alleles in the wrong habitat, it can counterbalance higher rates of migration, simply because all immigrants from the opposite habitat type are less fit. Following

Barton & Shpak (2000), N. Barton carried out numerical calculations for a symmetrical model with n = 5 to 20 unlinked selected loci assuming the above estimated influx of *B. bombina* alleles into populations with intermediate allele frequency ($m = 0.19$). He found that m = 0.2 can be counterbalanced by selection on 20 loci with $S \sim 1.7$, which implies a fitness of immigrant pure genotypes (p = 1) of 3.3% and of F1 individuals ($p = 0.5$) of 18%. It should be noted however, that this model only considers immigration from pure *B. bombina* sites. Naturally, there will also be equal immigration of *B. variegata*-like individuals (and the surrounding *B. variegata*-like sites might act as a single continuous population) which will reduce the swamping effect of *B. bombina* immigration and also contribute to linkage disequilibrium. Therefore, maintaining differences at selected loci might actually need less selection than $S \sim 1.7$ over 20 loci. The strong selection forces implied in these models are not implausible, given that we deal with two strongly diverged taxa. We have thus no reason to doubt the stability of clines at selected loci in Apahida.

But what circumstances will maintain divergence at neutral loci for as long a time as possible? Our observation that all four neutral loci are associated with the habitat index means that they are embedded into a matrix of selected loci. Since neutral divergence at a single locus should dissipate quite quickly, at a rate of $1/m = 5$ generations, introgression of neutral traits must be slowed by selection against linked alleles which needs to be considerably stronger than $S \sim 1.7$ and to involve many more loci. Specific predictions are difficult without knowledge of how exactly selection acts.

When trying to obtain an estimate of the age of the Apahida hybrid zone, two hypotheses were considered: a) that the hybrid zone originated after the last ice age, 10,000 years ago and b) that *B. bombina* moved into the area originally only inhabited by *B. variegata* after it was deforested in the 14th century (Pounds 1979). The much weaker selection that would be needed to counterbalance migration given a more recent origin render the latter more plausible.

3.5 Summary

In this Chapter I summarized the analysis presented in the paper "The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*" (Vines et al. in press). The *Bombina* hybrid zones in

Apahida (Romania) and Pescenica (Croatia) differ considerably in their spatial pattern of hybridization. In the former we observed a mosaic lacking a clinal component whereas in the latter, a cline in allele frequencies was found, with a habitat mosaic in the center. We explained this difference with the different availability of the two habitat types: an environmental gradient in Pescenica and an intermingled pattern in Apahida where habitat preference is stronger. Despite the difference in spatial pattern, both zones resemble each other in their genetic pattern (F_{IS} and D). From the observed maximum linkage disequilibrium in Apahida, we estimated a migration rate of $\overline{m} = 0.19$ of pure *B. bombina* into intermediate habitat types. While neutral divergence is probably collapsing, with this migration rate adaptive divergence requires selection around $S \sim 1.7$ over 20 loci. We assumed that the origin of the Apahida hybrid zone is most likely not at the end of the last glaciation, but may be dated to the beginning of the deforestation of Transylvania in the 14th century. Two conclusions emerged from our analysis. First, it implies the ongoing breakdown of neutral divergence in this hybrid zone at a more rapid rate than in the clinal hybrid zones in Poland, Pescenica and the Ukraine. Second, measurements of the mode and strength of selection in the field are necessary for more precise statements about the age and history of this hybrid zone as well as for predictions concerning its fate.

4 INTRINSIC SELECTION

4.1 Introduction

Natural selection is the non-random survival and/or reproductive success among variable phenotypes (Darwin 1859). The response to directional natural selection can be directional changes in trait frequencies over time, and both natural selection and the population response to it constitute the process of evolutionary adaptation. The prerequisites for adaptive evolution are that a trait is variable among the individuals of a population, that it is related to the fitness of an individual, and that it is heritable, i.e. at least partly determined at the DNA level. With directional selection, the trait frequency in the offspring will differ from that in the parental generation (Darwin 1859, Endler 1986) while stabilizing selection reduces the variance and offspring (before selection) may thus have a higher trait variance than adults (after selection). In hybrid zones, depending on the strength of natural selection, the outcome of hybridization ranges from the formation of a new hybrid species to ongoing diversification with reproductive isolation (see 1.3.2). *B. bombina* and *B. variegata* have been diverging for over 2 million years without attaining full postzygotic reproductive isolation, which is evident from the abundance of hybrid genotypes among adults in the Apahida hybrid zone (see Chapter 2). Nevertheless, in Chapter 3 we found that a strong selective barrier against *B. bombina* alleles migrating out of ponds into the surrounding *B. variegata*-like hybrid population is required to maintain divergence at selected loci. Even stronger selective forces are to be postulated in order to maintain the observed divergence at neutral loci for more than a few tens of generations. Without detailed knowledge of natural selection in the field, we cannot judge how relevant these predictions from theory are. The barrier to gene flow in the Apahida hybrid zone may be influenced by several different mechanisms. For example, assortative mating within sites may significantly reduce the reproductive success of single immigrant *B. bombina* adults who chose temporary habitats by mistake. However, Vines (2002) found no assortment in five focal sites. Instead, natural selection may dominate the barrier to gene flow. Natural selection must be acting on at least one of the different stages in the amphibian life cycle: egg, larva, juvenile or adult. It may act in different ways, such as against hybrids or as a function of the habitat against certain alleles in the wrong environment.

In the remainder of this thesis, I attempt to quantify natural selection acting on *Bombina* offspring in the Apahida hybrid zone. In this Chapter, I look at hybrid egg families that were removed from 14 sites in the field so that intrinsic fitness effects could be studied in a uniform setting. In the next Chapter, I investigate extrinsic fitness effects in the remainder of the egg batches that were allowed to develop in situ.

4.1.1 Intrinsic selection in hybrid zones

Hybrid zones offer an ideal opportunity to investigate the relative contributions of different individual mechanisms that reduce gene flow between the hybridizing taxa (Harrison 1990). For example, hybrid unfitness may be the primary factor maintaining divergence in stable hybrid zones as Barton & Hewitt (1985) concluded after a survey of almost 150 case studies. However, such tension zones tend to stabilize at environmental gradients restricting dispersal and are therefore hard to distinguish from ecotonal hybrid zones (see 1.3.3). Furthermore, the assumption of generalized hybrid unfitness (Barton & Hewitt 1985) was not based on direct evidence, but on the indirect argument that it is the most parsimonious explanation for the coincident clines that were observed in many cases. As I outlined in 1.3.3, observations from the field and laboratory experiments on hybrid unfitness sometimes produce contradictory results. This is not unexpected given the artificial constraints of laboratory studies and the inherent difficulties of measuring fitness in the field. Furthermore, a combination of intrinsic and extrinsic selection affecting hybrids is not unlikely in a hybrid zone between two taxa that diverged in allopatry. Harrison (1990) lists 28 studies of hybrid zones only 11 of which provide evidence for hybrid unfitness. Arnold & Hodges (1995) review a number of detailed hybrid zone studies and conclude that hybrids are not uniformly unfit but rather fall into several distinct hybrid classes that may have equal, lower or higher fitness relative to their parents. Thus there does not seem to be a general rule regarding hybrid unfitness (Barton 2001), and the debate over the relative importance of intrinsic versus extrinsic selection in preventing gene flow between two taxa continues.

4.1.2 Intrinsic selection in *Bombina*

If two taxa have been diverging for over 2 million years and exhibit markedly different phenotypes, one might expect incompatibilities between their genomes when they are

brought together as hybrids. However, data on hybrid dysfunction in *Bombina* hybrid zones are ambiguous. Data collected on the hybrid zone near Cracow revealed hybrid dysfunction in the form of increased embryonic mortality until gastrulation, and between gastrulation and independent feeding in central populations of the zone (Koteja 1984). These data fit to predictions from theory about hybrid unfitness and were offered as explanation for the coincidence of clines in the Cracow transect (Szymura & Barton 1986, 1991). However, Kruuk (1997) re-analyzed the data and showed that increases in embryonic failure rates towards the center of the zone were "primarily due to two data points" and that this effect was not evident in the second of the two developmental intervals examined. In a different, large scale laboratory breeding experiment with animals from the Pescenica hybrid zone, Nürnberger et al. (1995) found no evidence that offspring from hybrid populations have generally reduced viability compared to offspring from pure populations. Instead, they found different degrees of survival in different parts of the hybrid zone and a dichotomy in survival among F1 families some of which even showed above average survival rates. Kruuk (1997) suggested that data from this breeding experiment were potentially confounded by laboratory-genotype interactions, since ovulation was hormone-induced, which could have introduced noise into measures of tadpole mortality if different genotypes responded differently to the hormone. Therefore, Kruuk (1997) measured hybrid dysfunction in eggs laid naturally in breeding sites. She found significant increases in mortality at the egg and the larval stage and in developmental abnormalities in samples from hybrid populations around the center of the Pescenica hybrid zone. On the other hand, there was no evidence for differential survival apparent as shifts in allele frequencies, heterozygote deficit or linkage disequilibrium in an adult size cohort that was monitored over four years in 19 sites.

4.1.3 Measuring intrinsic selection

The study by Kruuk (1997) suggests that embryonic mortality may be used as a measure of fitness in *Bombina* hybrid zones. In general, the reproductive strategy in amphibians is adapted to high mortality rates in the egg and larval stage of the life cycle. Amphibians typically produce a large number of eggs most of which are destined not to survive. High mortality rates suggest that early stages of the life cycle may be seen as an arena of natural selection. Travis et al. (1987) found that almost all embryonic mortality in the treefrog *Hyla crucifer* occurred during gastrulation and neurulation, at a stage in

which interactions between maternally inherited cytoplasmic elements and the zygote is most intense. This effect will probably be magnified by the degree of divergence between the parental genomes. In addition, early hybrid dysfunction may be related to incompatibilities between nuclear parental alleles in the zygote that are likely to cause severe developmental problems and result in the abortion of the embryo.

Kruuk (1997) measured embryonic mortality in egg batches taken from the field in Pescenica. However, she related it to the mean hybrid index of the respective adult population. This might be in error, since the genotypic range of adults in a site may not represent the gene pool of the individuals that actually breed there. Adults may visit a site before moving on to a more suitable one in which they actually reproduce. For example, Vines (2002) found that the parents he inferred from egg data were significantly more *B. variegata*-like than the overall adult sample in temporary sites, implying strong breeding site preference. Therefore, intrinsic selection is better investigated at the level of families rather than at the population level. In this Chapter, I relate embryonic mortality to hybrid indices across families to test for a correlation between fitness and heterozygosity.

Second, to test for intrinsic selection within families, I take an approach in which I infer the parental genotypes from the family and compare the observed number of offspring per possible genotype to the expectations from Mendelian segregations without selection. This test for intrinsic selection within families can only identify effects that are physically linked to the loci in question, because correlations between genotype and phenotype within families always imply physical linkage. I refer to the entity of the inferred parental genotypes as “joint parental genotype”. It is the listing of the most likely parental genotypes across loci and families, where genotypes are coded by the number of *B. variegata* alleles present. For example, the occurrence of all three genotypes (0, 1 and 2) within a family suggests that both parents were heterozygous at this locus (1,1). The joint parental genotype across all four loci might be, for example, ((1,1)(0,0)(1,2)(0,2)). Note that for unlinked marker loci one may not determine which per-locus genotype came from which parent.

There are two ways in which intrinsic selection may act. First, alleles of one taxon may have an intragenomic selective advantage and preferentially be passed on to the next generation, resulting in a shift in segregation ratios, but not in heterozygote deficit. The selective advantage of single alleles may be determined in a heterozygous parent during

gamete production before any zygote is formed (akin to meiotic drive) or afterwards through selection against individual zygotes carrying a certain proportion of alleles of the "wrong" taxon. Meiotic drive favors a certain allele irrespective of the genetic background, whereas selection in the zygote depends on the allelic state of other genes in the genome. This mechanism is de facto frequency dependent because it disfavors alleles of the "minority taxon". Note that the occurrence of meiotic drive is not very likely a prominent force in a hybrid zone. If consistent patterns across marker loci are found one would have to assume that each one of them is linked to a driver locus despite the fact that same-taxon suppressor alleles need to have recombined away from the driver alleles and that drivers occur in only one taxon. Second, intrinsic selection may act against heterozygous individuals due to genetic incompatibilities, leading to heterozygote deficit in the offspring through selective deaths in early developmental stages.

Before any analysis of intrinsic selection can be attempted, it is important to exclude genotyping errors and errors that arise from mistakenly analyzing mixed families or the same family more than once. In *Bombina* in particular, the identification of true families is not trivial due to the adults' spawning habits. During spawning, pairs in amplexus move around the water body, and the females often deposit the eggs in several locations. Eggs are usually attached to plants which are sometimes limiting, especially in ephemeral puddles. Therefore, an egg batch that appears homogenous may in fact contain eggs from more than one family, while full siblings may be distributed over several separate egg batches. Genotyping errors and undetected mixed families would widen the range of genotypes present in a family. This would erroneously increase the overall number of heterozygous parents inferred from the data. A genotyping error may only become evident as a locus-specific, single aberrant genotype within a family, which I refer to as a "singleton". Initially, I assume Hardy-Weinberg equilibrium and test whether the observed frequency of singletons across the data set agrees with Mendelian expectations. An excess could have two explanations: genotyping errors or non-random segregation, which can be tested with repeated laboratory analyses. The presence of one family in various egg batches is indicated when compatible joint parental genotypes occur across all loci. In most sites, information on a highly variable microsatellite marker locus that allows family assignments is available, which enables the identification of repeatedly sampled and of mixed families. In two sites without genotypes for this locus, finding true families is more indirect. There, mixed families

can be detected by a significant association between allelic states at different loci, since no such associations are expected between physically unlinked loci among full siblings. To test for this, I apply two different statistical methods described below. Finally, I infer the maximum likelihood estimate of segregation ratios within loci across all families. This estimate will be used to compute the expected ratio of heterozygotes, which is compared to the observed number, to test for heterozygote deficit in the egg families.

4.2 Methods

4.2.1 Selection of sites

For the adult survey presented in Chapters 2 and 3, a wide range of sites was visited over both seasons. In this survey, all available egg batches were sampled, which gave a set of 34 sites. For the study presented in this and the next Chapter, it would not be worthwhile to genotype eggs in sites with almost pure *B. variegata* adults. Therefore, I concentrated the analysis on 14 sites that i) produced a reasonable number of egg batches over the season, ii) produced surviving tadpoles at the end of the season for the cohort study in Chapter 5, iii) covered a broad range of habitat types and iv) contained a wide range of adult genotypes. An estimate for adult genotypes may be obtained in the field from their belly pattern as described in Chapter 2, which facilitates the choice of suitable sites in the field.

4.2.2 Egg collection

All 14 sites were visited once every three to four days which should insure that all egg batches were found before tadpoles could hatch. Whenever detected, 10 to 16 eggs were collected per batch, and the developmental stage (Gosner 1960) was recorded. Batches from which eggs had been removed were flagged to prevent redundant sampling on later visits. Batches containing less than 10 eggs were taken entirely. When collecting eggs in the field one has to take into account other anuran species whose eggs resemble those of *Bombina*. For example the European tree frog, *Hyla a. arborea* lays eggs of a similar color and diameter in the same type of habitat as *B. bombina*, and also the mean number of eggs per batch is very similar in both species. However, when subsequently raised in the laboratory, *Hyla a. arborea* tadpoles may be recognized easily by their

violin-shaped body and protruding eyes. Frogs (*Rana* species) lay much larger egg batches than *Bombina*, but occasionally, small clutches become separated and may then be confused with *Bombina* egg batches. Since *Rana* and *Bombina* tadpoles resemble each other in early stages, I relied on amplification failure in the subsequent genotyping procedure to exclude mistakenly sampled *Rana* families from the study. The amount of erroneously sampled batches that turned out to be of species other than *Bombina* can be seen in Table 4.1. The problem was restricted to permanent sites, since *Hyla a. arborea* and *Rana* do not breed in temporary habitat. After excluding 53 foreign egg batches, 105 *Bombina* egg batches remained with 1016 eggs in total.

Table 4.1: Site numbers, habitat types and numbers of erroneously sampled egg batches of *Hyla a. arborea* (which were detected as tadpoles) and *Rana* species (which were detected as amplification failures).

Site number	Habitat type	*Hyla a. arborea* egg batches	*Rana* species egg batches
204	artificial pond	-	-
256	puddle	-	1
257	ditch	1	10
258	small pond	2	2
271	puddle	-	-
272	puddle	-	-
274	puddle	-	-
276	puddle	-	-
282	small pond	18	7
290	small pond	3	1
315	ditch	2	1
317	puddle	-	-
318	puddle	-	-
330	small pond	3	2
Sum		29	24

4.2.3 Rearing scheme and measuring viability

Eggs were taken to the laboratory where each batch was divided into groups of three to five eggs; these groups were raised at a temperature range of 22°C to 25°C in 200 ml

plastic cups filled with dechlorinated tap water. The water was topped up every day. Tadpoles were not fed, since they were raised only for about ten days until they had reached Gosner stage 25, which means that they still had a yolk sack. Rearing tadpoles for such a short time should ensure that any mortality due to genotype-specific competitive effects (i.e. a confounding selection process) is negligible. Within the rearing period, egg hatching failures and dead tadpoles were recorded and removed daily. Hatching failure and developmental abnormalities, which resulted in subsequent death in all cases, were treated summarily as tadpole mortality. At Gosner stage 25, surviving tadpoles were anaesthetized and preserved in 0,5 ml Eppendorf tubes filled with 99.9 % ethanol.

4.2.4 Genotyping

From the 105 *Bombina* egg batches, 922 tadpoles survived and were genotyped in the same way as adults for the four unlinked, neutral marker loci *Bb7.4*, *Bv12.19*, *Bv24.11* and *Bv24.12* (see Chapter 2). After genotyping, 896 tadpoles in 105 batches were left for the analysis. Except for sites 204 and 257, additional genetic information on the microsatellite marker locus *Bv41.11* (Acc # AF472428) was provided by Marlies Frenzel and Bruni Förg-Brey. This locus is highly polymorphic and therefore allows the assignment of individuals to families. However, it is not informative for the hybrid index, since its alleles have not been assigned to either *Bombina* taxon. After assigning individuals to families, the sum of *B. variegata* alleles across all four marker loci was computed for every animal to obtain the hybrid index *HI*. This hybrid index was rescaled as the *B. variegata* allele frequency *p* to vary between 0 and 1. The *B. variegata* allele frequency *p* was calculated per joint parental genotype as representative of a family and as the unweighted average of family means, $\bar{p}$, per site.

4.2.5 Preparing the data set

Detecting genotyping errors as singletons

A preliminary inspection of the genotype data revealed that most segregations seemed to be in Mendelian ratios. However, the number of singletons stood out. For example, family number 17 in site 257 shows a highly unlikely segregation of one homozygous *B. bombina*, 12 heterozygous and one homozygous *B. variegata* eggs at locus *Bv24.12* (Appendix 4.2). The inferred joint parental genotype for this segregation is (1,1), i.e. the

mating of two heterozygous individuals. Without the singletons the inferred parents would be homozygotes of either taxon (0,2). Thus the presence of singletons may inflate the number of heterozygous individuals inferred to be parents (see above). To exclude singletons caused by genotyping errors, I tested for an excess of singletons across all loci and batches by comparing the numbers observed with the numbers expected under Mendelian segregations with no errors. For this computation, I used the inferred joint parental genotypes for each of the four marker loci. For any mating involving one homozygous and one heterozygous parent (i.e. either a (0,1) or a (1,2) mating) with a family size *n*, the probability of getting one singleton of either the heterozygous or the homozygous genotype among the offspring is

$$\text{P(singleton)} = \frac{2n\ p(1-p)^{n-1}}{1-2\,p^n} = \frac{2n\ p^n}{1-2\,p^n}$$

where $p = 0.5$, i.e. the expected frequency of homozygous and heterozygous offspring in this type of mating. The numerator represents the probability of seeing either singleton amongst the offspring. The denominator rescales the overall probability by excluding the cases where only one genotype occurs because, in these cases, one would infer a mating of two homozygous individuals instead. In the case of two heterozygous parents (1,1), one expects a ratio of 0.25:0.5:0.25 between homozygous *B. bombina*, heterozygous and homozygous *B. variegata* genotypes among the offspring. Additionally, in a (1,1) mating either one singleton or two singletons of either homozygous genotype might occur. Each constellation has a separate probability for observing a singleton. For the allele with frequency *p*, the probability is

$$\text{P(one homozygote)} = \frac{2n\left(p^2\right)\left(2pq+q^2\right)^{n-1}}{1-2\left(2pq+q^2\right)^n+(2pq)^n}$$

The overall number of expected singletons in a sample of *N* egg batches is the sum over all within-batch probabilities. To obtain null distributions, 10,000 random segregations were generated with the same number of batches and mating types, using the actual sample sizes per egg batch. The test statistic u was the proportion of randomized batches that had a higher number of singletons. Initially, the overall number of singletons in the data set was 71, which significantly exceeded the expected number of 53.4 singletons ($u < 0.001$). The 25 least probable singletons (maximum $u = 0.303$) were re-amplified, corrected if necessary, and ambiguous cases were excluded. A total

of 63 singletons remained, which still deviated significantly from the now expected 47.6 (u = 0.001). However, since this may be an effect of mixed families and/or intrinsic selection, the singletons were left in the data set.

Detecting families present in more than one egg batch

To avoid any bias in statistics or pseudo-replication, it was necessary to minimize the probability of analyzing an individual family more than once. Given the *Bombina* spawning habits (see above), it is not unlikely that a single mating is sampled in more than one egg batch. Therefore, the joint parental genotype was inferred per locus for each egg batch, and all egg batches within a site that shared compatible joint parental genotypes over all loci were merged into one family. This method is conservative because it aims at obtaining the smallest possible sample size of families. Some egg batches with identical joint parental genotypes may in fact represent separate families, but are only analyzed once. Based on joint parental genotypes, I grouped two egg batches into one family in eight cases and three egg batches into one family in one case.

Detecting mixed families

The presence of undetected mixed families within an egg batch would artificially inflate the number of heterozygous parents inferred for this batch (see above). In most sites, data on locus *Bv41.11* allowed easy identification of 14 mixed families. For sites 204 and 257 however, where data on this locus were not available, a more complicated approach for the detection of mixed families was undertaken. The mixing of families generates correlations between alleles at two different unlinked loci. This is similar to the generation of linkage disequilibrium by migration between genetically distinct populations. This fact is used in tests for detecting mixed families when the parents are unknown. The test applied here comprises two measures for mixed families. The first measure is the variance in the hybrid index *HI*, which is given by an individual's number of *B. variegata* alleles summed over all loci (see Vines 2002). The variance in hybrid index should detect the mixing of families with divergent genotypes. The observed variance in the hybrid index in a family of size *n* genotyped at *k* loci is

$$\mathrm{Var}(HI) = \sum_{i=1}^{k}\sum_{j=1}^{n}\frac{\left(x_{ij}-\bar{x}_i\right)^2}{n},$$

where x_{ij} is the number of *B. variegata* alleles in individual *j* at locus *i* and $\bar{x}_i$ is the mean for that locus and family. No covariance terms appear in this expression as these

must be zero for unlinked loci. To obtain null distributions, the genotypes per locus within a family were randomized 10,000 times and the variance in hybrid index was computed for each randomization. The test statistic u was the proportion of randomized batches that had a higher variance in their hybrid index than the observed one. This test revealed no significant excess in variance in hybrid index in any egg batch.

The second test measure is the squared covariance between alleles, summed over all pairs of loci (Vines & Barton in press). This may detect mixing even if both families have an identical hybrid index because it considers all observed alleles individually instead of classifying them as either *B. bombina* or *B. variegata*, which renders the locus essentially biallelic. The power of this test increases with the number of alleles per locus, since it becomes less likely that two segregations in a mixed batch involve the same alleles. The significance of the measure was again assessed by comparing the original value to a null distribution generated by 10,000 randomizations of the alleles at each locus between individuals of a family. Randomizing alleles between individuals should remove any associations between loci in mixed families. Therefore, the test statistic u is the proportion of squared covariance values in randomizations that exceed the observed squared covariance of the egg batch. This test revealed significant excess in one batch (batch one in site 257; u = 0.01) which was subsequently split into two families. Splitting the batch removed any excess in the squared covariance between alleles.

4.2.6 Tadpole mortality

As the number of sites sampled is not sufficient to detect a significant trend in tadpole mortality across the allele frequency spectrum, especially at its *B. bombina* end, the *B. variegata* allele frequency *p* per joint parental genotype as a representative of a family was related to the “purity”, using a folded hybrid index *p*’ defined as

$p' = p$ if $p <= 0.5$ and

$p' = 1 - p$ if $p > 0.5$.

The proportion of tadpoles failing to survive was arcsine transformed (Sokal & Rohlf 1995) before testing for a correlation with *p*’ with Spearman’s rank correlation coefficient.

4.2.7 Test for intrinsic selection

Egg batches that consisted of fewer than four individuals were discarded since batches of this size contain too little information to make reliable inferences about the joint parental genotypes. In principle, intrinsic selection may only become evident in families involving at least one heterozygous parent, because homozygous parents produce homogenous gametes. Therefore, only (0,1), (1,2) and (1,1) matings were investigated.

In the following, I first consider the test whether segregation ratios deviate from the expected 0.5. For the (0,1) and (1,2) mating type the probability for the observed segregation of *n* offspring with *k B. variegata* gametes provided by the heterozygous parent was computed as

$$\text{P(segregation in one heterozygous parent)} = \frac{\binom{n}{k} p^k (1-p)^{n-k}}{1 - p^n - (1-p)^n}$$

In a mating between two heterozygous parents (1,1), both segregations need to be considered. The probability for the observed distribution of *k B. variegata* homozygotes and *m* heterozygotes in a family of *n* offspring is:

$$\text{P(segregation with two heterozygous parents)} = \frac{\binom{n}{k\,m} \left(p^2\right)^k (2pq)^m \left(q^2\right)^{n-k-m}}{1 - \left(2pq + q^2\right)^n - (p^2 + 2pq)^n + (2pq)^n}$$

where $q = 1 - p$. The correction factor in the denominator now must account for the possibility that *p* is different from *q* and therefore list the cases that there is no *B. bombina* homozygote and no *B. variegata* heterozygote with their possibly different probabilities separately. The likelihood of a given frequency of *B. variegata* alleles *p* was determined per family as

$L[p] = P$ [observed data | p]. Over i segregations

$$\log L[p] = \sum_i \log L_i[p]$$

The segregation ratio *p* was varied between 0 and 1, and the segregation ratio producing the maximum likelihood max(log*L*) was computed. The distribution of 2log*L* follows approximately a χ^2 distribution, and if 2max(log*L*) differed from log*L* ($p = 0.5$) by 3.84

or more, the deviation from a segregation ratio of 0.5 was considered significant ($p = 0.05$).

Second, I tested for heterozygote deficit within families to detect intrinsic selection against hybrids. The maximum likelihood segregation ratios were used to compute the expected number of heterozygous individuals per family. Since segregation ratios deviating from 0.5 produce different numbers of heterozygous offspring in different mating types, the expected number of heterozygotes was determined as p, $2pq$ and $(1-p)$ for the mating types (0,1), (1,1) and (1,2), respectively. The likelihood of any ratio h of heterozygotes was determined as

$L[h] = P$ [observed data | h]. Over all i segregations

$$\log L[h] = \sum_i \log L_i[h]$$

The heterozygote ratio h was varied between 0 and 1, and the ratio producing the maximum likelihood max($\log L$) was computed. Any deviation from 0.5 was again tested with a χ^2 distribution.

4.3 Results

4.3.1 The data set

After splitting and merging egg batches into families and after the exclusion of families containing less than four eggs, 886 eggs in 90 families were left for the analysis of intrinsic selection (Table 4.2). The egg genotypes can be found in Appendix 4.1 and 4.2. In the latter, a family's segregation at each locus is given by the number of individuals that are homozygous for the *B. bombina* allele, heterozygous or homozygous for *B. variegata* alleles in curly brackets, e.g. {0,0,10} for ten pure *B. variegata* eggs.

Table 4.2: Site numbers, numbers of egg families and individual eggs. Tadpoles were genotyped as indicated in the text, giving the mean *B. variegata* allele frequency $\bar{p}$ per site as the unweighted average of family means.

Site Number	Number of families	Number of eggs	$\bar{p}$ over all families
204	7	75	0.865
256	5	60	0.777
257	25	251	0.759
258	3	30	0.558
271	3	29	0.783
272	2	26	0.907
274	5	52	0.863
276	3	24	0.578
282	1	4	0.438
290	16	181	0.694
315	5	30	0.825
317	4	44	0.809
318	2	16	0.759
330	9	64	0.667
Sum	90	886	0.742

The distribution of genotypes among all 90 families is indicated in Figure 4.1. The sample is dominated by *B. variegata*-like hybrid families so that the mean *B. variegata* allele frequency $\bar{p}$ over all loci and families is skewed towards the *B. variegata* end of the allele frequency spectrum with no value below 0.4 and 13 out of 90 families with $\bar{p}$ ranging between 0.4 and 0.5.

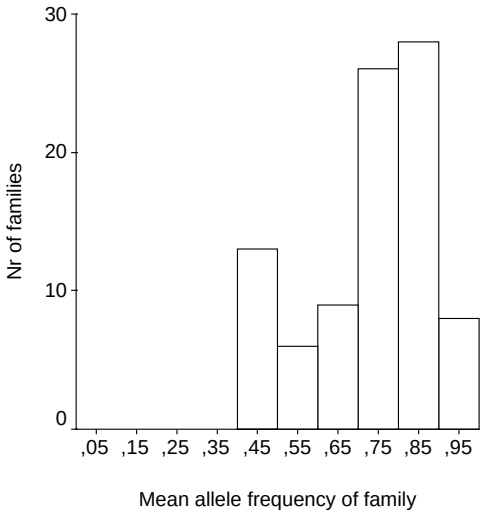

Figure 4.1: Number of families with a mean *B. variegata* allele frequency *p* between 0 and 1 in steps of 0.1. Overall $\overline{p}$ = 0,742; S.D. = 0.15; N = 90.

4.3.2 Tadpole mortality

The egg batches were monitored before information on sibships was available. Therefore, the analysis of mortality rates could only involve families that were still consistent with originally sampled egg batches after family assessments. However, the nine cases in which two or three egg batches had been merged into one family were included in the analysis, and tadpole mortality was calculated as the mean over the original batches. The analysis then comprised 83 families. Note that due to the over-representation of *B. variegata*-like families in *p*' (see above), the results hold only for *B. variegata*-like hybrid egg batches.

Overall survival rate was high, with 90.4% of all tadpoles surviving until Gosner stage 25. However, tadpole mortality occurred in 43.4% of the 83 batches monitored. Figure 4.2a gives the individual mortality rates in relation to *p*' for each batch. The maximum mortality rate affecting an egg batch was 70% at *p*' = 0.025. No correlation between the (arcsine transformed) tadpole mortality rate and *p*' could be detected (r_{SP} = - 0.216; p = 0.059). It has to be noted though that families may vary in their degree of hybrid generation with the most recombined families offering the least reliable prediction from marker genotypes about hybrid unfitness. This means that families may be quite heterogeneous in the degree to which alleles at marker loci are associated with alleles at selected genes. Therefore, tadpole mortality due to genetic incompatibilities may be

hidden when analyzed at the family level. However, when relating tadpole mortality to the population mean *B. variegata* allele frequency, similar results emerge as at the family level (Figure 4.2b).

At both the family and the population level there is the unexpected and nearly significant trend that slightly introgressed *B. variegata*-like families have a higher mortality than intermediate ones. Under the equal environmental conditions given in this experiment, tadpole mortality in early stages may be mediated by intrinsic selection within families, either against certain alleles or against hybrid individuals. I explore this issue in the next section.

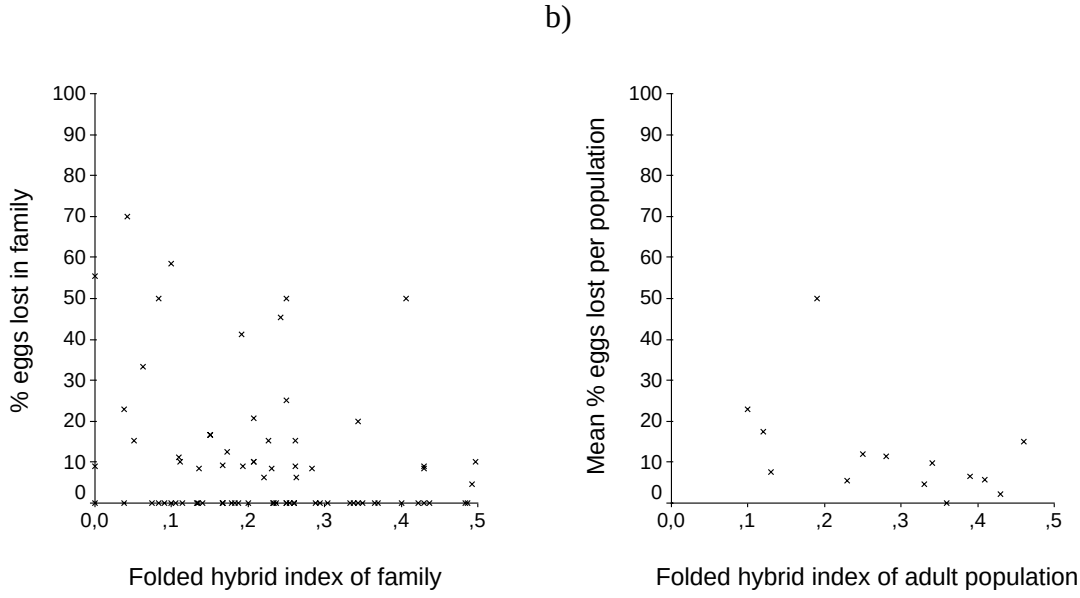

Figure 4.2: Tadpole mortality rate related to a) the egg family folded hybrid index (N = 83) and b) the adult population folded hybrid index (N = 13). Folded hybrid index $p' = p$ if $p <= 0.5$ and $p' = (1 - p)$ if $p > 0.5$.

4.3.3 Intrinsic selection

Only family/locus combinations involving one or more heterozygous parents were considered in the analysis of intrinsic selection because no segregations occur in homozygous parents. The overall pattern in the number of families involving at least one heterozygous parent is similar between loci (Table 4.3). The (0,1) mating type is rare and even absent in locus *Bv12.19*. Most common is the (1,2) mating type, which

reflects the predominance of *B. variegata*-like animals in the adult population in Apahida.

Table 4.3: Number of families generated by a homozygous *B. bombina* and a heterozygous parent (0,1), by a homozygous *B. variegata* and a heterozygous parent (1,2) and by two heterozygous parents (1,1) per locus. The last column gives the number of segregations within heterozygous animals over all 90 families for the respective locus. Note that in a (1,1) mating, segregations occurred in both parents.

Locus	Mating type (0,1)	Mating type (1,1)	Mating type (1,2)	Segregations
Bb7.4	5	27	11	54
Bv24.11	8	37	14	73
Bv12.19	0	25	14	53
Bv24.12	9	33	18	78

Segregation ratios

Table 4.4 shows the maximum likelihood estimates max(log$L$) of *B. variegata* allele frequencies p per locus. The maximum likelihood frequency of *B. variegata* alleles is closest to the expectation of $p = 0.5$ for locus *Bv24.12*, while the highest positive deviation is observed for locus *Bv24.11*.

Table 4.4: Maximum likelihood estimates of mean *B. variegata* allele frequencies p per locus and across all loci.

	Bb7.4	*Bv24.11*	*Bv12.19*	*Bv24.12*	Observed (across all loci)
p	0.551	**0.557***	0.567	0.476	**0.535****
max(log$L$)	-96.706	-111.126	-72.790	-109.600	-396.601
log$L$(p = 0.5)	-99.394	-115.657	-76.962	-110.343	-402.357

The single most likely segregation ratio across all loci is $p = 0.535$, which differs significantly from the expected segregation ratio of $p = 0.5$ ($\chi^2 = 11.512$, 1 df, $p < 0.005$), indicating an overall excess of *B. variegata* alleles in offspring of *B. variegata*-like families that could arise either at the gamete or fertilization stage or from differential offspring survival to stage 25. Moreover, there is significant

heterogeneity in the segregation ratios among loci: the sum of the log*L*-values for the most likely *p* estimates per locus is -390.356, which represents a much better fit to the data than the single estimate of $p = 0.535$ ($\chi^2 = 12.49$, 3 df, $p < 0.01$). After adjusting the α level for multiple test ($\alpha' = 0.0125$), locus *Bv24.11* individually shows a significant excess of *B. variegata* alleles ($\chi^2 = 9.062$, 1 df).

Heterozygote deficit

Table 4.5 shows the ratio of heterozygotes *h* per mating type and locus that gave maximum likelihood values max(log*L*). The expected ratio of heterozygotes *h* was computed using the maximum likelihood segregation ratio *p* per locus determined above. It also gives joint estimates across all loci based on the overall $p = 0.535$.

Table 4.5: Maximum likelihood estimates of the ratio of heterozygotes *h* per locus and over all loci for the three possible mating types involving at least one heterozygous parent.

Locus	Mating type (0,1)		Mating type (1,2)		Mating type (1,1)	
	h (obs)	*h* (exp)	*h* (obs)	*h* (exp)	*h* (obs)	*h* (exp)
	log*L*	log*L*	log*L*	log*L*	log*L*	log*L*
Bb7.4	0.690	0.551	0.495	0.449	0.465	0.495
	-7.555	-9.739	-57.625	-58.760	-20.035	-20.500
Bv24.11	0.605	0.557	0.420	0.443	0.550	0.496
	-13.224	-13.605	-71.160	-71.508	-23.005	-23.677
Bv12.19	-	-	0.380	0.433	0.415	0.491
			-40.929	-42.083	-30.548	-31.877
Bv24.12	0.430	0.476	0.505	0.524	0.470	0.499
	-15.630	-15.917	-52.918	-53.205	-37.350	-37.626
p across	0.565	0.535	0.455	0.465	0.470	0.498
loci	-41.178	-41.566	-228.327	-228.677	-113.151	-113.786

Over all mating types, h does not deviate significantly from the expected ratio of heterozygotes ($p = 0.25$), meaning that there is no evidence for selection against heterozygous individuals within families.

4.4 Discussion

I now first cover the initial checking of the egg data for genotyping errors and the presence of mixed families and of repeatedly sampled families. I then consider whether intrinsic selection is operating in *Bombina* and discuss my findings in the context of the existing knowledge of selection in *Bombina* hybrid zones.

I found an overall highly significant excess of singletons within families. This could be explained by genotyping errors or by non-random segregations. Genotyping errors that introduce a novel genotype into a clutch of siblings inflate the number of heterozygous parents inferred and may bias estimates of segregation ratios within heterozygous parents. It is therefore important to exclude genotyping errors from the data. This was done by repeated laboratory analysis of the singletons in question. Even after corrections were made for genotyping errors, the excess of singletons remained highly significant. Remaining error might be caused by genotyping errors that failed to produce the singleton's genotype that is actually present in other individuals within the family. This would lead to an underestimate of heterozygosity. However, this type of error is entirely inconspicuous and would only be found if one re-analyzed the entire data set. Since this is not feasible given the enormous amount of work, it only remains to take the excess of singletons as an effect of non-random segregations.

Another potential problem is the mixing of families from separate matings, which is rather likely in *Bombina* and which can produce considerable bias when inferring parental genotypes from egg data. In two sites, I applied two methods that use the variance in hybrid index within families and the covariance among alleles within loci to detect mixing. In this way, I found only one family that was probably the product of mixing two egg batches. In the remaining 12 sites, data on a highly polymorphic locus revealed a variety of mixed families, approximately four times as many as detected with the indirect variance and covariance techniques. This finding shows that the mixing of different families in *Bombina* poses a more serious problem than had been initially assumed. More importantly, tests using indirect techniques seem to have too little power if four loci, with up to six alleles, are applied to detect mixing, especially if there are only one or two foreign eggs in a batch. The third potential problem with field data in *Bombina* is the repeated sampling of the same family over two or more egg batches. However, compared to the analysis of mixed families, this is less of a problem since it does not cause severe bias when segregation ratios are analyzed within families. Since

the consequences of joining unrelated families are worse than leaving batches of the same family apart, the test for repeatedly sampled families needs to be rigorous. The highly polymorphic locus *Bv41.11* allows good insights into true families while an analysis based entirely on the other four loci would not provide much resolution.

I now consider intrinsic selection in *Bombina* from Apahida. The first step of analysis comprised mortality in egg families and its relationship to the families' genetic purity. In a second step, I estimated the maximum likelihood segregation ratio and heterozygote deficit within families.

In a sample of 83 *B. variegata*-like hybrid families no correlation between the mortality rate within the family and the mean *B. variegata* allele frequency *p*' could be detected both if embryonic mortality was related to the inferred parental genotypes across families and to the adult genotypes across populations. However, deaths occurred in almost half of the families monitored. For comparison, in Kruuk's (1997) study conducted in Pescenica, only 20.3% of all 167 egg batches monitored were affected by embryonic mortality or larval developmental abnormalities. These were concentrated around the population *p*' = 0.4, causing a highly significant positive correlation between tadpole mortality and *p*' across populations. The maximum rate in hatching failure per egg batch reached 50% around *p*' = 0.4, whereas I observed similar and even higher mortality rates (maximum: 70%) across the entire spectrum in *p*' across families and populations. So while mortality in eggs and tadpoles is correlated with the population's hybrid index in Pescenica, high tadpole mortality rates are observed over the entire *B. variegata*-like genotype spectrum in Apahida. Do the predominantly *B. variegata*-like hybrid populations around Apahida suffer a generalized increase in early tadpole dysfunction compared to pure populations? One cannot answer this question without knowing egg failure and tadpole mortality rates in both pure taxa, which are more difficult to come by given that these are located at considerable distance (*B. bombina* 100 km to the north-west in the Hungarian plain and *B. variegata* around 40 km to the south-west in the Apuseni mountains). The difference between Apahida and Pescenica might be explained by the difference in the spatial distribution of habitat and populations. Immigration of pure *B. bombina* may happen sporadically in almost any temporary habitat in the broad mosaic in Apahida, so that alleles causing dysfunction on a largely *B. variegata*-like background may segregate in most of these populations. In contrast, the clinal setting of Pescenica implies that they are concentrated in populations

of intermediate allele frequencies and are considerably less likely to diffuse much further into the opposite gene pool.

There was good evidence for consistent bias in segregation ratios as *B. variegata* alleles were in excess at three of the four marker loci. Locus *Bv24.12* showed a significant excess of *B. bombina* alleles, but the overall mean was shifted towards *B. variegata*. After considerable rescoring, I exclude systematic scoring errors as potential cause for this bias, especially, since scoring error mainly increases the number of segregations and leads to an apparent shortage of heterozygotes, but it does not cause a bias in favor of one or the other allele. It is extremely unlikely that *B. variegata* alleles were consistently mistaken for *B. bombina* alleles across three out of four loci. Therefore, the observed bias in segregation ratios may be caused by systematic selection against *B. bombina* alleles at loci linked to the three marker loci. It is presently impossible to distinguish between meiotic drive or selection against *B. bombina* alleles through cyto-nuclear interactions in the zygote or through nuclear incompatibilities in early embryonic stages. However, it is unlikely to observe meiotic drive by the analysis of four marker loci in a hybrid zone holding many hybrid generations, as one of these would have to be linked to each marker (see above). The effect of epistatic interactions between nuclear loci may be frequency-dependent, in that there is selection against *B. bombina* alleles in early embryonic stages of predominantly *B. variegata*-like hybrid families. Frequency-dependent selection against *B. bombina* alleles could explain the unexpected trend that embryonic mortality across slightly introgressed families is almost significantly higher than across more intermediate families. Is the bias in segregation ratios a strong enough barrier to halt the influx of *B. bombina* alleles into *B. variegata*-like sites, as was postulated in Chapter 3? From the argument there, this is at least the more effective scenario compared to selection against heterozygotes. It is also consistent with the idea that many selected loci must be spread across the genome, which are more likely to be detected with such a small number of neutral markers. Interestingly, the heterogeneity found between the four marker loci supports the intuitive scenario that parts of the genome are subject to a range of different strengths and directions of selection. Simulations should be a useful tool to explore the relationship between the pattern of recombinants and the selection against *B. bombina* alleles found in this study.

There was no consistent evidence for selection against heterozygous individuals within families from the segregation patterns. I discuss the following plausible reasons. If selection against heterozygotes is acting during the embryonic stage, it might be too weak to have been discerned with this data set. Alternatively, the marker loci might happen not to be linked to loci that are affected by selection against heterozygotes, selection might act at a later stage of the life cycle, or it might be mediated by the environment rather than through intrinsic mechanisms.

Fitness differences at the family level can only be detected with marker loci if these are physically linked to selected genes. Heterozygote deficit within families may be hidden if alleles at marker loci are recombined away from alleles at genes that generate incompatibilities in heterozygotes. The probability for this increases with the degree of hybrid generation, and hybridization might have occurred in Apahida for around 200 generations, since the 14th century (see Chapter 3). However, there has been constant influx of pure individuals so that "early" hybrid generations are continuously regenerated. Furthermore, the marker loci were sufficient to detect selection against *B. bombina* alleles due to epistatic interactions. However, it is important to note that selection against heterozygotes is less easily detected with neutral markers than epistatic interactions, because a *B. bombina* allele at any locus linked to and in phase with the marker can have negative epistatic interactions with any number of loci across the genome and thus be selected against, while for the detection of selection against heterozygotes one needs two marker alleles in phase with the alleles at a specific selected and linked locus.

Selection against heterozygotes may occur in later stages of the life cycle, after embryonic development. For example, this form of hybrid dysfunction may set in during metamorphosis. This is not unlikely since metamorphosis is a phase of revolutionary changes in anatomy and physiology, and many toadlets die during and shortly after metamorphosis for unclear reasons (personal observation). It is very difficult however, to investigate intrinsic selection during late larval stages in the laboratory without inflicting artificial constraints and introducing confounding effects of food availability, light, or temperature.

Hybrid unfitness may be mediated primarily through extrinsic selection and thus not manifest itself in the laboratory. For example, the proportion of hybrids between the cyprinid fish species *Notropis cornutus* and *N. chrysocephalus* decreases over

successive age classes in the field (Dowling & Moore 1985). F1 fitness is primarily ecological in hybrid sticklebacks from the species complex *Gasterosteus aculeatus* (Hatfield & Schluter 1999) and in Darwin's finch hybrids, where hybrid fitness depends on cyclically changing feeding conditions (Grant & Grant 1992, 1996). Most selective disadvantages of hybrids in post-embryonic stages of the life cycle will only be relevant in the environmental context in which they arise. Measuring selection in the field requires sophisticated techniques, for example a cohort analysis, in which the same population is sampled at least twice in successive stages of the life cycle and the allele frequencies are compared between them. I attempt this in the next Chapter.

4.5 Summary

In this Chapter, I considered intrinsic selection in *Bombina* during early development. Predominantly *B. variegata*-like egg batches were collected from 14 sites in the Apahida hybrid zone and tadpoles were raised for ten days in the laboratory. Deaths were recorded and the remaining tadpoles were genotyped subsequently, which gave joint parental genotypes per egg family. There was no correlation between embryonic mortality and a folded hybrid index across 83 families and across 14 populations. However, there was an unexpected though non-significant trend towards higher embryonic mortality in slightly introgressed families and populations compared to more intermediate ones. The difference between these results and findings from the Pescenica hybrid zone (where embryonic mortality is positively correlated with the folded hybrid index) may be explained by different spatial patterns in the distribution of habitat and introgressed populations.

To test for intrinsic selection within families, maximum likelihood segregation ratios were compared to the likelihood of the expected segregation ratio of 0.5 per locus. There was good evidence for selection in favor of *B. variegata* alleles at three of the four marker loci (overall $p = 0.535$), most likely caused by epistatic interactions in the nuclear genomes of zygotes or early embryos. This mechanism would also explain the higher embryonic mortality across slightly introgressed families compared to intermediate ones. Second, the observed maximum likelihood segregation ratio was used to compute the expected rate of heterozygotes within families. Comparison of its likelihood with the maximum likelihood ratio of heterozygotes revealed no evidence for heterozygote deficit within families.

5 EXTRINSIC SELECTION

5.1 Introduction

5.1.1 Breeding habitat preference and differential extrinsic selection

In this Chapter, I investigate extrinsic natural selection in the form of differential survival in tadpole cohorts across 14 sites in Apahida. The reference point for shifts in allele frequencies are the parental genotypes. Therefore, the initial step in the analysis is to investigate breeding habitat preference in the form of a shift in allele frequencies from adults to the parental genotypes. The two toad species prefer opposing breeding habitats: *B. bombina* occupies densely vegetated permanent ponds whereas *B. variegata* is typically found in temporary puddles (Arntzen 1978, MacCallum et al. 1998). Intensive hybridization, as observed in Pescenica as well as in Apahida, should eventually break down parental allele combinations between different loci, with the alleles for habitat choice recombining away from other traits of the same parental species. However, a strong association between habitat type, morphological features and neutral DNA markers of individuals, including hybrids, has been observed in both regions (Nürnberger et al. 1995, MacCallum et al. 1998, Vines et al. in press). Comparisons among transects nevertheless suggest that the structure of a *Bombina* hybrid zone may be importantly influenced by the distribution of the habitat types so that a smooth cline in allele frequencies is not the only possible outcome (Vines et al. press, Szymura & Barton 1986, Szymura 1993). The association between habitat type and the allelic state at neutral DNA markers in adults (i.e. after dispersal) is more plausibly maintained by active habitat preference than solely by unrealistically strong habitat-specific selection (see Chapter 3). It is unclear yet whether breeding site preference in *Bombina* is based on the same criteria as preference for feeding and resting habitat. Breeding habitat preference results in assortative mating and reduces the frequency of hybridization events. It thus constitutes a prezygotic isolation mechanism. It is expected to confer an adaptive advantage since it means a restriction in resource use which can only be outweighed by higher fitness in the preferred habitat (Rice & Hostert 1993). This is most clearly seen in the pure *Bombina* taxa, which tend to use ephemeral and permanent habitat for breeding even where the distribution ranges of the

two taxa do not overlap. Since the aquatic habitat chosen by the parents for breeding affects most directly eggs and tadpoles, adaptive advantages of adult habitat preference should apply primarily to them.

The different habitats favored by the two *Bombina* taxa are characterized by their permanence. Due to their relative longevity, permanent ponds contain dense aquatic vegetation and are regularly invaded by potential aquatic predators, both invertebrate and vertebrate. Many insects have aquatic predatory larvae restricted to constantly high water levels. In Pescenica, the density of potential tadpole predators is significantly higher in ponds than in puddles (Kruuk 1997, Kruuk & Gilchrist 1997). Many studies have demonstrated the importance of predation on anuran larvae in determining species composition (Semlitsch 1993, Morin 1995, Walls 1995, Wellborn et al. 1996, Azevedo-Ramos & Magnusson 1999). Predation is considered the predominant selective agent at the permanent end of the aquatic habitat gradient. On the opposite end of this gradient, the drying of puddles is the main danger for anuran larvae; across 46 sites within a region, desiccation has been shown to cause up to 79% premetamorphic mortality in *B. variegata* per season (Barandun & Reyer 1997). Additionally, competition among growing tadpoles for decreasing quantities of food intensifies over the season (Smith & Van Buskirk 1995) and may lead to reduced rates of, and smaller size at, metamorphosis (Smith 1983, Pfennig et al. 1991, Semlitsch 1993). Due to the fitness trade-off in tadpoles between risk of predation and risk of desiccation, most anuran species have adapted to a specific region in the permanence gradient. There are very few generalist species; instead, ecological specialization often occurs between closely related species pairs (Morin 1995, Wellborn et al. 1996). Predation on the one hand, and desiccation risk on the other, thus have the potential to generate differential selection on anuran larvae and different survival strategies may be favored in ephemeral vs. permanent habitat. If these are genetically determined, there will be differential survival of different taxon-diagnostic alleles along the ecological gradient. With strong selection and continual immigration of pure types, linkage disequilibrium may be maintained between tadpole fitness traits and adult breeding habitat preference loci despite considerable hybridization and recombination. Thus ecological isolation between the two *Bombina* taxa can be maintained since breeding habitat preference is associated with assortative mating within the habitat.

5.1.2 Methods for the detection of natural selection in the field

Many methods are applied to detect natural selection, and Endler (1986) divides them into ten categories. Having different properties, they vary in their ability and directness to demonstrate natural selection, and some are more sufficient or work better than others in a given species. The most direct but also the most laborious method is a cohort analysis. Related but easier is the comparison among different life-history stages within a population at the same time and place. I use both methods in this Chapter and briefly characterize their features in the following.

In a cohort analysis, an attempt is made to obtain detailed information on survivorship, fertility, fecundity or mating ability within a cohort of individuals subsequently at two or more stages of the life cycle. Therefore, this method requires the marking of individuals within a cohort and resampling at least once after an appropriate amount of time. A cohort analysis tests whether particular trait frequencies vary between different stages of the life cycle more than is expected by chance. A cohort analysis may not only demonstrate selection, but can also provide measures of selection coefficients. Cohort studies are a common method in medical research. Yet, because of the work and time involved, very few attempts at cohort studies have been made in the field, e.g. in hybrid zones. For comparison, I consider the following four examples:

1) Dowling & Moore (1985) performed a cohort analysis on successive adult age classes of hybrids between the cyprinid fish species *Notropis cornutus* and *N. chrysocephalus*. The authors reported a constant loss of hybrids from a regression of the mean hybrid index, which was obtained from a combination of morphological and electrophoretic diagnostic traits on age. Considering only allozyme loci, different selection strengths were postulated for different linkage groups.

2) In a cohort study in the hybrid zone between two species of leopard frogs (*Rana berlandieri* and *R. utricularia*) in Texas, Kocher & Sage (1986) reported high mortality of hybrid compared to pure individuals between the larval and the juvenile stage of the life cycle, though no measure of statistical significance was given. The argument that hybrid mortality was increased was based on the absence of individuals in two intermediate genotype classes, although the frequency within these classes was already very low at the beginning of the study. There was an overall increase of *R. berlandieri*

genotypes at the expense of *R. utricularia* which might indicate extrinsic selection; however, the authors did not mention this.

3) Howard et al. (1993) performed a one-year-cohort analysis in the mosaic hybrid zone between the ground crickets *Allonemobius fasciatus* and *A. socius* in the United States. They showed generally higher survival of one parental genotype relative to the other, with hybrids being intermediate. The relative survival of *A. fasciatus* was higher in one and that of *A. socius* was greater in four of the five populations. Though not discussed, this may indicate extrinsic selection which implies that four of the five localities were situated at one side of the ecological gradient.

4) A ten-year survey of Darwin's finch hybrids between *Geospiza fortis* and *G. scandens* on the Galapagos island Daphne Major (Grant & Grant 1996) revealed partial hybrid superiority following an El Nino event, coinciding with a long-term change in food availability towards small seeds. Hybrid finches have intermediate beak sizes and consumed intermediate sized seeds. The finding indicates that fitness in these Darwin's finch hybrids is ecological rather than genetic.

A less direct method for the detection of natural selection is to compare trait frequencies between different age classes or stages of the life cycle within a population. With this approach it is not required to keep track of individuals, and a single sampling event suffices, preferentially across many localities. The null hypothesis is that different age classes differ in trait frequencies only by chance, and the alternative hypothesis states that differential response to selection causes significant differences between age classes. Depending on the component of selection one wishes to investigate (e.g. mating success, gamete competition, fecundity), any particular interval of the life cycle may be studied. The drawback of this method is that, unlike a cohort analysis, it does not provide data on the relative success of individuals and cannot quantify the individual variance in fitness, because different environmental conditions especially during development may have contributed to differences among the cohorts. Ideally, a cohort study applying a wide range of marker loci can be used to quantify the number of loci that determine an individual's fitness.

5.1.3 The study approach

I begin the investigation of extrinsic selection with an assessment of the habitat that considers additional variables aside from those that are included in the habitat permanence gradient *H* (see Chapter 2). These additional variables may be relevant for selection on tadpoles, e.g. predator abundance, temperature etc. Next, I test for non-random reproduction among adults by comparing the adult sample per site with the inferred parents based on the samples of egg families (see Chapter 4). Finally, I attempt a cohort study on the larval stage: the remaining eggs of each clutch are allowed to develop in situ, and a subsequent sample is taken towards the end of the larval period. I a) look for consistent changes in the genetic composition of the larval cohort across all sites and b) test in each site individually whether the observed shifts in allele frequencies could have arisen by chance.

In Chapter 3, it was suggested that selection acts on a large number of loci throughout the genome. Most of these are not likely to be linked to the marker loci. Therefore, any response to selection as a shift in marker allele frequencies at the population level is mainly due to linkage disequilibrium between selected and marker loci, especially if the measure of genotype is the mean hybrid index.

Ideally, all egg batches within a site would be sampled and the exact number of remaining family sizes known so that the inference of selection would be most direct. This was the original aim of this study. However, it soon became clear that it was extremely difficult to detect every single egg batch, especially in densely vegetated sites. Moreover, due to the *Bombina* spawning habits of depositing eggs in multiple small batches (see Chapter 4), individual mating events cannot be easily discerned. Therefore, it is likely that some families remain unsampled at the egg stage but are then represented in the late stage tadpoles. This might introduce noise in the analysis of allele frequency shifts. To remedy this problem, genotypes of a highly polymorphic locus that allow the assignment of individuals to families (see Chapter 4) are added to the data set. As we will see, there are some late stage tadpoles that cannot be assigned to any of the egg families sampled. Also, unsampled neighboring batches imply that initial family sizes cannot be simply determined by counting the remaining eggs in the sampled ones. Since the analysis of cohorts of family-assigned tadpoles reduces the sample size considerably and lowers the chance to detect an effect of selection, I apply two levels of resolution. I compare the joint parental genotypes first to all tadpoles found in a site and

second only to the family-assigned tadpoles. At the first level, a shift in allele frequencies may be due to selection or to variance in family sizes but also due to effects of non-represented egg families, while the latter effect is excluded at the second level. Nevertheless, consistent shifts in allele frequencies in relation to habitat would be a strong evidence for selection, unless the egg batches of one taxon are inherently harder to find. Still a confounding effect of non-random variance in family size cannot be excluded unless information on egg batch sizes is incorporated in the analysis.

5.2 Methods

5.2.1 Site descriptions

This cohort study was conducted in the 14 sites that were the focus of Chapter 4. Initially, eggs were sampled in every site visited during the adult survey described in Chapter 2. However, genotyping capacities were restricted, and the 14 sites were chosen according to the criteria listed in Chapter 4. I summarize the features of the focal sites here. The sites can be grouped roughly into four categories (see Chapter 2).

Small ponds

Sites 258 (H = 0.424) and 290 (H = 0.370) were typical small ponds measuring 6 m long and 5 m wide and around 0.6 m deep. While pond 290 was densely covered by aquatic vegetation, pond 258 was less so but adjoined closely a reedy area covered by *Juncus* species. Both sites were in no immediate proximity to big ponds containing pure *B. bombina* populations. Pond 290 was maintained by an adjoining deep well. Sites 282 (H = 0.359) and 330 (H = 0.527) were similar ponds in flooded meadows measuring around 20 m long, 12 m wide and 0.3 m deep. There was little aquatic vegetation, especially in pond 330 which contained grass mainly. Site 282 was 100 m from the Gadalin river, while site 330 was an isolated pond on the hilltop above Visea and was about 800 m from a big pond in Coasta to which it was connected through a valley.

Artificial pond

Site 200.4 (H = 0.538) was an excavated pool measuring 5 m long, 4 m wide and 1.5 m deep. There was no aquatic vegetation and no shallow water zone. It was part of a series of 15 artificial pools some of which were much older, densely covered by aquatic vegetation and contained a considerable number of *B. bombina*-like adults.

Ditches

Sites 257 (H = 0.694) and 315 (H = 0.345) were ditches around 50 m long, 0.6 m wide and 0.4 m deep, though both dissolved into a range of puddles during the season before they finally dried out. Ditch 315 was much more densely covered by reeds which were absent from site 257 but abundant in the drainage ditch into which it ended. Site 315 was across the road from the range of artificial ponds including site 200.4 while site 257 was about 500 m from the big pond in Apahida, which harbored a pure *B. bombina* population in 2000.

Puddles

All remaining sites comprising 256 (H = 0.570), 271 (H = 0.831), 272 (H = 0.723), 274 (H = 0.772), 276 (H = 0.512), 317 (H = 0.688) and 318 (H = 0.649) were puddles in wheel ruts and measured around 2 m long, 0.3 m wide and 0.15 m deep. Sites 271, 317 and 318 were located between the series of 15 artificial ponds including 200.4. Site 256 was found 300 m from the big pond in Apahida. Site 272 connected to a wide reedy area south of Cara, while sites 274 and 276 were isolated sites in the Zapodie and the Gadalin valley. All puddles contained little vegetation.

5.2.2 Ecological habitat data

The discriminant function axis H, computed in Chapter 2, was used as habitat permanence index. The four retained variables were the width of the water body, % emerged vegetation, the depth of the water body, and % submerged vegetation, and the discriminant score H was rescaled to run from 0 (ponds) to 1 (puddles). In the 14 sites that are the focus of this Chapter, the density of known tadpole predators was estimated by the number of predators in five standardized sweeps with a kitchen sieve (18 cm diameter). Data were collected whenever a site was visited for the first time and the following predator families or genera were considered: Libellulidae, Lestidae, *Dysticus*, *Notonecta* and *Nepa*. Based on visual inspection, the presence of *Triturus* species was recorded separately. Newts are known to be extremely severe tadpole predators (personal observation, Semlitsch 1993, Morin 1995). Additional ecological data were registered every three to four days, though only in the nine sites studied in 2001 (Table 5.1).

Table 5.1: List of additional habitat parameters. Except for measurements on predator density, data are available for nine sites that were visited in 2001.

Habitat parameters
O_2-Content
Min-/Max Temperatures
Algae cover
Ratio of visible depth to total depth
Presence of *Triturus*
Amount of invertebrate tadpole predators in 5 standardized sieve sweeps

5.2.3 Egg sampling

As described in detail in Chapter 4, beginning on 25.04. (day 1), all selected sites were visited once every 3-4 days and searched for egg batches. Whenever detected, a sample of 10-16 eggs was taken per batch to ensure that all alleles per locus and family were detected. All eggs were taken if a batch contained less than ten eggs. The number of eggs remaining in the site was recorded for each batch. The sampled eggs were reared for ten days in groups of 3-5 in plastic cups with tap water. After ten days the tadpoles had hatched and reached approximately Gosner (1960) stage 25. They were dried and stored in a 0.5 ml Eppendorf tube with 99.9% ethanol for the genetic analysis.

5.2.4 Tadpole sampling

Surviving tadpoles were collected in every site after as long a selection period as possible between 8. May and 19. June. (day 55). Due to the unusual drought in both years, this was just before the site dried and so always long before metamorphosis was reached. Thus tadpoles had been exposed to selection for different amounts of time (1 to 52 days, see Figure 5.1). Despite this difference in time, selection operated always to the point of near drying of the sites which here serves to standardize the total selection across habitats. Note, however, that age-specific habitat effects among genotypes may have been missed, when e.g. one cohort never reached a certain critical age. Since it was difficult to judge the exact date of drying, some sites were sampled more than once for tadpoles. They were collected with a kitchen sieve or a net and transferred to the lab in

plastic boxes with pond water. Tadpoles were gutted and stored in 1 ml Eppendorf tubes with 99.9% ethanol. Due to desiccation, none of the tadpoles would have survived if left in the site.

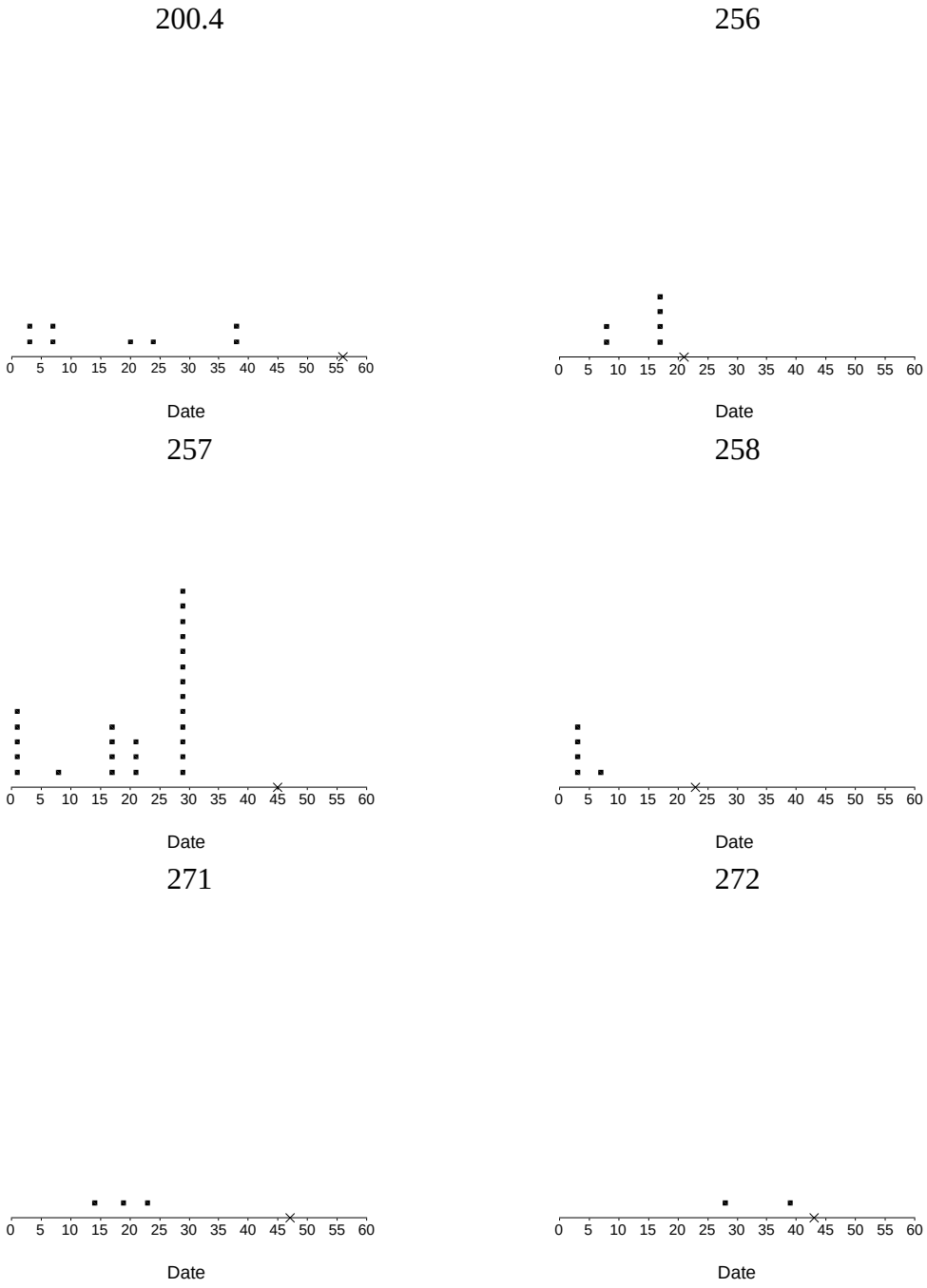

Figure 5.1: Egg and tadpole sampling scheme per site. Sampling took place between the 25. April (Date 1) and 24. June (Date 60). Indicated are the number of egg batches sampled (squares) and the date on which tadpoles were collected (crosses). Due to drought, tadpoles were exposed to selection for different amounts of time.

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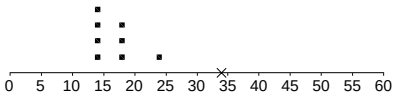

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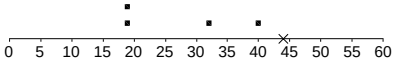

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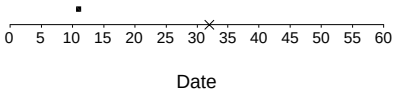

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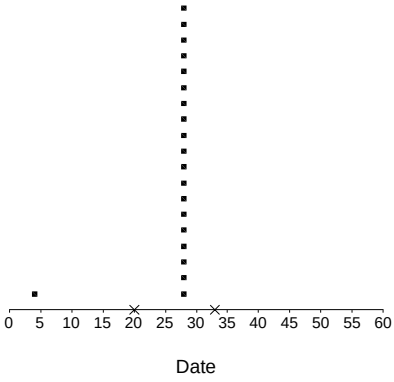

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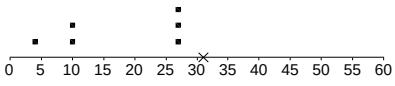

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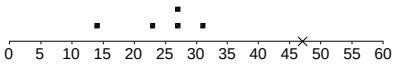

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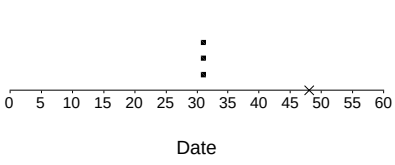

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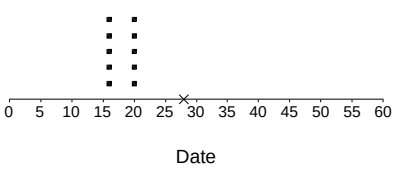


Figure 5.1: continued.

5.2.5 Genotyping

In total 922 eggs (see Chapter 4) and 380 tadpoles were genotyped in the same way as adults for the marker loci *Bb7.4*, *Bv12.19*, *Bv24.11* and *Bv24.12* (see Chapter 2). Except for sites 200.4 and 257, additional genetic information on the microsatellite marker locus *Bv41.11* (Acc # AF472428) was provided by Marlies Frenzel and Bruni Förg-Brey. This locus is highly polymorphic and therefore allows the assignment of individuals to families. However, it is not informative for the hybrid index since its alleles have not been assigned to either *Bombina* taxon. The hybrid index is the number of *B. variegata* alleles across the remaining four loci and was rescaled to represent the mean *B. variegata* allele frequency from 0 (*B. bombina*) to 1 (*B. variegata*).

Table 5.2: Number of egg families, eggs left behind to develop in situ, family-assigned and non-assigned tadpoles per site. Note that no family assignments were done in sites 200.4 and 257.

Site	Habitat type	*H*	Nr of sampled eggs (families)	Nr of eggs left in site	Nr of tadpoles assigned (to Nr of families)	Nr of non-assigned tadpoles
204	artificial pond	0,538	75 (-)	121	-	10
256	puddle	0,570	60 (3)	196	7 (3)	16
257	ditch	0,694	251 (-)	646	-	42
258	small pond	0,424	30 (3)	14	10 (3)	32
271	puddle	0,831	29 (3)	75	8 (2)	4
272	puddle	0,723	26 (2)	66	8 (2)	3
274	puddle	0,772	52 (5)	128	34 (5)	16
276	puddle	0,512	24 (3)	97	8 (3)	27
282	small pond	0,359	4 (1)	7	2 (1)	10
290	small pond	0,370	181 (12)	295	3 (2)	9
315	ditch	0,345	30 (5)	35	6 (3)	8
317	puddle	0,688	44 (4)	201	38 (1)	4
318	puddle	0,649	16 (3)	126	21 (2)	7
330	small pond	0,527	64 (5)	38	6 (4)	41
Sum			886		151 (31)	229

After assigning eggs to families, the mean *B. variegata* allele frequency *p* of the parents was calculated from the joint parental genotypes (jpg, see Chapter 4) per family and as the unweighted average of family means, $\bar{p}$, per site. Table 5.2 shows the number of egg families, assigned and non-assigned tadpoles per site. In three ponds (282, 258, 330) the number of eggs left behind is exceeded by the number of surviving tadpoles found after the larval period. This means that some egg batches must have been missed, since in these densely vegetated ponds eggs are difficult to spot.

5.2.6 Statistics

Variables were transformed (arcsine for percentages, log for continuous variables; Sokal & Rohlf 1995). Since data were still not normally distributed, means between categories were compared with a Mann-Whitney U test. Correlations were tested for significance with Spearman's rank correlation coefficient. Bonferroni techniques were used for adjusting significance levels. Least squares regression curves were fitted and r^2 values compared between different categories. To compare differences in the goodness-of-fit of regressions of mean allele frequencies at different life stages on the habitat variable, *F* was computed from the ratios of residual variances. In case of correlation between the variables, F was modified according to Snedecor & Cochran (1980):

$$r_{DS} = (F-1)/\sqrt{(F+1)^2 - 4r^2F}$$

where r^2 is obtained from a regression between the independent variables. ANCOVA was applied to test for significant influences of the habitat permanence or stage of the life cycle on the distribution of allele frequencies.

5.3 Results

5.3.1 Habitat ecology and predator density

The habitat axis *H* is roughly associated with additional ecological parameters that were recorded in the sites chosen for the cohort analysis: temporary sites (high *H* values) have a tendency towards lower means in water transparency, O_2 content and

invertebrate predator density (Table 5.3) though none of the correlations are significant at the α' level 0.0073.

Table 5.3: Association between arcsine transformed habitat index *H* and other ecological factors. r_{SP}: Spearman rank correlation coefficients (α' = 0.0073). Bold: significant difference or correlation.

Ecological parameter	Habitat axis *H*
Presence of *Rana* / *Triturus* (n = 14)	M-W U = 0.006 U = 2.00
Predator density in 5 sieve sweeps (n = 14)	p = 0.050 r_{SP} = -0.577
Visible depth / total depth (n = 9)	p = 0.011 r_{SP} = -0.655
Mean O_2 content (n = 9)	p = 0.026 r_{SP} = -0.728
Algae coverage on surface (n = 14)	p = 0.488 r_{SP} = -0.221
Mean minimal temperature (n = 9)	p = 0.500 r_{SP} = 0.259
Mean maximal temperature (n = 9)	p = 0.187 r_{SP} = -0.483

Ponds contain newts and many invertebrate predators which were absent in temporary puddles (Table 5.4). Three newt species were found: *Triturus cristatus*, *T. alpestris* and *T. vulgaris*. Anisoptera larvae are mainly representatives of the species *Libellula depressa* which prefers muddy, open water bodies for the larval development (Honomichl 1998). However, they only occur in the more permanent sites along with newts, Zygoptera larvae and *Dysticus marginalis*.

Dragonfly and *Dysticus marginalis* larvae are sit-and-wait predators that hide in dense vegetation and feed on other water insects, small fish and tadpoles by visual and tactical orientation (Honomichl 1998). The same strategy applies in the water scorpion *Nepa rubra* (Honomichl 1998) which was found in only one site. The only predator in ephemeral puddles is the water bug *Notonecta glauca* which in turn occurs only in very low numbers in ponds. *Notonecta glauca* is an actively hunting predator that finds tadpoles and water insects by visual and tactical orientation (Honomichl 1998). Site 330 has an intermediate predator density since it does not harbor any newts, but all invertebrate predators that were otherwise found in ponds.

Table 5.4: Predator density per site. *H*: Discriminant function index. Presence (+) of *Triturus* species was recorded by eyesight. Listed is the number of invertebrate predators found in five standardized sieve sweeps.

Site	Habitat	H	*Triturus spec*	Anisoptera larvae	Zygoptera larvae	*Dysticus marginalis* larvae	*Notonecta glauca*	*Nepa rubra*
204	artificial pond	0,538	+	-	-	-	-	-
256	puddle	0,570	+	1	4	-	-	-
257	ditch	0,694	-	-	-	-	-	-
258	pond	0.424	+	-	11	2	-	-
271	puddle	0.831	-	-	-	-	1	-
272	puddle	0,723	+	2	-	-	-	-
274	puddle	0,772	-	-	-	-	-	-
276	puddle	0,512	-	1	-	-	-	1
282	pond	0.359	+	3	-	2	-	-
290	pond	0.370	+	1	-	8	1	-
315	ditch	0.345	+	-	3	-	-	-
317	puddle	0.688	-	-	-	-	3	-
318	puddle	0.649	-	-	-	-	4	-
330	pond	0.527	-	2	-	2	1	1

5.3.2 Breeding habitat preference

The *B. variegata* allele frequency in the adult population is highly significantly correlated with the habitat discriminant index *H* over all sites (r_{SP} = 0.833; $p < 0.001$; see Chapter 3). While breeding habitat preference may be based on the same criteria as resting or feeding habitat preference, it may be more stringent because habitat preference in adults should mainly affect the tadpoles' fitness. Surprisingly, there is no significant correlation between the *B. variegata* allele frequency based on the inferred joint parental genotypes, which represent the input of egg genotypes into a site, and the

habitat discriminant function *H* (r_{SP} = 0.497; p = 0.07). Figure 5.2 compares the respective allele frequencies of adults and joint parental genotypes in relation to *H*. Regressions per category are shown in Figure 5.3. Over the range of habitats considered here, the habitat discriminant function is a much better predictor for the mean *B. variegata* allele frequency in adults (r^2 = 0.741) than for the joint parental genotypes (r^2 = 0.266). The residual variance around the regression is significantly higher in joint parental genotypes than in adult genotypes (*F* = 3.00; r_{DS} = 0.657; p = 0.02). Since genotype data were corrected for sample sizes, these results indicate that breeding habitat preference might be based on other criteria than resting and feeding habitat preference.

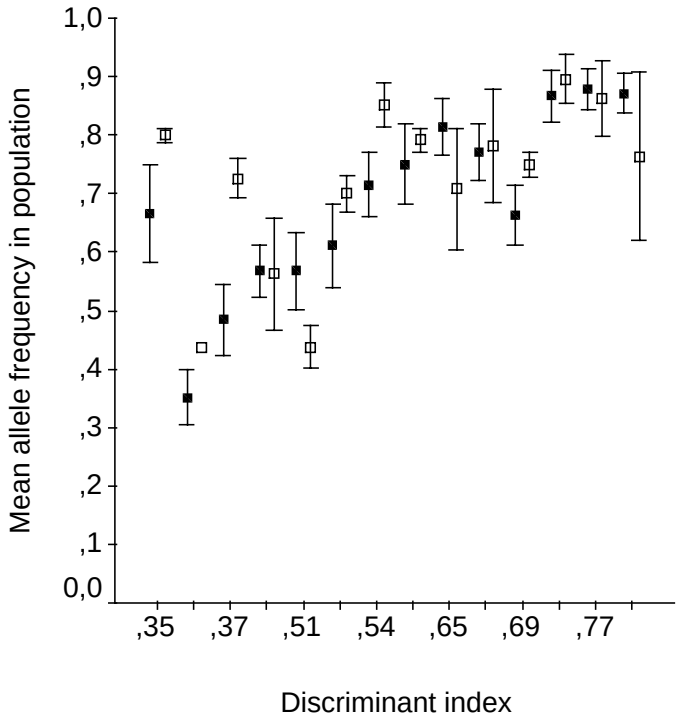


Figure 5.2: *B. variegata* allele frequencies per site in relation to the habitat discriminant function *H* for the overall adult sample (filled squares) and the joint parental genotypes (empty squares), which represent the input of egg genotypes into a site. Error bars represent one standard error of the mean per site.

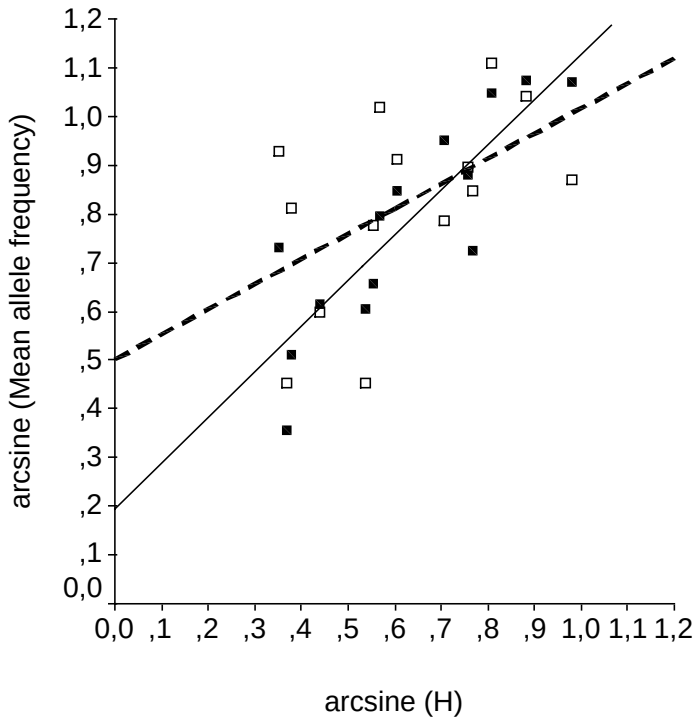


Figure 5.3: The mean *B. variegata* allele frequency per site as a function of the habitat discriminant index *H* for the overall adult sample (solid squares and line; y = 0.195 + 0.934 * *H*; n = 14; F = 34.28; p < 0.001) and the joint parental genotypes (empty squares, broken line; y = 0.501 + 0.515 * *H*; n = 14; F = 4.35; p = 0.07).

A different aspect of breeding habitat preference in Apahida would be a consistent one-directional bias. For example, though a wide range of adult genotypes is found in intermediate habitat, only one taxon may actually breed there, if breeding habitat is chosen according to other criteria than resting habitat. To test this, the mean *B. variegata* allele frequency of the adult sample in a site was subtracted from the *B. variegata* allele frequency over the joint parental genotypes. The result was divided by the allele frequency of the adult sample to obtain comparable values over all sites. The mean of these statistics deviates significantly from zero over all sites ($\bar{x}$ = 0.212; t = 3.633; p = 0.003; n = 14). So the mean allele frequency over the eggs deposited in a site is generally more *B. variegata*-like than the adult genotypes found there, indicating that the range of habitat investigated here is preferred for breeding by *B. variegata*-like adults and seems to be less acceptable for *B. bombina*-like ones (Figure 5.4).

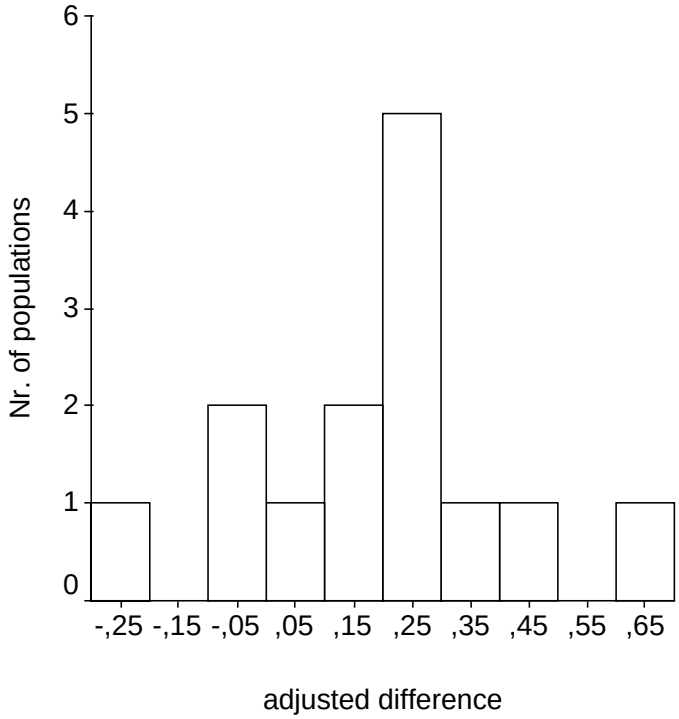


Figure 5.4: The difference between the joint parental genotypes and the overall adult sample divided by the adult genotypes per site. Except for three sites, the quotient deviates positively from zero, meaning that the proportion of *B. variegata* alleles is overall higher in the eggs as compared to the adult sample ($t = 3.633$; $p = 0.003$; $n = 14$).

5.3.3 Extrinsic selection in tadpoles

Habitat effect on allele frequencies

If there is habitat permanence correlated selection on tadpoles, the response should be a shift in allele frequencies between the egg and the late tadpole stage of the life cycle across all sites. Any shift in allele frequencies might however, be biased by an effect of underrepresented egg batches in the joint parental genotypes and by variance in family sizes as illustrated above. I account for these effects in the following analysis.

Three scenarios are possible for a difference in allele frequencies between joint parental genotypes and late stage tadpoles. The first would be a steepening regression line between genotype and habitat. This would mean that selection for one or the other taxon's alleles changes direction across the habitat gradient. An artificial effect of family size would only be possible if the females adjusted their egg batch sizes to the habitat as a function of the genotype. Similarly, not represented egg batches would be a problem if one missed *B. bombina*-like clutches in permanent and *B. variegata*-like clutches in temporary habitat. The second scenario would be that along the habitat range

considered here, a consistent shift in allele frequencies towards *B. variegata* is found, as in adult habitat preference. This could be caused by selection, unless it was an effect of family size with *B. variegata*-like animals laying consistently larger clutches, which is not very likely (see Rafinska 1991). The problem of unsampled egg batches should be unimportant, because one would have to assume that *B. bombina*-like clutches were consistently underrepresented in the joint parental genotypes. The third scenario is a reduction in variance around the regression line of late stage tadpoles compared to joint parental genotypes. If an effect of sample sizes can be excluded, this would be a strong indication of selective optima that vary with the habitat permanence. As indicated above, there are two different levels of resolution concerning the representation of egg batches, and I consider the comparison of i) the joint parental genotypes and all tadpoles found within a site and ii) the joint parental genotypes and the tadpoles that can be assigned to the egg families within a site. While the first level offers a bigger sample size, the second level is more exact since it excludes the possibility that a shift in allele frequencies is biased by tadpoles from unsampled egg batches. Significant shifts in allele frequencies at both steps of analysis would give good evidence of selection, if an effect of family size can be excluded (see above).

I first consider regressions between genotype and the habitat permanence axis. Figure 5.5 compares the *B. variegata* allele frequencies between joint parental genotypes and tadpoles in relation to the habitat discriminant index *H*, and Figure 5.6 shows the relationship between genotype and habitat permanence per site per tadpole category. While the habitat discriminant index *H* is a good predictor for the allele frequency over all tadpoles (r^2 = 0.521) it is not for the assigned tadpoles (r^2 = 0.180) and the joint parental genotypes (r^2 = 0.266; see above).

The regression between genotype and *H* is highly significant in the first tadpole category and comparable to the situation in the adult sample (compare Figures 5.3 and 5.6a). This could be explained by extrinsic selection during the larval stage which favors *B. variegata* alleles in temporary and *B. bombina* alleles in semi-permanent habitat. However, ANCOVA weighted for sample sizes does not reveal a significant influence of the age class ($F = 3.11$; $p = 0.090$), or of an interaction between age class and habitat ($F = 2.56$; $p = 0.123$) on the genotype regression line. Therefore, the difference in significance between the regression lines cannot be attributed to habitat-dependent selection. Also, the variance around single regression lines does not differ

significantly between joint parental genotypes and either of the tadpole levels (all tadpoles: F = 1.691; r_{DS} = 0.330; p = 0.2; assigned tadpoles only: F = 2.095; r_{DS} = 0.459; p = 0.1), implying that selection does not favor intermediate genotype optima that vary with the habitat permanence.

a) All tadpoles b) Only family-assigned tadpoles

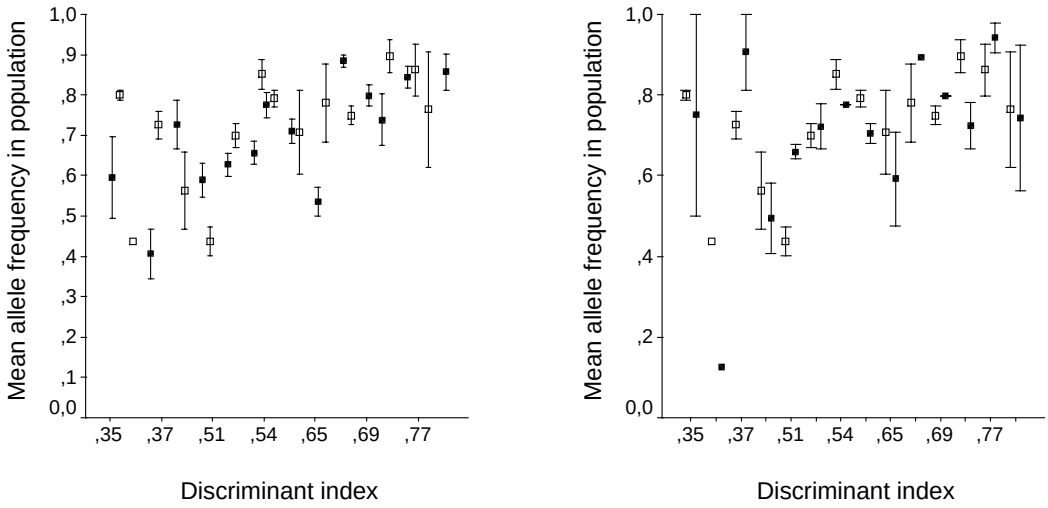


Figure 5.5: Mean *B. variegata* allele frequencies per site in relation to the habitat discriminant function *H* for the joint parental genotypes (empty squares), which represent the input of egg genotypes into a site, and late stage tadpoles (filled squares). a) all tadpoles found in the site; b) family-assigned tadpoles per site. Error bars represent one standard error of the mean.

a) All tadpoles b) Only family-assigned tadpoles

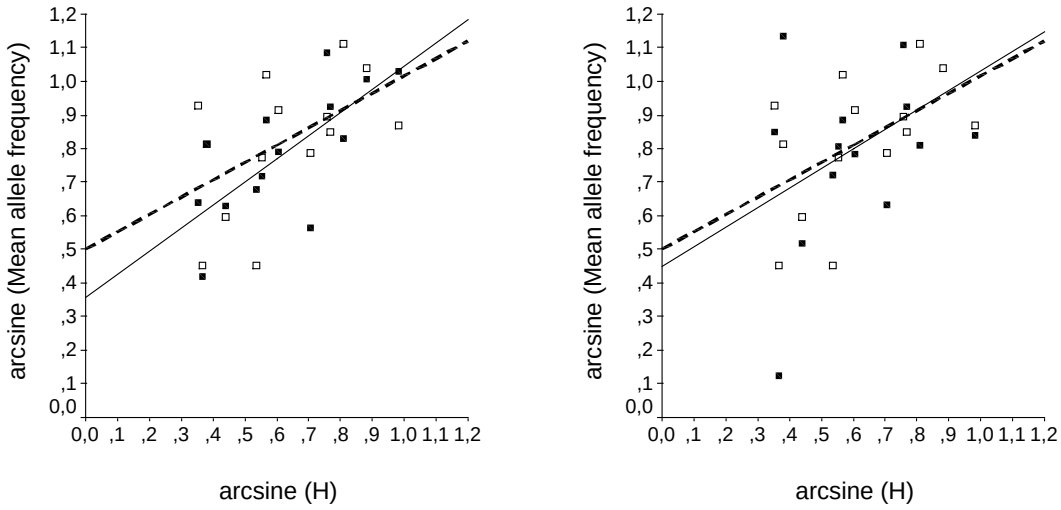
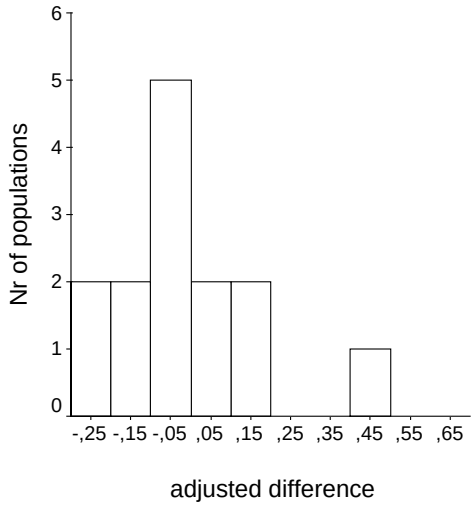


Figure 5.6: The mean *B. variegata* allele frequency per site as a function of the habitat discriminant index *H* for joint parental genotypes (empty squares, broken line) and tadpoles (filled squares, solid line). a) all tadpoles found in a site (y = 0.359 + 0.688 * *H*; n = 14; F = 13.061; p = 0.003) and b) only family-assigned tadpoles (y = 0.450 + 0.581 * *H*; n = 14; F = 2.637; p = 0.203).

To test for an overall shift in *B. variegata* alleles in the larval stage, the allele frequency of the joint parental genotypes was subtracted from the frequency over tadpoles and then divided by the latter. There is no consistent shift in allele frequencies as the sum of quotients does not differ significantly from zero (all tadpoles and jpg: $\bar{x}$ = -0.016; t = -0.332; p = 0.745; n = 14; assigned tadpoles and jpg: $\bar{x}$ = -0.028; t = -0.382; p = 0.709; n = 14; Figure 5.7). Taking out the outliers (site 276 in the second and 282 in both categories) does not give significance (all tadpoles and jpg: $\bar{x}$ = -0.050; t = -1.445; p = 0.174; n = 13; assigned tadpoles and jpg: $\bar{x}$ = -0.015; t = -0.392; p = 0.703; n = 12). This finding indicates that, unlike breeding habitat preference, selection during the larval stage does not favor alleles of one taxon across the range of habitat considered here.

a) All tadpoles



b) Only family-assigned tadpoles

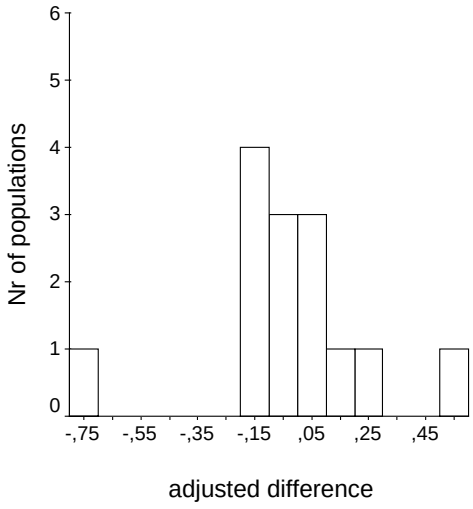


Figure 5.7: The difference in *B. variegata* allele frequencies between the tadpoles and the joint parental genotypes divided by the joint parental genotypes per site. a) all tadpoles included and b) family-assigned tadpoles only.

Any significant shift in allele frequencies between eggs and tadpoles may be confounded by a genotype effect on the size of egg batches. In general, *B. bombina* lays more eggs than *B. variegata* (Rafinska 1991). Therefore, the *B. variegata* allele frequency over all eggs within each site may be significantly lower if batch sizes are

accounted for. Comparing allele frequencies between the egg and the tadpole stage, one may detect a significant shift when accounting for egg batch sizes that might be hidden in the analysis above. I therefore computed the joint parental genotypes again, this time including information on the number of eggs that were known to belong to a family and were left in the site. There is no correlation between joint parental genotypes and egg batch size (r_{SP} = -0.043; p = 0.7), and the new computation did not give a better correlation between joint parental genotypes and *H* or confer a significant shift in allele frequencies between joint parental genotypes and late stage tadpoles.

Non-random survival

I now explore whether, on a per site basis, surviving tadpoles may have been sampled randomly from the egg batches. Non-random survival is seen in a significant shift between the expected and the observed *B. variegata* allele frequencies in tadpoles. Again I consider assigned tadpoles and all tadpoles as two different levels of resolution in the analysis (see above). Null distributions of allele frequencies per site were computed by drawing repeatedly the observed number of surviving tadpoles from randomly generated offspring based on the joint parental genotypes per site (1000 replicates). The expected mean *B. variegata* allele frequency per site is the mean over all replicates and includes information on the number of eggs that had remained in the site.

Significant shifts in allele frequencies in both categories are apparent in four puddles (Table 5.5). However, these shifts are twice positive (274, 317) and twice negative (272, 318). Significant shifts occur in two ponds in the assigned tadpoles category (282, 290). Site 315 exhibits a significant negative shift in allele frequency when all tadpoles are considered and a non-significant negative shift towards the smaller sample of assigned tadpoles. While an allele frequency shift would be expected towards *B. variegata* in temporary and towards *B. bombina* in permanent habitat if selection on tadpoles was based on factors associated with the habitat permanence, the non-consistent shifts in the puddles indicate that criteria other than the habitat permanence may be additionally responsible for tadpole survival.

Table 5.5: Expected and observed mean *B. variegata* allele frequencies in late stage tadpoles per site. Expected means are based on 1000 replicates of random survival over all joint parental genotypes, including information on the number of eggs that had remained in the site. The sites are ordered by their habitat permanence index *H*.

Site	H	expected $\bar{p}$ in tadpoles +/- S.D.	observed $\bar{p}$ (assigned tadpoles)	p (shift significant)	observed $\bar{p}$ (all tadpoles)	p (shift significant)
315	0.345	0.814 +/- 0.049	0.750	n. s.	0.596	p < 0.001
282	0.359	0.446 +/- 0.133	0.125	p = 0.012	0.406	n. s.
290	0.370	0.720 +/- 0.010	0.906	p = 0.018	0.726	n. s.
258	0.424	0.500 +/- 0.054	0.495	n. s.	0.589	n. s.
276	0,512	0.544 +/- 0.078	0.660	n. s.	0.627	n. s.
330	0.527	0.668 +/- 0.053	0.723	n. s.	0.657	n. s.
204	0,538	0.852			0.775	n. s.
256	0,570	0.789 +/- 0.045	0.706	n. s.	0.710	n. s.
318	0.649	0.717 +/- 0.036	0.592	p < 0.001	0.526	p < 0.001
317	0.688	0.758 +/- 0.034	0.895	p < 0.001	0.884	p < 0.001
257	0,694	0.750			0.799	n. s.
272	0,723	0.933 +/- 0.024	0.724	p < 0.001	0.739	p < 0.001
274	0,772	0.789 +/- 0.023	0.942	p < 0.001	0.845	p = 0.003
271	0.831	0.785 +/- 0.070	0.743	n. s.	0.858	n. s.

5.4 Discussion

In this Chapter, I aimed at detecting breeding habitat preference and extrinsic selection in tadpoles in the Apahida hybrid zone. Breeding habitat preference in adults is not strictly correlated with the habitat permanence gradient. Instead, over the habitat range studied here, the egg genotypes are consistently more *B. variegata*-like than the adult genotypes, indicating that per site the more *B. variegata*-like individuals have a higher propensity to reproduce. In contrast to this, no effect of extrinsic selection in tadpoles could be detected as a shift in allele frequencies between joint parental genotypes and late stage tadpoles. However, genotype and habitat permanence are significantly

correlated in tadpoles, as in adults (see Chapter 3), hinting at differential adaptation of different genotypes in tadpoles to habitat permanence.

According to which criteria do adults choose a side for breeding? One criterion might be the degree of the habitat's permanence. One could say that all sites outside big ponds were essentially temporary in both seasons. This means that the divide between permanent ponds and ephemeral habitat cannot simply be drawn at $H = 0.5$, but at some value much below that. If the toads judge this distinction similarly, they must have perceived the entire habitat range considered in this study as temporary. This would explain why it was preferred for breeding by *B. variegata*-like adults. But then some *B. bombina*-like individuals do obviously reproduce in these sites (see Chapter 3). Do they avoid ponds as a consequence of strong competition for territory or mating partners? Or are alleles at the marker loci recombining away from alleles for breeding habitat preference? This issue needs clarification, and a more extensive survey of eggs and local adults that is currently under analysis by Tim Sands, will hopefully shed more light on breeding habitat preference in Apahida.

Breeding habitat preference in adults should be correlated with differential selection at the larval stage, since traits characteristic of either *Bombina* taxon are in strong linkage disequilibrium within hybrid populations in Apahida (see Chapter 3). As illustrated in the introduction, permanent habitat is more likely to contain tadpole predators, and development under the risk of predation creates a trade-off between resource acquisition and predator avoidance in tadpoles (Werner & Anholt 1993, Skelly 1995). Low activity rates reduce vulnerability to visually hunting predators, but also decrease foraging rates and hence, growth and development rates. Kruuk (1997, Kruuk & Gilchrist 1997) showed that, when exposed to a predator, both *Bombina* species reduced their activity levels significantly. However, *B. variegata* tadpoles were still more active and spent more time feeding than *B. bombina* ones. Consequently, *B. variegata* tadpoles suffered higher mortality rates in predator choice experiments. On the other hand and presumably influenced by high foraging activity, the larval period in *B variegata* is 87% that of *B. bombina* (Nürnberger et al. 1995) which means less time at risk of desiccation.

I now consider the potential of habitat ecology to exert extrinsic selection on *Bombina* tadpoles in the Apahida hybrid zone. The results confirm the prediction that the abundance of newts and, though not significantly, the overall predator density is higher

in semi-permanent ponds than in temporary puddles. How can the non-significant correlation between predator density and habitat permanence be explained? Kruuk (1997, Kruuk & Gilchrist 1997) performed an ecological survey on the abundance of tadpole predators in five ponds and puddles respectively in Pescenica and showed that the density of newts, dragonfly and damselfly larvae, diving beetles, and salamander larvae was substantially higher in ponds. So, their and my results agree, at least concerning newts. However, no information is given about the range of habitat permanence investigated in Pescenica except that the most diverging habitat types were chosen from opposing ends of the hybrid zone. I therefore assume that the ponds were more permanent than the semi-permanent sites in my study all of which dried out during the season. Including the large ponds in the analysis would probably have produced a significant correlation between predator density and habitat permanence.

Kruuk's (1997, Kruuk & Gilchrist 1997) findings are consistent with the hypothesis that the tadpoles of the two *Bombina* taxa have adapted to diverging breeding habitats. However, no direct evidence for extrinsic selection was found in the cohort study here. There was no consistent shift in allele frequencies between joint parental genotypes and late stage tadpoles and no reduction in variance around the regression between genotype and habitat permanence index. Furthermore, allele frequencies are not correlated significantly with the habitat discriminant index in joint parental genotypes or family-assigned tadpoles, and the regression line did not steepen significantly towards late stage tadpoles. The observed trend in this direction was not significant as seen by the lack of an interaction between age class and *H*. The lack of significance may be explained by the following issues.

A statistical problem may be given by insufficient variation in the environment and in allele frequencies. The range of habitat considered here is quite narrow in that almost all sites contained predators but were affected by drought at the same time. Additionally, the input of egg genotypes across sites consists mainly of *B. variegata*-like families. If variation in trait frequencies or environment is small, even strong selection may not be detected (Endler 1986), especially if one tries to detect selection via linkage disequilibrium between neutral markers and selected loci. A way to explore this issue would be to introduce pure *B. bombina* egg batches into temporary sites thus widening the range of genotypes present. The aim of this study was however, to detect natural selection in the setting of the hybrid zone without artificially influencing the input of

genotypes. As discussed in Chapter 3, strong selection against *B. bombina* alleles should be postulated in this range of habitat and genotype input.

Additionally, selection may be too weak to detect even with a sample size equal to the population size. Weak selection is not very likely though; many studies have clearly demonstrated the potential of the aquatic habitat to generate divergent selection and define alternative adaptations in tadpoles of many different anuran species including *B. bombina* and *B. variegata* (see above). Furthermore, for the stability of the Apahida hybrid zone, strong selection was postulated (Chapter 3). Nevertheless, as many tadpoles died of desiccation, the sample size might have been too small to detect a significant effect of selection. If most individuals die, selection will not be detectable or distinguishable from random genetic drift (Krimbas & Tsakas 1971).

Selection might be strongest during later stages of the larval period than the ones investigated here, e.g. during metamorphosis. An inherent difficulty of this field study was the severe drought which affected even the biggest ponds and forced me to collect tadpoles long before metamorphosis. However, many examples from the literature strongly suggest that selection on tadpoles at least due to predation concentrates in early developmental stages. I tentatively conclude that the lack of evidence of extrinsic selection has most likely been caused by low sample size due to too high mortality.

In four puddles there were diverging non-random shifts in allele frequencies from eggs to tadpoles, suggesting that factors other than our measure of habitat permanence and predator density might be additional determinants of tadpole survival. These might include genotype-environment interactions, frequency-dependent selection or intrinsic selection within families. There is also the possibility that despite the high linkage disequilibrium, alleles at the marker loci have recombined away from alleles at loci that mediate fitness in tadpoles.

To conclude, considering all tadpoles regardless of family-assignments, there is a significant correlation between genotype and habitat which approximates the situation in adults, while joint parental genotypes are not significantly correlated with habitat. This is at least an indirect clue that there may be habitat-permanence correlated selection on tadpoles, favoring opposing genotypes in opposing ends of the habitat permanence gradient.

Another aspect of natural selection on *Bombina* tadpoles is differential phenotypic adaptation, e.g. in growth rates and phenotypic plasticity which I investigate in the next Chapter.

5.5 Summary

This Chapter focused on extrinsic selection which may be the ultimate factor underlying in breeding habitat preference and may be apparent in shifts in allele frequencies in a tadpole cohort over time. The joint parental genotypes inferred in Chapter 4 were the reference point for detecting selection in tadpoles. A comparison to all adults present revealed a consistent shift towards *B. variegata* in breeding habitat preference across the range of 14 semi-permanent to temporary sites considered here. This type of habitat might not be acceptable for *B. bombina*-like animals for breeding. The remainder of the egg batches that have been the focus of Chapter 4 were counted and allowed to develop in the sites. After as long a time as possible, surviving tadpoles were collected, in every case just before the site dried out. With the aid of a highly polymorphic locus, tadpoles were assigned to egg families. Not all tadpoles could be assigned which means that some families must have been missed at the egg stage. Although the density of potential tadpole predators was, though not significantly, positively correlated with habitat permanence, no habitat-dependent effect of extrinsic selection on tadpoles could be detected. However, genotype and habitat permanence were significantly correlated considering all late stage tadpoles. This indicates that there may be habitat-permanence correlated selection on tadpoles, favoring opposing genotypes in opposing ends of the habitat permanence gradient. I attributed lack of evidence to the low sample size as desiccation caused high mortality among tadpoles. On a per site basis, there was non-random survival in four cases, indicating that factors other than habitat permanence or predator density might additionally influence tadpole survival.

6 GROWTH, DEVELOPMENT AND PHENOTYPES

6.1 Introduction

6.1.1 Differential extrinsic selection on tadpoles and adaptive strategies

In addition to the survival rates addressed in Chapter 5, differential selection may also be apparent in differential growth and development and in the phenotype of surviving tadpoles which I investigate in this Chapter. As shown in Chapter 5, the breeding habitats favored by the two *Bombina* taxa differ in their degree of permanence and, related to the permanence, in their density and diversity of potential tadpole predators. In the fitness trade-off in tadpoles between risk of predation in permanent habitats on the one hand and risk of desiccation in temporary habitats on the other hand, most anuran species have adapted their activity levels and hence, growth and development rates to a specific region in the permanence gradient. In ephemeral sites, high foraging activity is vital in that it enhances growth and development and competitive ability. To minimize the risk that desiccation occurs before the onset of metamorphosis, tadpoles in ephemeral habitats should maximize their growth rates by optimizing food acquisition, food processing and metabolic rates. In contrast, permanent habitat favors traits that reduce the predation risk. These are a more quiescent lifestyle, which implies lower growth rates since food intake is reduced (Werner & Anholt 1993) and a different body shape including a higher tail fin which tends to be part of an often complex phenotypic response to predator presence.

6.1.2 Phenotypic plasticity

While permanent and truly ephemeral habitats exhibit stable conditions in terms of the predictability in the mode and strength of selection on tadpoles, the situation is complicated in intermediate and temporary habitats. These show great intra- and inter-seasonal variation in species composition due to high variation in the hydroperiod and are therefore in the long term unpredictable with respect to predation risk (Wellborn et al. 1996). However, if a reliable cue indicates the state of the environment in the short term and if there is a trade-off in the fitness of phenotypes in different environments,

phenotypic plasticity is expected. Phenotypic plasticity is defined as the capability of one genotype to generate different phenotypes in response to different environmental conditions. Many studies have demonstrated the capability of anuran tadpoles to adapt their activity levels and phenotype in response to locally differing predation risk. Phenotypic defenses may include body and tail shape (van Buskirk & Relyea 1998, Lardner 2000, Relyea & Werner 2000, Vorndran et al. 2002) and tail coloration (in *Acris crepitans*: Caldwell 1981). Tadpoles encountering predators most often reduce their activity level and develop a relatively higher tail fin (e.g. Relyea & Werner 2000). Why a higher tail fin increases the probability of survival remains to be fully explained (van Buskirk & McCollum 2000). The costs of induced behavioral as well as of morphological defenses seem to be a decrease in size and a longer time to metamorphosis in some but not all cases (van Buskirk & Relyea 1998, Vorndran et al. 2002) which may be fatal in quickly desiccating sites. Size at metamorphosis is correlated with size at first reproduction in the salamander *Ambystoma talpoideum* (Semlitsch et al. 1988) and with age at maturity in the chorus frog *Pseudacris triseriata* (Smith 1987) and is therefore linked to long term reproductive success at least in these species.

6.1.3 Adaptive growth rates and phenotypic plasticity in *Bombina*

Earlier work by Vorndran et al. (2002) investigated phenotypic plasticity and mortality rates in genetically pure *Bombina* tadpoles grown in the presence of predators. According to predictions from life history theory the authors found a higher degree of phenotypic plasticity in the species adapted to temporary habitats, *B. variegata*, compared to *B. bombina* tadpoles which showed high tail fins even when grown in the absence of predators. In predation trials, predators did not discriminate between induced morphs of the two taxa. So the question as to whether inferior adaptation to high predation risk keeps *B. variegata* out of ponds was left unanswered. However, Kruuk & Gilchrist (1997) showed that *B. variegata* tadpoles from the *Bombina* hybrid zone in Pescenica grown in the absence of predators (naïve) have higher activity levels than naïve *B. bombina* tadpoles though both species reduce their activity when exposed to predators. In their experiment, *B. variegata* tadpoles suffered a higher predation rate. The critical question that emerges from these experiments might be whether early stage *B. variegata* tadpoles in true ponds in nature have enough time to develop their phenotypic response or to reach a size refugium before they are eaten by predators. On

the other hand one could ask whether *B. bombina* tadpoles in puddles in nature can adjust their growth and development to the desiccation risk and to strong competition for food.

6.1.4 The study approach

In the Apahida hybrid zone, *B. variegata*-like tadpoles do encounter predators in temporary and intermediate habitats (Thiel 2001) and should not be considered naïve. This study focuses i) on predominantly hybrid *Bombina* tadpoles and ii) on intermediate, semi-permanent to temporary habitats as common in the Apahida *Bombina* hybrid zone. In this Chapter, I explore how growth, development and the body and tail shape of tadpoles are determined by genotype and its interaction with the environment. I focus here on the fitness components of growth rate and development rate, reduced by the predator-induced morphological defense which exerts a cost to tadpoles (Vorndran et al. 2002). Extrapolating from the data on the pure taxa, the expectation is that as an adaptation to ephemeral habitat, *B. variegata*-like tadpoles should have a higher maximal growth rate. Additionally, while *B. bombina*-like tadpoles should exhibit a rather fixed phenotype as an adaptation to the more uniform permanent habitat, *B. variegata*-like tadpoles should show phenotypic plasticity since they are adapted to less predictable temporary and intermediate environment. The display of predator-induced phenotypic features should exert a cost to tadpole fitness in terms of reduced growth and development rates.

Morphological data are collected from tadpoles from eight sites in the Apahida hybrid zone. Their age is estimated as the average spawning date per site (see below) and all tadpoles are genotyped. Body size is the first component of a PCA on five morphological traits and measures of shape are residuals after regression of individual measurements on body size. Growth and development rates are computed by regressions of size and developmental stage on age. Residuals and shape measures are then tested for significant effects of genotype and habitat.

6.2 Methods

6.2.1 Sites and ecological habitat data

Data for this Chapter were collected in the 2001 season in eight of the 14 sites that were the focus of Chapters 4 and 5. These eight sites comprise one ditch (315), four semi-permanent ponds (258, 282, 290, 330) and three puddles (271, 317, 318).

The habitat index *H* was computed in Chapter 2. The four retained variables were the width of the water body, % emerged vegetation, the depth of the water body, and % submerged vegetation and the discriminant score *H* was rescaled to run from 0 (ponds) to 1 (puddles).

6.2.2 Tadpole sampling

Beginning on 25. April (day 1), all sites were visited once every three to four days and intensively searched for egg batches. Whenever detected, a sample of 10-16 eggs was taken per batch and reared for about ten days before the then hatched tadpoles were stored in 99.9% ethanol for the genetic analysis. Surviving tadpoles were collected at every site (Table 6.1) after as long a selection period as possible, always shortly before the site dried out, between 8. May and 19. June (day 55).

Table 6.1: Number of surviving tadpoles found in each site.

Site Nr	Habitat	N Tadpoles
258	small pond	49
271	puddle	12
282	small pond	12
290	small pond	51
317	puddle	42
318	puddle	28
330	small pond	54
Sum		248

6.2.3 Estimates of tadpole age

The tadpoles had to be collected before the sites dried out. Due to the unusual drought this was in every case before metamorphosis was reached. Thus tadpoles had grown in the habitats for different time spans (1 to 52 days, see Chapter 5). The family-assignments in Chapter 5 would allow to determine the exact age of each tadpole. However, analyzing family-assigned tadpoles only would considerably reduce the sample size (see Chapter 5) and is not attempted here. Tadpole age was therefore estimated as one or two means per site. Since the variance in egg laying dates within sites is not very high (Table 6.2), the mean spawning date was computed per site, weighting the collecting days by the respective number of eggs. In two sites, it was possible to relate individual tadpoles to two distinct spawning dates since the site dried and refilled over the season (290) or according to two distinct size classes of tadpoles (315). Estimated tadpole age was ln-transformed to improve normality.

Table 6.2: Number of tadpoles, spawning date and mean age per site. The mean spawning date was computed weighing the collecting days by the respective number of eggs.

Site	No of tadpoles	mean spawning date (S.D.)	mean tadpole age (days)
258	49	3.80 (1.79)	18.64
271	12	18.67 (4.51)	28.34
282	12	11.00 (0)	21.00
290	51	4 (0); 28 (0)	12.00; 5.00
315	14	8.8 (3.46); 27 (0)	22.2; 4
317	42	24.40 (6.47)	24.20
318	28	31.00 (0)	17.00
330	54	18.00 (2.10)	8.68

6.2.4 Genotyping

This Chapter is based on the genotype data of tadpoles at the four unlinked, presumably neutral marker loci *Bb7.4, Bv12.19, Bv24.11* and *Bv24.12* (Chapter 5). The mean frequency of *B. variegata* alleles across all four marker loci was used as a hybrid index *HI* and scaled to vary from 0 (*B. bombina*) to 1 (*B. variegata*).

6.2.5 Morphometric data of tadpoles

Using a stereo microscope fitted with an eyepiece micrometer I determined the tadpoles' developmental stage (Gosner 1960) and made five morphological measurements known to reflect functionally important variation in tadpole size and shape (McCollum & van Buskirk 1996): body-height, -length, -width, tail-fin-height and height of tail muscle. Tail fin length was not determined because many tadpoles had injured tail tips. The five morphometric variables were ln-transformed to improve normality. They are linearly correlated (Table 6.3).

Table 6.3: Spearman Rank correlation coefficient r_{SP} pairwise between ln transformed variables. All correlations are highly significant ($p < 0.001$; N=248).

Variable ln (x)	tail muscle height	tail height	body width	body height
body length	$r_{SP} = 0.912$;	$r_{SP} = 0.896$;	$r_{SP} = 0.959$;	$r_{SP} = 0.951$;
body height	$r_{SP} = 0.908$;	$r_{SP} = 0.930$;	$r_{SP} = 0.979$;	
body width	$r_{SP} = 0.898$;	$r_{SP} = 0.908$;		
tail height	$r_{SP} = 0.895$;			

Single missing morphological values (8 of 248 cases) were estimated after regression on the most highly correlated variable. My measure of body size was the first component of a principal component analysis (based on a covariance matrix) performed on the five morphological measures for all individuals (Table 6.4). Measures of shape were the residuals of the five body and tail traits after regression on body size.

Table 6.4: Principle component analysis of data on tadpole morphology based on a covariance matrix. The table gives raw factor loadings and % of total variance explained for each component (N = 248).

Variable	PC 1	PC 2	PC 3
ln(body length)	0.345	0.000	0.053
ln(body height)	0.321	0.031	0.027
ln(body width)	0.320	0.026	0.045
ln(tail height)	0.325	0.066	-0.085
ln(tail muscle height)	0.390	-0.102	-0.035
% total variance explained	94.11	2.74	2.16

6.2.6 Statistics

Variables were ln-transformed to improve normality as needed. Measures of size and developmental stage were analyzed with MANCOVA and measures of shape in ANCOVA for influence of genotype, habitat axis and age and for their interaction. If age was significant, the residuals after regression of the respective variables on age were tested in MANCOVA / ANCOVA for significant influence of genotype class and habitat axis. The residuals of ANCOVA / MANCOVA were checked for normality using Kolmogorov-Smirnov tests.

6.3 Results

After genotyping, the dataset comprised 208 tadpoles across the eight sites. First, I describe the distribution of genotypes and the relationship between genotype, tadpole age and the habitat permanence. I then explore growth and development rates and differences in body shape i) between the two hybrid genotype classes of *B. bombina*-like and *B. variegata*-like individuals and ii) along the habitat axis *H*.

6.3.1 Genotype distribution in tadpoles

As in the adults and eggs (see Chapters 2 and 4), the mean frequency of *B. variegata* alleles is skewed towards the *B. variegata* side of the genotype spectrum. The overall mean *B. variegata* allele frequency is 0.67 (S.D. = 0.25; N = 208). Based on the hybrid index, tadpoles were grouped into two genotype categories: $HI <= 0.5$ (*B. bombina* N = 71) and $HI > 0.5$ (*B. variegata* N = 137). This grouping is feasible since hybrid adults exhibit intermediate genotypes and phenotypes (data not shown). Individuals of both genotype categories occur in all sites except for one (317) which contains only *B. variegata*-like genotypes among the tadpoles.

6.3.2 Tadpole age and genotype in relation to habitat

This field study was designed to detect differential growth rates and phenotypic plasticity in hybrid *B. bombina*-like and *B. variegata*-like tadpoles under natural conditions. In general, growth, development and shape are determined by genotype and age of tadpoles as well as by interactions with the habitat they live in. In field studies it

is impractical to vary a single variable while keeping others constant. Instead, all variables may be recorded under natural conditions. Therefore, it is necessary to discern correlations among the independent variables before analyzing their impacts on the dependent variables.

B. variegata-like tadpoles occur on average in less permanent habitats (*H* higher) than *B. bombina*-like tadpoles ($F = 34.18$, $p < 0.001$, $N = 208$, Figure 6.1). The tadpoles' age and the habitat axis *H* are also highly significantly correlated, with older tadpoles in more temporary habitats ($F = 18.89$, $p < 0.001$, $N = 208$, Figure 6.2). Note that the age values are means per site. The association occurs due to earlier spawning dates in the less persistent sites ($r_{SP} = 0.462$, $p < 0.001$, $N = 208$). Temporary sites might become available for egg laying earlier, e.g. by reaching a threshold temperature. Furthermore, there is a significant positive correlation between tadpole age and the genotype class ($F = 35.33$, $p < 0.001$, $N = 208$, Figure 6.3).

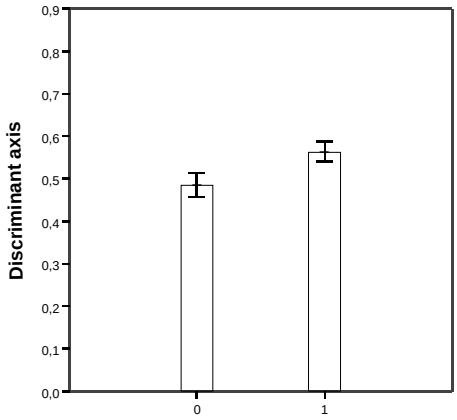


Figure 6.1: Relationship between the habitat axis *H* and the genotype class of *B. bombina*-like (0) and *B. variegata*-like (1) tadpoles ($F = 34.18$, $p < 0.001$, $N = 208$).

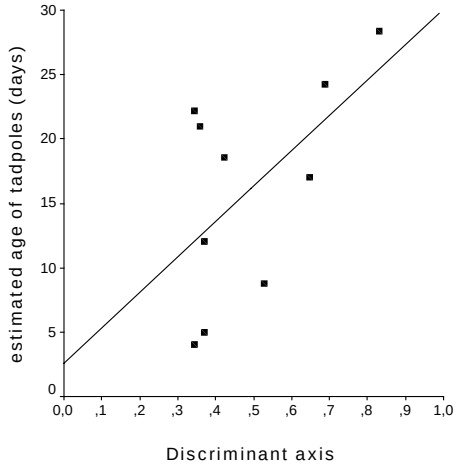


Figure 6.2: Relationship between habitat axis *H* and the age x of tadpoles ($F = 18.89$, $p < 0.001$, $N = 208$). Age values are means per site; in two cases, two means per site were determined (290, 315). Regression: $x = 3.32 + 24.78 * H$.

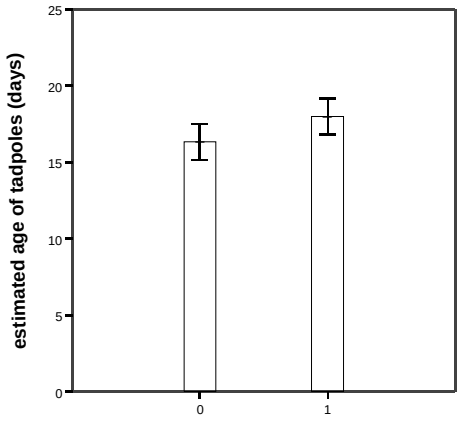


Figure 6.3: Relationship between tadpole age and genotype class for *B. bombina*-like (0) and *B. variegata*-like (1) tadpoles ($F = 35.33$, $p < 0.001$, $N = 208$). Age values are based on means per site.

6.3.3 Tadpole growth and development

To test for genotype-determined differences in growth and developmental rates I regressed both size (PC1) and developmental stage on age for each genotype class. One must bear in mind that age is correlated with the habitat permanence, which might have an age-independent additional effect on growth and development, but thus be hidden in the age effect. One site was excluded (317) since it contained only *B. variegata* individuals. Both size and developmental stage are significantly correlated with age (size and age: $r_{SP} = 0.643$; $p < 0.001$; $N = 208$; stage and age: $r_{SP} = 0.645$; $p < 0.001$; $N = 208$). Developmental stage is not linearly related to age. Initially, there is little change in stage and then it increases rapidly with age. The addition of squared age improves r^2 of the linear regression (linear model: $r^2 = 0.299$; $p < 0.001$; addition of age^2: $r^2 = 0.499$; $p < 0.001$).

The predicted age curves show that, at least in the range of habitats considered here, *B. variegata*-like tadpoles grow and develop faster than *B. bombina*-like tadpoles (size: M-W U = 2708.00; $p < 0.001$; stage: M-W U = 3128.50; $p < 0.001$; Figure 6.4). The crossing of the predicted time curves for size and stage might be explained by the sparse data and hence poor prediction for *B. variegata*–like hybrids at the lower end of the age scale.

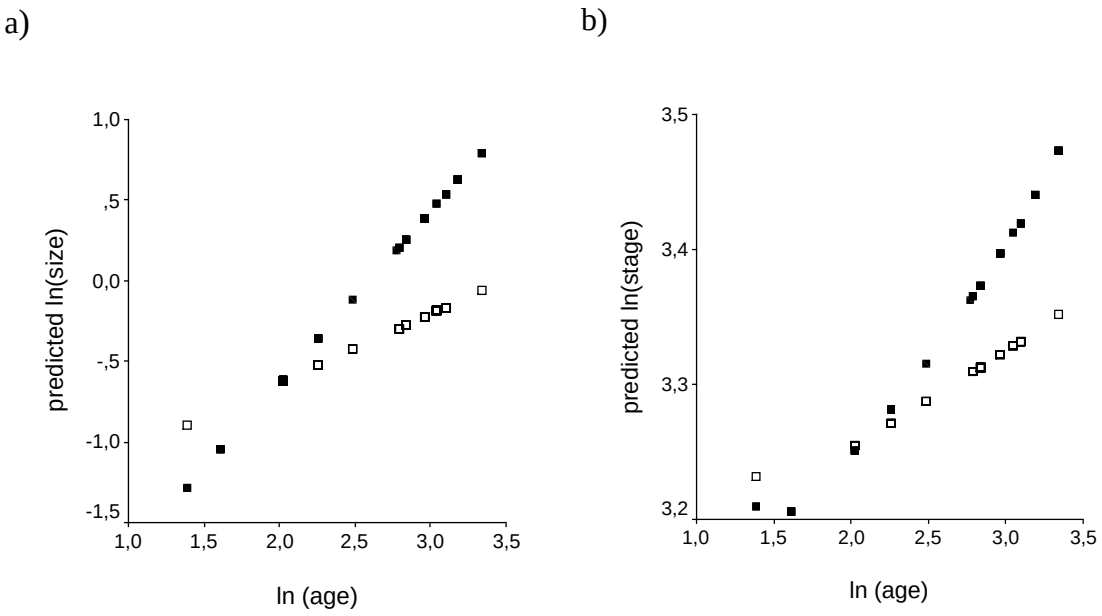


Figure 6.4: Predicted curves for (a) growth and (b) development in *B. bombina*-like (white) and *B. variegata*-like tadpoles (black). Predicted values were obtained by regression (a) of size (PC1) on age and (b) of stage on age + age² for each genotype. One site was excluded (317) since it contained only *B. variegata*-like individuals.

Bearing in mind this general difference between the two genotype classes, the next step is to look at the effect of habitat on growth and development. First, it is necessary to get rid of the age effect which was accomplished by linear or quadratic regressions of size or stage on age, respectively. A single regression for all tadpoles is needed here to take out the age effect, so that effects of habitat and genotype remain in the residuals. The residuals were tested for a correlation with *H* for each genotype class. The first impression is that *B. bombina*-like tadpoles seem to grow and develop faster in their respective habitat (low *H* values) than in more ephemeral sites, whereas *B. variegata*-like tadpoles obviously display no difference between sites. However, this trend is due to one site (318) in which all tadpoles were unusually small. If this site is excluded, only one site with an *H* value higher than 0.55 remains for the analysis. This renders regressions unconvincing (Figure 6.5), especially for *B. bombina* for which there is only one individual in the site with $H > 0.55$.

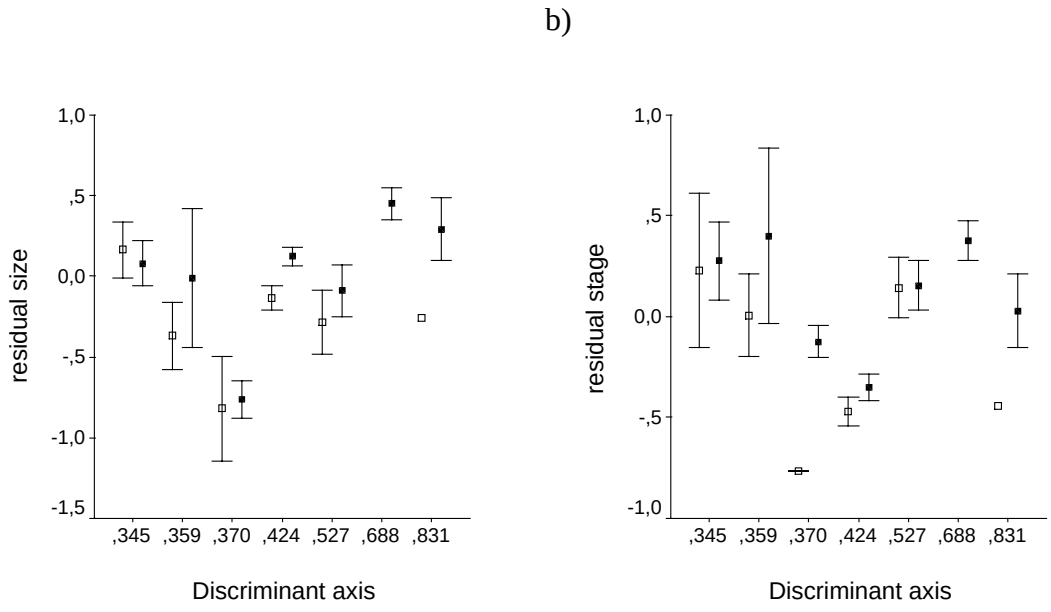


Figure 6.5: Relationship between a) age-corrected size and b) age-corrected stage and *H* for the two genotype classes (empty squares: *B. bombina*; filled squares: *B. variegata*). Error bars represent one standard error of the mean. Sites 317 and 318 were excluded.

There is a large amount of growth variation among sites that is not explained by the habitat axis. Within sites, the mean of age-corrected size of *B.variegata*-like tadpoles is greater than that of *B. bombina*-like individuals in all but one case. This implies a genotype effect on growth that is essentially constant across the investigated habitat range. However, one has to bear in mind that the original correction for age might still hide some habitat effects, as the younger animals tended to be in the relatively more permanent habitat and vice versa. I therefore conclude that *B. variegata*-like tadpoles grow and develop faster in the range of habitat considered here.

6.3.4 Phenotypic plasticity

Effects of age, genotype and habitat on tail and body shape were explored with MANCOVA. Independent of age, tadpoles in permanent sites grew higher tail fins and shorter bodies than in temporary sites, indicating significant phenotypic plasticity (Table 6.5, Figure 6.6). Since *H* is nearly significantly correlated with the presence of predators (see Chapter 5), higher tail fins and shorter bodies may be the response to cues signaling predator presence. No significant influence of the genotype classes could be detected; both classes exhibit an equal range of plastic responses. Interestingly, tadpoles show a continuous range of phenotypes rather than two alternative morphs,

with a site's permanence index *H* being correlated positively with tail fin height and negatively with body length. Body width is significantly related to age and there is a genotype effect on the way relative body height changes with age. However, these two measures cannot be related to a site's permanence index.

Table 6.5: Univariate analyses on morphological plasticity of tadpoles. Morphological measurements are corrected for body size (residuals ln (x) after regression on PC1). The table gives F (above) and p values (below). Bold: significance ($p < 0.05$).

	Body height	Body length	Body width	Tail height	Tail muscle height
Age	0.75	2.24	**7.60**	2.57	2.69
	0.39	0.14	0.006	0.11	0.10
Genotype class	0.09	0.46	0.77	0.22	0.28
	0.77	0.50	0.38	0.64	0.60
H	1.11	**22.96**	0.37	**28.77**	1.09
	0.29	**< 0.001**	0.54	**< 0.001**	0.30
Genotype class * *H*	0.02	0.77	0.24	0.76	0.08
	0.90	0.38	0.63	0.38	0.78
Genotype class * Age	**8.84**	**6.01**	3.16	0.17	0.48
	0.003	**0.02**	0.08	0.68	0.49

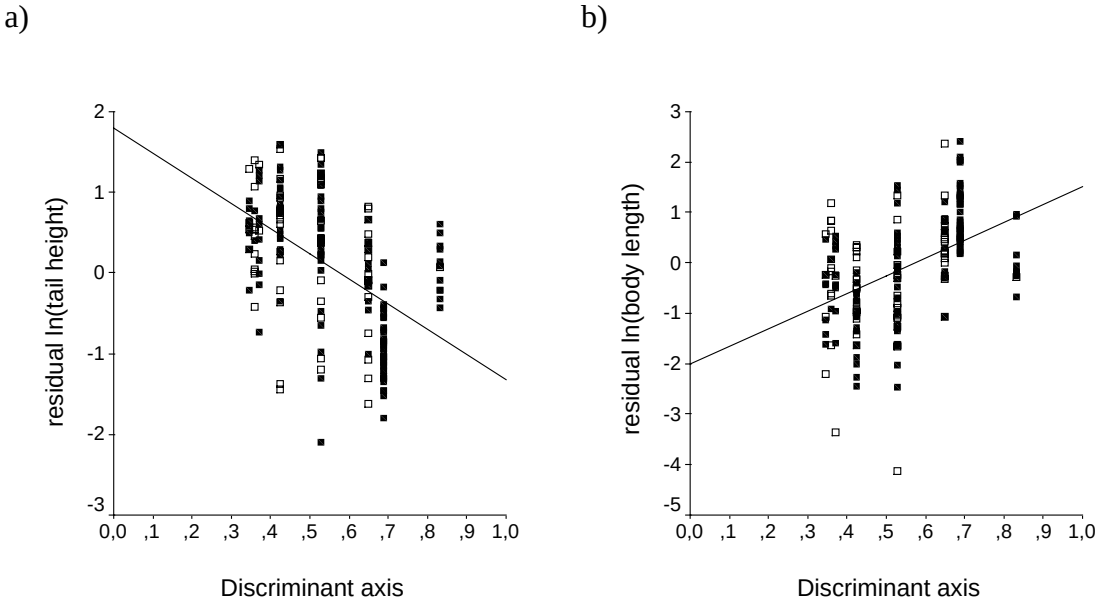


Figure 6.6: a) Tail fin height and b) body length as a function of *H* (white: *B. bombina*; black: *B. variegata*). Morphological measurements are corrected for body size (residuals after regression on PC1).

Finally, body size (PC1) and tail height are negatively correlated (r_{SP} = -0.157, p = 0.026, N = 207), but body size and body length are not (r_{SP} = 0.086, p = 0.224, N = 208), which might imply a cost in body size to the formation of a higher tail fin. The measurement of fitness differences between induced and non-induced tadpoles in the temporary habitat would be necessary to discern a cost to predator-induced phenotypic plasticity.

6.4 Discussion

In this Chapter, I investigated growth, development and phenotypic plasticity in *Bombina* tadpoles. This study differed from the typical laboratory experiments reviewed above in its complexity. On the one hand, I focused on hybrid tadpoles as these dominate the sites in Apahida. On the other hand, the sites enclosed in this study were intermediate rather than extremely temporary or permanent. This implies that both selection factors, the risk of desiccation and the risk of predation, affected tadpoles during their development. Therefore, tadpole phenotypes were probably influenced by selection for rapid growth counteracted by selection for a phenotypic response to predator presence. While most experimental studies focus on fitness and phenotypic plasticity of distinct genotypes in opposing artificial selection regimes, this is the first study concentrating on these issues in the natural setting of a hybrid zone. Two main results were found. First, *B. variegata*-like tadpoles grew and developed faster than *B. bombina*-like ones across the habitat range. Second, tadpoles surviving in more permanent habitats showed higher tail fins and shorter bodies than those collected in temporary sites, irrespective of the genotype. A continuous range in tail fin height and body length was observed across all tadpoles and sites, in accordance with a site's permanence.

Limitations of the data set

This study was designed to test hypotheses about adaptive morphology of hybrid tadpoles in the field. Growth, development and shape of tadpoles are in general determined by their genotype and age as well as by interaction with environmental factors. Since it was impractical to control for single independent factors, I recorded them separately and investigated them for associations. On average, *B. variegata*-like tadpoles were found in more temporary habitats (higher *H* values) and *B. bombina*-like ones in more permanent sites. Tadpoles in temporary habitats were significantly older

than in permanent habitats, due to earlier spawning in these sites. Consequently, *B. variegata*-like tadpoles tended to be older than *B. bombina*-like ones.

The following causal factors for the association between tadpole genotypes and habitat permanence are plausible: i) breeding habitat choice in the adults and ii) differential survival of tadpoles, depending on the genotype. It is difficult to estimate the relative importance of these factors without knowledge of phenotype distributions before the onset of selection. However, I have demonstrated in Chapter 5 that breeding habitat preference is very likely responsible for the predominant input of *B. variegata*-like eggs into the habitat range considered here while I have no evidence for selection on tadpoles by the habitat. It may suffice to keep in mind that genotypes are not distributed equally among the sites. That tadpoles were older in temporary as compared to permanent habitats was observed both in *B. bombina*-like and in *B. variegata*-like hybrids; this was related to earlier spawning dates in temporary sites. Since the sites were too close together to be exposed to different rain regimes, temporary sites might become available for egg laying earlier, e.g. they reach a threshold temperature earlier. Since tadpoles were not measured at a predetermined age, growth curves were fitted, and the residuals were then tested for an effect of habitat and genotype. Due to the correlation between age and habitat permanence mentioned above, environmental effects might have been hidden in the age effect after the regressions were performed.

Growth and development rates

B. variegata-like tadpoles grew and developed faster than *B. bombina*-like tadpoles. However, this finding is not independent of the habitat. Size and developmental stage were corrected for the mean age per site which was correlated with the habitat index. Do the differences in growth and development imply that *B. variegata*-like tadpoles enjoy a general adaptive advantage over *B. bombina*-like ones in ephemeral puddles? In a laboratory breeding experiment on tadpoles from the Pescenica transect, the average larval period for *B. variegata* was 87% of that for *B. bombina* (Nürnberger et al. 1995). So a comparatively short development time is at least in part genetically determined in *B. variegata*. Furthermore, the high desiccation risk even in semi-permanent sites was impressively demonstrated in this study by the drought in both years which would have led to 100% mortality in tadpoles had they been left in the sites. In a different study, desiccation caused 79% premetamorphic mortality in *B. variegata* tadpoles within a region (Barandun & Reyer 1997). Under these circumstances, adaptive strategies

comprising growth and metabolic rates should ensure that tadpoles gain independence of water as early as possible. On the other hand, the predator density in temporary and semi-permanent habitat varies considerably (Thiel 2001). In this study even the most ephemeral site was not predator-free. Werner & Anholt (1993) developed a model predicting that to minimize the ratio of mortality rate to growth rate when both rates are linear with activity levels, the latter should be either maximal or minimal depending on resource level and on the effects of activity on mortality. On the other hand, physiological constraints might impose diminishing returns on increases in development rate with activity, whereas the probability of encounter with a predator increases linearly. So the incremental benefit to development rate of increased activity would be lower than the incremental cost in terms of mortality risk (Werner & Anholt 1993). This means that a less active species will always fare better in an environment in which predation is a regulating factor, in spite of the longer larval period incurred. This model applies to adaptation to fixed extreme habitat types. To maximize fitness in variable habitats like the range investigated here and common in the Apahida hybrid zone, tadpoles need to evaluate the current predation risk and adjust their activity around the innate levels. That tadpoles reduce their activity levels if exposed to chemical cues from predators has lead to the risk allocation hypothesis (Lima & Bednekoff 1999), which states that prey adaptively allocate their foraging efforts, and thus their exposure to predation risk, across high risk and low risk situations.

If *B. bombina* has adapted to predator-rich environments in terms of a fixed, low foraging activity level and hence a low growth rate, it might have a competitive disadvantage in ephemeral sites where food and space are limited. In ephemeral puddles, competition is thought to be the limiting factor (Begon et al. 1990) and faster-developing, more active tadpoles have a competitive advantage (Petranka & Sih 1986, Semlitsch 1989, Scott 1990, Werner 1992). Since I cannot estimate differential survival of a range of size-classes that might have hatched I assume that the relatively small *B. bombina*-like tadpoles I collected are the best that this genotype category can afford in ephemeral habitats. This implies that *B. bombina*-like tadpoles are not able to increase their rates of foraging, food processing or metabolism sufficiently to match *B. variegata*-like ones in growth and development rate. The genetically-determined faster growth rate, probably mediated by a higher activity level, should afford an increase in fitness to *B. variegata*-like relative to *B. bombina*-like tadpoles in ephemeral habitats where competition and risk of desiccation are major selective agents. This

finding agrees with the trend in habitat preference towards *B. variegata* demonstrated in Chapter 5.

Differential phenotypes

Tail and body shape of tadpoles are influenced by the environment: individuals surviving in more permanent habitats had higher tail fins and shorter bodies than those collected in temporary sites. No significant genotype effect on tail and body shape could be seen. The results suggest that, irrespective of genotype, *Bombina* tadpoles surviving in more permanent habitats have relatively higher tail fins and shorter bodies than those in ephemeral sites. In the following, I address the questions: 1) Can the expression of higher tail fins and shorter bodies be interpreted as predator-induced defense phenotype? 2) Does the absence of a significant difference in the range of phenotypic plasticity imply that hybrid *B. bombina*-like and *B. variegata*-like tadpoles are equally well adapted to predation risk in semi-permanent sites?

In general, higher tail fins and shorter bodies are part of a whole suite of traits that respond to predator presence. Morphological plasticity is taken here as a representative measure with which to assess the degree of plasticity. It need not be the main trait on which a selective advantage is based. In striking similarity to this study, van Buskirk & McCollum (1999) found higher tail fins and shorter bodies in *Hyla versicolor* tadpoles that were collected in permanent, predator-rich habitats as compared to tadpoles from temporary sites. The presence of newts is significantly concentrated to permanent habitats, and the density of other tadpole predators is correlated (though not significantly) to habitat permanence (see Chapter 5). Kruuk & Gilchrist (1997) have also demonstrated that the predator density in ponds is significantly higher than in puddles in the Pescenica hybrid zone.

Is the morphological response an adaptation to the presence of predators? I have not shown that the induced phenotype indeed conveys a selective advantage when tadpoles face predators. At least, tadpoles with relatively high tail fins and short bodies survived in semi-permanent habitats. However, whether tadpoles that were killed by predators had a significantly different mean phenotype is unknown. The possibility exists that the phenotypic distributions were equal at the start of tadpole development and that any differences I observed stem entirely from differential survival. Given that phenotypic plasticity is so common in tadpoles (see above) this is not likely, but I cannot discount

that selection also had an effect on the differences in the data. To separate the effects cleanly, one would need to control for initial phenotypes before the onset of selection.

What is the benefit of the induced phenotype when tadpoles face predation risk? It has not yet been solved how a higher tail fin increases the probability of survival in tadpoles (van Buskirk & McCollum 2000). McCollum & Leimberger (1997) documented increased swimming speed in induced phenotypes and Watkins (1996) related burst swimming speed in tadpoles to success at escaping predation. Thrust for burst swimming is produced in the mid part of the tail fin where it has its highest point (Wassersug & von Seckendorf Hoff 1985). However, van Buskirk & McCollum (2000) could not find any deteriorated performance in tadpoles with experimentally cut-off tail fin portions. In their experiment, tadpoles could stand the loss of up to 30% of the tail length or depth without harm to the angle of escape or burst swimming speed. They proposed that predator-induced tadpoles are less vulnerable to predation for reasons other than enhanced swimming performance. Higher tail fins and shorter bodies might deflect predator strikes to the tail, away from the body and head. I found many tadpoles in the field with injured tail tips, suggesting that tadpoles are indeed frequently attacked at the tail. The defense strategy might be to reduce to size of the vulnerable body and instead enlarge the tail fin area of which parts may be sacrificed without fatal consequences (van Buskirk & McCollum 2000).

How can the graded dosage of phenotypic plasticity be explained? In semi-permanent habitats, development under the simultaneous risks of predation and site desiccation creates a trade-off between resource-acquisition and predator avoidance (Skelly 1995, Werner & Anholt 1993). While predator-induced phenotypes entail a fitness benefit in the presence of predators, they mean a selective disadvantage in habitats where competition and receding water are the main selective agents. Perhaps due to a competitive disadvantage, predator-induced tadpoles suffered from higher mortality rates compared to non-induced ones in a predator-free environment (van Buskirk & Relyea 1998). These studies suggest that in semi-permanent habitats, in which the predation risk varies considerably in space and time, predator-induced morphological responses should be reduced to the vital minimum, if the underlying fitness function is linear rather than shows a threshold. In this context it seems plausible that tadpoles show graded phenotypic plasticity continuously in correlation with predation risk (*Bombina*: this study, *Rana lessonae*: van Buskirk & Arioli 2002). So far, experimental

studies have always contrasted a predator rich with a predator-free environment without restrictions on hydroperiod. In the present case, tadpoles had the much more difficult task to balance the opposing demands of predator avoidance and desiccation risk within a given site.

How can the absence of a significant difference in the range of phenotypic plasticity between *B. bombina*-like and *B. variegata*-like tadpoles found here be interpreted? Life history theory predicts that the species adapted to temporary habitat, *B. variegata*, should possess a higher variability in anti-predator defenses since their habitat is locally and temporally much less predictable in terms of predation risk than permanent habitat (Wellborn et al. 1996). In accordance with this prediction, Vorndran et al. (2002) documented less phenotypic plasticity in *B. bombina* tadpoles which displayed high tail fins even when raised without predators. The discrepancy between their and my findings may be related to some factors that differed between the two studies.

First, the number of surviving *B. variegata* tadpoles in my study by far exceeds the one of *B. bombina* tadpoles, especially in temporary habitats. So, I might have had too few *B. bombina* left for ANCOVA to detect a significant effect of genotype on shape. Second, tadpoles in this study were younger in more permanent sites than in temporary sites whereas Vorndran et al. (2002) measured all tadpoles at predetermined ages. They found that differences between induced and non-induced *B. bombina* tadpoles were higher in early and late phases of the larval period than in intermediate ones. Due to the association between habitat index and tadpole age I might simply have observed the difference between old *B. bombina* tadpoles with relatively low tail fins (in temporary habitats) and young conspecifics with higher tail fins (in permanent habitats). However, ANCOVA did not reveal an effect of age on shape, which weakens this point.

Third and most important, Vorndran et al. (2002) investigated genetically pure individuals whereas the individuals I grouped in the *B. bombina*-like genotype class exhibited up to 50% *B. variegata* alleles at neutral loci, and only 5.7% of the *B. bombina*-like individuals had a pure 0 hybrid index. Within Apahida hybrid populations, the maximum linkage disequilibrium R is 0.38 among neutral loci (see Chapter 3). If the correlation among marker loci applies also to the correlation between hybrid index and a given trait, then this figure gives an idea how closely correlated traits are within populations. The question then is, how does a given trait map across the hybrid index gradient? One could imagine one for growth which is highly correlated

with the hybrid index (hence the difference between *B. bombina*-like and *B. variegata*-like tadpoles), and one for phenotypic plasticity which is non-linear so that even *B. bombina*-like hybrids already behave essentially like *B. variegata*.

Implications for the Apahida *Bombina* hybrid zone

Differences in tadpole growth and susceptibility to predation have been invoked in some anuran taxa to explain species' distribution along environmental gradients (Kats et al. 1988, Skelly 1995, Lardner 2000). I do not know of any other system with two regularly interbreeding species in which differential selective advantages in opposing breeding habitats are associated with the maintenance of reproductive isolation through habitat preference. In accordance with Vorndran et al. (2002), I did not find restrictions to phenotypic plasticity in *B. variegata*. However, this species' selective disadvantage in permanent habitats might be due to an activity-mediated increased susceptibility to predation in true ponds (Kruuk & Gilchrist 1997). In *B. bombina*-like tadpoles, slower growth and development might be seen as inferior adaptation to desiccation risk and competition for limited resources in ephemeral sites. If the above linkage disequilibrium applies similarly for the hybrid index and other traits, selection on differences at the larval stage should cause a correlated advantage of habitat preference in adults. Concerning growth and development of tadpoles, this would be in accordance with the trend in breeding habitat preference towards *B. variegata* in the sites studied here (see Chapter 5). However, it is questionable whether the demonstrated effects are strong enough to stabilize the Apahida hybrid zone. This might be investigated with the aid of simulations.

6.5 Summary

This Chapter focused on differential phenotypic plasticity as a function of genotype and habitat in *Bombina* tadpoles. Across eight sites in the Apahida hybrid zone, hybrid tadpoles were collected, genotyped, morphological measurements were made and the tadpoles' age estimated as site means using the spawning data. Body size was the first component of a PCA on five morphological traits and measures of shape were residuals after regression of individual measurements on body size. Growth and development rates were computed by regressions of size and developmental stage on age. Residuals and shape measures were then tested for significant effects of genotype and habitat.

There is clear trend that *B. variegata*-like tadpoles grew and developed faster than *B. bombina*-like tadpoles across the range of investigated habitat. Faster growth and development should confer an advantage to *B. variegata* in temporary habitats with high risk of desiccation. Second, tadpoles surviving in more permanent habitats showed higher tail fins and shorter bodies than those collected in temporary sites, irrespective of the marker genotype. Interestingly, tadpoles of both genotype categories seem to possess a continuous range of responses in tail fin height and body length, in accordance with a site's permanence. The trait for phenotypic plasticity might be distributed non-linearly across the genotype map so that even *B. bombina*-like hybrids already behave essentially like *B. variegata*. At minimum the growth and development data are in accordance with the bias towards *B. variegata* in breeding habitat preference across the habitat spectrum considered here (see Chapter 5).

7 CONCLUSIONS

This thesis describes empirical data on the maintenance of reproductive isolation in a *Bombina* hybrid zone. In this Chapter, I summarize the results and conclusions and point out several potential areas for future research. As I outlined in the beginning, the speciation process is often a consequence of different forms of divergence between populations that each contribute to reproductive isolation. Understanding the process of speciation requires investigating how reproductive isolation mechanisms accumulate and are maintained between diverging populations. Hybrid zones are the ideal study system for this research as they offer a unique opportunity to investigate the interaction between gene flow and selection in incipient species in a natural context.

The main focus of this thesis was the identification of selection factors that help to maintain the *Bombina* hybrid zone. At our field site in Apahida, Romania, this issue is particularly critical given the unusual structure of the hybrid zone there. In general, the dynamics of hybrid zones are best understood in the case of clinal transitions in allele frequencies between two very large, i.e. essentially infinite, divergent gene pools (Barton 1979, Barton 1983, Barton & Hewitt 1985). Until recently, the *Bombina* hybrid zone served as a classic example of this phenomenon. In our joint field work, T. Vines and I discovered instead a broad mosaic structure. In this mosaic, *B. bombina* was restricted to scattered ponds while *B. variegata*-like hybrids dominated the surrounding, more abundant semi-permanent to ephemeral sites. A steep genotype gradient could not be detected, which was in striking contrast to hybrid zones in Poland, Croatia and the Ukraine where steep clines had been found along altitude transitions.

The difference in the distribution of the underlying habitat is a very plausible explanation for this difference, though other factors may contribute. Maintaining divergence in a broad mosaic is much more difficult than in a narrow cline where dispersal from nearly infinite pools of the pure taxa on either side constantly re-creates high levels of linkage disequilibrium. Strong introgression at neutral marker loci was found in Romania and there were hardly any pure *B. variegata* populations.

Does this indicate the breakdown of divergence? If so, how many generations would be required until the two taxa have completely merged? And is this process slowed by some mechanism counteracting gene flow? Linkage disequilibrium and heterozygote

deficit are strongest in *B. bombina*-like hybrid populations and therefore seem to be driven by the migration of pure *B. bombina* adults out of ponds into the surrounding populations in intermediate habitat. Inferences for the selection strength necessary to maintain divergence at neutral and selected loci were drawn from the estimated migration rate by N. Barton. Differences at neutral loci could only be maintained by implausibly strong selection and are therefore probably collapsing. These inferences raise the issue of the age of the hybrid zone in Apahida. More generally, we see here that taxon barriers may be temporary as long as interbreeding occurs and fertile hybrids are produced.

The selection strength postulated to prevent the swamping of locally adapted *B. variegata* alleles by alleles adapting *B. bombina* to ponds was not implausibly high, provided that it acts against immigrant alleles rather than heterozygotes. In which way could this selection act? Selection against adults in the wrong habitat is unlikely, and no evidence was found for assortative mating within populations (Vines 2002). One mechanism that might reduce the frequency of hybridization is active habitat preference.

Interestingly, habitat preference among adult toads was twice as strong in Romania than in Croatia. So is the strong habitat preference a characteristic feature of the toads in Romania and a prerequisite for the sympatry found there? Or did habitat preference strengthen as a response to competition, once the two taxa got into contact? This issue is worth further investigation. First, it is essential to explore the strength of habitat preference outside the zone of contact to discern how it is influenced by competition. Second, with the aid of a simulation model, Kruuk (1997) showed that considerable habitat preference would allow the two taxa to exist in sympatry. The model considers a gradient in habitat distribution. It should be extended to an interspersed pattern like the one in Romania. Then one may decide whether the habitat preference found there is an important factor for the maintenance of adaptive divergence in the broad mosaic.

So far, the adult habitat preference had been established via a correlation between adult genotypes and the habitat score. However, a more critical stage at which habitat preference can be expressed is reproduction itself. For this second component of habitat preference, I showed a considerable shift towards *B. variegata* alleles in parents relative to sampled adults in intermediate habitat. How do adults assess the suitability of a habitat for breeding? A study investigating this is currently under way. So far, one may conclude that partial reproductive isolation is maintained by strong adult habitat

preference and a greater propensity of *B. variegata*-like individuals to reproduce in intermediate sites. However, the overall abundance of introgressed genotypes implies that this prezygotic isolation mechanism cannot be the sole barrier to gene flow. Additionally, breeding habitat preference can only be understood in the light of a fitness gain of the juveniles in the preferred habitat that must outweigh the restriction to a certain habitat range.

Therefore, natural selection during the tadpole stage of the life cycle is one potentially important source of partial reproductive isolation in this hybrid zone and was the main aspect of this thesis. I investigated two components: intrinsic selection (differential survival as a function of the genotype) and extrinsic selection (differential survival of different genotypes as a function of the habitat). Further on, I drew inferences from the analysis of phenotypes in the field after selection.

I found direct evidence for a considerable fitness reduction of *B. bombina* alleles in the primarily *B. variegata*-like genetic background of lab-reared embryos at three out of four marker loci. At the same time, there was no sign of selection against heterozygote individuals. Assuming neutrality of the markers themselves, this implies that *B. bombina* alleles at loci physically linked to the markers are disfavored. It would clearly be of interest to investigate the segregation of *B. variegata* alleles in a mostly *B. bombina*-like genetic background. Heterozygote disadvantage, even though not found in this study, may, of course, contribute to the barrier elsewhere in the genome.

Now, to assess whether the selection strength found can quantitatively prevent the swamping of *B. variegata*-like populations by *B. bombina* alleles at selected loci, a modeling approach is needed that incorporates an estimate of the number of loci under selection. The estimate of the number of loci and their distribution across the genome requires more marker loci and a QTL approach with which one may locate the selected loci. This would then allow us to estimate the number of generations required for the current level of introgression. Reduced fitness of *B. bombina* alleles in a *B. variegata*-like genetic background certainly constitutes an intrinsic barrier to gene flow. However, it is probably just one postzygotic component of partial reproductive isolation.

The adult preference for opposing aquatic habitat types strongly suggests that extrinsic factors constitute a similarly important component. Here, the investigation would have been most direct if all egg batches could have been sampled and remaining family sizes

known. The only unknown factor would have been the amount of linkage disequilibrium between selected loci and neutral markers. However, linkage disequilibrium has been quantified among neutral markers. On the population level, we know that there are strong correlations between markers, belly score and the habitat permanence. So, a correlation of allele frequency shifts as a function of habitat is a plausible expectation. The original scheme turned out to be difficult because i) not all egg batches could be sampled in densely vegetated sites and ii) egg batches turned out not to be equivalent to families. This issue was solved in the following way. Eggs from batches within a site were assigned to families with the help of a highly variable locus. Also surviving tadpoles were assigned to these families.

The comparison of regressions between mean allele frequencies at various life stages on the one hand and the habitat score on the other hand showed that the mean allele frequencies of surviving tadpoles are, in contrast to the joint parental genotypes, significantly correlated with the habitat permanence, implying that selection by the habitat on *Bombina* tadpoles is a plausible expectation. Further work on extrinsic selection would be interesting. For example, a within-family QTL approach could treat each family as a separate experimental unit and investigate shifts in allele frequencies as a function of genotype and habitat. In this way, it may be possible to uncover selection effects at linked loci directly.

There was non-random survival in some sites which could not be explained by the environment. Assuming that the count of remaining eggs per inferred family gives adequate estimates of family sizes, these results suggest that other factors such as perhaps intrinsic selection of varying strength among families additionally determine survival. It would be interesting to investigate exactly what site specific components determine survival in tadpoles, including habitat permanence and fine-scaled predator density. For this, one would have to take a more detailed look at the habitat ecology and at the corresponding absolute survival among all families per site.

At the phenotypic level, I investigated surviving tadpoles after selection. I showed that *B. variegata*-like tadpoles grow and develop faster than *B. bombina*-like ones in the range of habitat investigated. An enhanced growth rate, mediated by higher food processing activity, should confer an advantage to *B. variegata* in habitat where tadpoles face a high risk of desiccation and should gain independence of water as quickly as possible. Further on, I found that the range of phenotypic plasticity in

tadpoles was continuous along the range of habitat permanence, suggesting that the underlying fitness function is linear rather than threshold. This phenomenon has only been found in one anuran species other than *Bombina* so far (*Rana lessonae*: van Buskirk & Arioli 2002). Additionally, the range of phenotypic plasticity was not smaller in *B. bombina*-like tadpoles compared to *B. variegata*-like ones. This was not expected since *B. bombina* tadpoles are adapted to fixed permanent habitat with a constantly high density of tadpole predators while *B. variegata* tadpoles encounter much more variable conditions to which they must adjust their phenotype.

An explanation for the difference between the gradients in growth rate and phenotypic plasticity might be that the alleles of some strongly selected loci, e.g. phenotypic plasticity map differently across the gradient in allele frequencies seen in neutral loci. So, in some respects *B. bombina*-like individuals may essentially behave like *B. variegata* ones. To explore this issue we need a deeper insight into the location and distribution of selected and neutral marker loci in the *Bombina* genome using a QTL approach (see above).

To summarize, it has been shown that a *Bombina* hybrid zone can take different structures. Whether a cline or a mosaic forms depends most likely on the underlying distribution of the different habitat types. Habitat preference is an important prezygotic component for the maintenance of divergence between the two hybridizing *Bombina* taxa. It is plausible as the tadpoles have adapted their growth rates to opposing ecological conditions. Still, considerable introgression occurs, mainly through the flow of *B. bombina* alleles into *B. variegata*-like populations. This is in part counteracted by selection against alleles in the minority within the genome. Extrinsic selection certainly plays a similarly important role and needs further investigation. These findings may contribute to a wider understanding of the roles of habitat preference and natural selection in the evolution and maintenance of reproductive isolation in incipient species.

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Appendix 2.1

Ecological data for all sites in the Apahida hybrid zone. The column 'type' refers to the habitat type (1 = true pond; 2 = small pond; 3 = ditch; 4 = artificial pool; 5 = artificial hole; 6 = puddle), and '*H*' is the habitat score. 'Height' is the height above sea level. The following three columns describe the dimensions of the water body. '% em' and '% su' are the percent of the surface area covered by emergent and submerged vegetation respectively. The 'bk' columns refer to the percentage of bank cover less than 15 cm, between 15 and 50 cm, and more than 50 cm high respectively.

Sites	type	*H*	height	width	depth	length	% em	% su	bk < 15	bk 15-50	bk >50
100	1	0,16	360	25	0,6	30	30	10	20	50	30
200.3	4	0,44	300	2,7	2	5,5	10	5	40	10	30
200.4	4	0,54	300	3,2	0,7	7,1	5	5	30	10	0
200.5	4	0,48	300	3	1,2	8	10	5	10	20	10
200.6	4	0,44	300	2	1,7	5	20	20	20	40	40
200.7	4	0,41	300	4	1,5	5	20	20	30	20	10
200.8	4	0,44	300	4	1	5,2	20	30	70	20	10
200.9	4	0,56	300	3,1	0,6	6,3	0	20	25	25	0
200.10	4	0,45	300	4,4	1,2	6,4	10	20	40	20	10
244	6	0,65	300	1,5	0,4	4,2	5	0	100	0	0
245	3	0,46	390	3	0,4	9	50	20	100	0	0
246	3	0,51	390	1,5	0,4	10	50	5	20	0	10
247	6	0,72	310	3	0,1	15	5	0	70	0	30
248	2	0,67	320	3,6	0,1	5,3	10	40	100	0	0
249	2	0,53	300	1,5	1,2	3,9	5	5	80	0	0
250	3	0,71	340	1,2	0,2	40	15	5	80	10	0
251	6	0,56	360	5,2	0,1	6,8	30	60	80	10	10
252	6	0,75	360	1,5	0,1	3,1	35	0	100	0	0
253	6	0,62	370	1,3	0,2	2,4	10	80	50	30	0
254	6	0,8	370	0,4	0,2	1,3	5	0	0	90	10
255	6	0,53	370	1,2	0,2	2,2	60	40	15	45	40
256	6	0,57	320	1,2	0,3	15	50	5	80	10	10
257	3	0,69	320	0,9	0,2	11	15	10	70	30	0
258	2	0,42	320	8	0,5	15,4	40	20	80	0	0
259	6	0,86	310	0,2	0,1	10,2	10	0	80	10	0
260	6	0,77	310	0,6	0,2	1,2	5	5	60	20	20
261	6	0,89	300	0,2	0,1	1,3	0	0	20	40	40
262	6	0,69	360	0,2	0,2	0,5	50	5	80	20	0
263	6	0,76	340	0,6	0,2	2,1	5	0	40	0	0
264	6	0,77	350	0,4	0,2	12,2	5	0	80	0	0
265	6	0,91	280	0,4	0,1	1	0	0	10	0	0
266	6	0,62	280	3,6	0,1	5,2	40	0	50	0	30
267	6	0,8	360	0,6	0,1	6	10	0	30	20	10
268	2	0,41	360	7,6	0,4	16	45	40	10	80	10
270	2	0,43	350	8	0,3	11	50	20	70	10	0
271	6	0,83	300	0,4	0,1	2	5	5	30	10	0
272	6	0,72	360	0,3	0,4	3,1	5	5	10	80	0
273	6	0,75	370	0,3	0,1	1,2	30	50	90	10	0
274	6	0,77	370	0,5	0,2	4,9	0	0	60	0	0
275	6	0,83	350	0,3	0,1	0,3	10	10	60	30	5
276	6	0,51	360	6	0,4	8	20	10	70	10	0
277	6	0,78	310	1	0,1	5,4	5	0	50	40	0
279	6	0,82	310	0,5	0,1	9	5	0	0	10	0
280	6	0,78	280	0,7	0,1	8	10	10	10	0	30

Sites	type	*H*	height	width	depth	length	% em	% su	bk < 15	bk 15-50	bk >50
281	6	0,87	280	0,2	0,1	0,2	0	5	50	0	0
282	2	0,36	300	15	0,5	128	50	10	20	60	20
283	3	0,67	300	0,5	0,1	2	50	30	5	55	0
284	3	0,71	310	0,5	0,4	0,5	0	0	5	75	0
285	5	0,69	380	1	0,3	1,2	0	5	0	5	0
286	5	0,62	300	2,5	0,4	5	0	5	0	0	2
287	5	0,83	360	0,5	0,1	1	0	5	70	0	0
288	6	0,79	360	0,5	0,2	0,5	5	0	20	60	20
289	3	0,53	300	2,5	0,6	5	20	0	10	20	60
290	2	0,37	300	6	0,5	11	50	60	0	30	70
291	6	0,77	370	0,5	0,2	1,3	5	20	0	45	0
292	3	0,64	460	5	0,2	1,5	0	5	30	50	20
293	1	0,2	460	70	2	80	20	40	85	5	5
294	2	0,1	420	50	2	70	60	50	95	0	5
295	3	0,65	440	0,3	0,2	15	50	30	0	50	50
296	6	0,62	430	2	0,1	8	40	30	85	15	0
297	5	0,6	510	1,5	0,4	1,5	5	50	20	50	20
298	6	0,91	350	0,2	0,1	0,8	10	20	10	40	50
299	6	0,84	370	0,4	0,1	2	5	0	30	60	0
300	6	0,74	370	1,5	0,1	2	5	20	10	90	0
301	3	0,7	370	1,5	0,2	2,5	0	10	60	30	0
302	3	0,72	300	0,3	0,2	2,9	20	80	50	45	0
303	6	0,7	350	0,5	0,4	1,4	0	10	40	30	30
304	6	0,67	370	1,3	0,3	1,9	5	0	50	30	5
305	2	0,38	340	6,8	2	7,9	10	10	40	0	5
306	6	0,87	340	0,2	0,1	0,4	5	0	40	50	10
307	6	0,87	350	0,2	0,1	0,3	5	0	90	0	0
315	3	0,35	300	1,8	0,3	120	90	5	5	5	90
317	6	0,69	300	0,4	0,1	2	1	0	50	0	50
318	6	0,65	300	0,6	0,3	1,6	2	0	90	10	0
321	3	0,35	320	4,1	0,3	36	85	5	100	0	0
327	6	0,58	310	0,3	0,1	0,3	50	0	100	0	0
330	2	0,53	380	16	0,4	26	10	5	100	0	0
333	1	0,22	290	60	3	700	85	5	20	70	10
334	3	0,47	300	5	0,2	80	50	10	100	0	0
335	1	0,34	300	45	0,4	220	70	0	40	60	0
342	6	0,39	360	2,1	0,3	8,4	80	0	90	10	0
344	6	0,59	320	0,3	0,1	0,3	50	0	100	0	0
345	6	0,58	350	2,2	0,1	1,8	25	10	100	0	0
347	3	0,67	300	0,8	0,1	1	2	2	95	5	0
372	5	0,63	350	1	0,4	1,3	0	2	100	0	0
374	2	0,38	360	2	0,3	12,7	80	10	100	0	0
377	6	0,44	380	4,9	0,2	6,8	45	55	2	98	0
379	6	0,5	380	2	0,1	3	60	0	100	0	0
380	5	0,62	310	1,4	0,3	1,4	2	1	70	30	0
385	6	0,64	360	5	0,1	4	0	1	80	20	0
388	6	0,59	370	2,6	0,5	4	0	0	30	70	0
389	2	0,49	370	0,9	0,2	2	60	10	10	60	30
396	6	0,59	340	3,4	0,4	8,2	0	0	0	0	100
397	2	0,56	350	1,9	0,4	3,8	15	15	10	60	30

Appendix 2.2

The adult genotypes per individual and locus. For *Bb7.4*, *Bv24.11*, *Bv12.19* and *Bv24.12* 'v' alleles are *B. variegata* and 'b' alleles are *B. bombina*. In some cases, genotyping did not yield results.

Sites	ind	7.4	24.11	12.19	24.12
100	1	bb	vv	bb	bb
100	2	bb		bb	bb
100	3	bv	vv	bv	bv
100	4	bb	bv	bb	bb
100	5	bv	bb	bb	bv
100	6	bv	bb	bv	bv
100	7	bb	bb	bv	bb
100	8	bv		bb	bv
100	9	bb	bb		bv
100	10	bv	bb	bb	bb
100	11	vv	bb	vv	vv
100	12	bb	bb	bb	bb
200.3	1	bv	vv	bv	vv
200.3	2	vv	vv	vv	bv
200.3	4	vv	vv	bv	vv
200.3	5	vv	vv	vv	vv
200.3	6	bv	bv		bv
200.3	7	vv	bb	vv	vv
200.3	8	vv	vv	vv	vv
200.3	9	vv	bv		vv
200.3	10	vv	bv	vv	vv
200.3	11	vv	bv	vv	vv
200.3	12	bb	bb		bv
200.3	13	vv	bv	bv	vv
200.4	1	vv	vv	vv	vv
200.4	2	vv	vv	vv	bv
200.4	3			bv	vv
200.4	4	vv	vv	bv	vv
200.4	5	vv	vv	bv	vv
200.4	6	bb	bv	vv	bv
200.4	7	vv	bb	bv	vv
200.4	8	vv	bv	bv	vv
200.4	9	bv	bv	vv	vv
200.4	10	bv	bb		bv
200.4	11	bv	vv	bv	vv
200.4	12	bb	vv	bv	bv
200.5	1	vv	vv	vv	vv
200.5	2	vv	vv	vv	bv
200.5	3	vv	vv	bv	vv
200.5	4	bv	bv	vv	bv
200.5	5	bb	bv	bv	bv
200.5	6	bv	bv	vv	bv
200.5	7	vv	bv	bv	vv
200.5	8	vv	bv	vv	vv
200.6	1	vv	bv		vv
200.6	2	vv	bv	vv	vv
200.6	3	vv	bv	bv	vv
200.6	4	vv	vv	vv	bb
200.6	5	vv	vv	bv	vv
200.6	6	vv	vv	bv	vv
200.6	7	vv	bv	vv	bv
200.7	1	vv	bv	vv	bv
200.7	2	bv	bv	bv	bv
200.7	3	vv	bv	vv	vv
200.7	4	bb	bv	vv	bb
200.7	5	bb	bb	bb	bb
200.7	6	vv	bb	bb	bv
200.8	1	bb	vv	vv	vv
200.8	2	bb	bv	bv	vv
200.8	4	bb	bb	bb	bb
200.8	4	vv	bv	vv	bv
200.8	5		bb	bb	bb
200.8	7	vv	bv	vv	vv
200.8	8	vv	vv	bb	bv
200.8	9	vv	bb	bv	bb
200.8	10	vv	bv	vv	bv
200.9	1	vv	vv	bv	bv
200.9	2	vv	bb	vv	vv
200.9	3	vv	bb	bv	vv
200.9	4	vv	bv	vv	vv
200.9	5	vv	vv	bv	vv
200.9	6	bv	bv	vv	vv
200.10	1	vv	vv	bv	vv
200.10	2	bv	bb	bv	bb
200.10	3	vv	vv	vv	vv
200.10	4	vv	vv		bv
200.10	5	bv	vv		vv
244	1	bb	vv	bv	vv
244	2	bv	bv	bv	bv
244	3	vv	bv	vv	vv
244	4	vv	bv	vv	vv
244	5	vv	vv	bv	vv
244	6	bv	vv	bv	vv
245	1	bv	bb	bv	bb
245	2	vv	bv	vv	vv
245	3	vv	bv		vv
245	4	bb	bv	bb	bb
245	5	bv	vv		vv
245	6	bb	bb	bv	bv
245	8	vv	vv	vv	vv
246	1	bv	bv	bv	bv
246	2	bb	bb	bv	bb
246	3	vv	vv		bv
246	4	bv	bb	bb	bb
246	5	bb	bb	bb	bb
247	1	bv	bv	vv	vv
247	2	bv	bb	bb	bv
247	3	bv	vv	bv	bv
247	4	vv	vv	vv	vv
247	5	vv	vv		bv
247	6		bv	bv	bv
247	7	vv	bb	bv	bv
247	8	bv	bv	bv	bb
247	9	vv	bv	bv	
247	10	bv	bv	vv	bv
247	11	vv	vv	vv	vv
247	12	bb	bv	bv	bv
248	1	vv	vv	bv	bv
248	2	bb	vv	bv	bv
248	3	vv	vv	vv	vv
248	4	vv	vv	vv	bv
248	5	bv	vv		bv
248	6	vv	bv		vv

Sites	ind	7.4	24.11	12.19	24.12
248	7	vv	bv	vv	vv
248	8	bv	bb	bb	bb
248	9	bv	bv	bv	bv
248	10	vv	bv	bv	bv
249	1	bb	bb	bb	bb
249	2	bv	vv	bv	vv
249	3	vv	vv	vv	bv
249	4	bv	bv	bv	vv
249	5	bb	vv		bv
249	6	vv	vv		vv
249	7	bv	bv		bv
249	8	bv	bv	vv	bb
250	1	bb	bv	bv	bv
250	2	vv	vv		vv
250	3	vv	vv	vv	vv
250	4	bv	vv	vv	bv
250	5	bv		vv	bv
250	6	vv	bv		bv
250	7	vv	vv		vv
250	8	vv	vv	vv	bv
250	9	bv	bv		vv
250	10	vv	vv		bv
250	11	bv	vv	vv	bv
250	12	bv	bv	bb	bv
251	1	vv	bv	vv	vv
251	2	bb	bb	bv	bv
251	3	bv	bv	bv	bv
251	4	bv	vv	bv	vv
251	5	bb	bv	vv	bb
251	6	bb	bb	bb	bb
251	7	bb	bb	bb	bb
251	8	bv	bv	bb	bb
251	9	bb	bb	bv	bb
251	10	bv	bv	bv	vv
251	11	bv	vv	vv	bv
251	12	vv	bv	bv	vv
252	1	bv	bv	bv	bb
252	2	vv	bv	vv	vv
252	3	vv	bb	vv	vv
252	4	vv	vv	vv	vv
252	5	vv	vv	vv	bv
252	6	bv	vv	vv	vv
252	7	vv	bv	vv	vv
253	1	bv	bv	bv	bv
253	2	vv	bv	bb	bb
253	3	vv	vv		vv
253	4	bv	bv	vv	vv
253	5	bb	bb	bv	bv
253	6	bv	bv	bb	bb
253	7	vv	vv	bv	bv
253	8	vv	vv	bv	vv
254	1	bv	bv	vv	vv
254	2	vv	vv	bv	bv
255	1	vv	vv	vv	vv
256	5	vv	vv	bb	bv
256	6	vv	vv	vv	vv
256	7	vv	bb	vv	vv
256	12	bb	bv	bb	bv
256	13	vv	vv	vv	vv
256	18	vv	bv	vv	bv

Sites	ind	7.4	24.11	12.19	24.12
256	19	vv	bb	vv	vv
256	20	vv	vv	vv	vv
256	21	bv	bv	bb	bv
256	22	vv	bv	vv	bv
256	23	vv	vv	bv	vv
256	24	bv	vv	vv	vv
257	1	bb	bv	bv	bb
257	2	vv	bv	vv	bv
257	3	bb	bv	bb	bb
257	4	bv	bv	bb	bv
257	5	bv	bv	bv	bb
257	6	vv	bb	bv	bb
257	7	bv	vv	vv	bv
257	8	bv	vv	vv	bv
257	9	vv	vv	bv	vv
257	10	vv	bv	bv	vv
257	11	vv	vv	vv	vv
257	12	vv	bv	vv	bv
257	13	bv	bv	bv	bv
257	14	vv	vv		bv
257	15	bb	bb	bv	bv
257	16	bv	bv	vv	bv
257	17	bb	bv	vv	bv
257	18	vv	vv	vv	vv
257	19	bv	bv	vv	vv
257	20	vv	bv		bb
257	21	vv	bv	vv	bv
257	22	vv	bv	vv	bv
257	23	vv	vv	vv	vv
257	24	vv	vv	vv	vv
257	25	vv	vv	vv	bb
257	26	vv	vv	vv	vv
257	27	bv	bb	bv	bb
257	28	vv	vv		vv
258	1	vv	vv	vv	bv
258	3	bb	bb	bb	bb
258	4	bb	bb	bb	bb
258	5	bv	bb	bv	vv
258	6	bb	bb	bb	bb
258	7	bb	bb	bb	bb
258	8	vv	vv	vv	bv
258	9	vv	vv	bv	bv
258	10	bv	bv	bv	bv
258	11	vv	vv	vv	vv
258	12	vv	bv		bv
258	13	vv	vv	bv	vv
258	14	bv	vv	vv	bv
258	15	vv	vv	bv	bv
258	16	vv	vv	bb	vv
258	17	bv	vv	bv	bb
258	18	vv	bv	vv	bv
258	19	bb	bb	bb	bb
258	20	vv	bv		vv
258	21	bv	bb	bv	bv
258	22	bb	bv	bv	bv
258	23	vv	bv		vv
258	24	bv	bv	bv	vv
258	25	bv	bv	bv	bv
258	26	vv	bv	vv	vv
258	27	bv	bv	bb	bv

Sites	ind	7.4	24.11	12.19	24.12
258	28	bv	bb	bb	bb
258	29	vv	vv	vv	bv
258	30	vv	bv		vv
258	31	vv	vv	bv	vv
258	32	bb	bv	bb	bv
258	1	bv	bv		vv
258	2	bv	bv	bv	vv
258	3	bv	bv	bv	vv
258	4	vv	vv	bv	vv
258	5	vv	bv	bv	bv
258	6	bv	vv	bv	bv
258	7	bv	bv	bv	bv
258	8	vv	bv	vv	vv
258	9	bb	bv	bv	bv
258	11	vv	bb	vv	vv
258	12	bv	bb	bb	bb
258	13	vv	vv	bv	bb
259	1	vv	vv	vv	bv
259	2	vv	bv	bv	vv
259	3	vv	vv	bv	vv
259	4	bv	bv	bv	vv
259	5	bb	bv	bv	bv
259	6	bv	bv	bv	bv
259	7	vv	vv	vv	vv
259	8	vv	vv	vv	vv
259	9	bv	bv		bv
259	10	bb	vv	vv	vv
260	1	bb	bb	bb	bv
260	2	vv	vv	vv	bv
260	3	vv	bv	vv	bv
260	4	vv	vv	vv	vv
260	5	bv	vv	vv	vv
260	6	vv	bv	bv	bv
260	7	bv	bb		bv
260	8	vv	vv	vv	vv
260	9	vv	bv	vv	
260	10	bv	bv	vv	vv
260	11	vv	vv	vv	vv
260	12	vv	bv		vv
261	1	vv	bv	vv	vv
261	2	vv	bv	vv	vv
261	3	vv	vv	vv	vv
261	4	vv	bv	vv	vv
261	5	vv	vv	vv	vv
261	6	vv	vv	bb	vv
261	7	bv	vv	vv	vv
262	1	vv	bv	vv	vv
262	2	vv	vv	vv	bv
262	3	vv	vv	vv	bv
262	4	vv	vv	vv	vv
262	5	vv	vv	bv	bv
262	6	bv	vv	vv	bv
262	7	vv	bb		vv
262	8	vv	vv	bv	vv
262	9	vv	vv	vv	vv
262	10	vv	bb	vv	vv
262	11	bv	bv	bv	vv
262	12	bv	vv		vv
263	1	bv	bv		vv
263	2	bb	bv		vv

Sites	ind	7.4	24.11	12.19	24.12
263	3	vv	bv	vv	vv
263	6	vv	bv	vv	vv
263	8	bv	bv	vv	bv
263	10	bv	bv	vv	bv
263	11	vv	vv	bv	vv
263	12	vv	vv	vv	bv
264	1	bb	bv	vv	vv
264	2	vv	bv	bv	bv
264	3	vv	vv	vv	bv
264	4	bv	bv	vv	vv
264	5	vv	bv	bv	vv
264	6	vv	bv	bb	bv
264	7	vv	bv	vv	vv
264	8	bv	vv	vv	vv
264	9	vv	vv	vv	vv
264	10	vv	vv	bv	vv
264	11	bb	bv		bv
264	12	vv	vv	vv	vv
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265	2	vv	bv	vv	vv
265	3	vv	vv	vv	vv
265	4	vv	vv	bv	vv
265	5	vv	vv	vv	vv
265	6	vv	bv	vv	vv
265	7	vv	vv	vv	vv
265	8	vv	vv	vv	vv
265	9	vv	vv	vv	vv
265	10	vv	vv	vv	vv
265	11	vv	vv	vv	vv
265	12	vv	vv	vv	vv
266	1	vv	vv	bv	vv
266	2	vv	vv	bv	vv
266	3	vv	vv	bv	vv
266	4	vv	bv	vv	vv
266	5	vv	vv	vv	vv
266	6	vv	vv	vv	bv
266	7	vv	vv	vv	vv
266	8	vv	vv	vv	vv
266	9	bv	vv	vv	vv
266	10	vv	vv	vv	bv
266	11	vv	vv	vv	vv
267	1	vv	vv	vv	vv
267	2	bv	bv		vv
267	3	vv	vv	vv	vv
267	4	vv	bv	bv	vv
267	5	vv	vv	bb	vv
267	6	vv	vv	vv	vv
267	7	vv	bv	vv	bv
267	8	vv	bv	vv	vv
267	9	vv	vv	vv	vv
267	10	vv	vv	vv	vv
267	11	vv	vv		bv
267	12	vv	vv	vv	vv
268	1	vv	vv	vv	vv
268	2	vv	vv	vv	vv
268	3	vv	bv	vv	bb
268	4	vv	bv	bb	vv
268	5	vv	vv	vv	vv
268	6	bv	vv	vv	vv
268	7	vv	vv	vv	bv

Sites	ind	7.4	24.11	12.19	24.12
268	8	vv	vv	vv	vv
268	9	vv	vv	vv	vv
268	10	vv	vv	bb	vv
268	11	vv	vv	bb	bv
268	12	vv	bb		vv
270	1	vv	vv	vv	bv
270	2	vv	vv	vv	vv
270	3	vv	bv	vv	vv
270	4	bv	bv	vv	vv
270	5	bv	vv	vv	vv
270	6	vv	bv	bv	bv
270	7	bv	bv	vv	vv
270	8	vv	vv	vv	vv
270	9	vv	vv	bv	bv
270	10	vv	bb	vv	vv
270	11	vv	bv	vv	vv
270	12	vv	vv	vv	bv
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271	2	vv	vv	bv	bv
271	3	bv	bv	vv	vv
271	4	vv	vv	vv	vv
271	5	vv	bv	bv	vv
271	6	vv	vv	vv	vv
271	7	vv	bv	vv	bv
271	8	vv	vv	vv	vv
271	1	vv	vv	bv	vv
271	2	vv		vv	vv
271	3	bv	bv	vv	vv
271	4	vv	vv	vv	vv
272	1	bv	bv	vv	vv
272	2	vv	vv	vv	vv
272	3	vv	vv	vv	bv
272	4	vv	vv	vv	bv
272	5	bv	bv	bv	vv
272	6	vv	vv	vv	vv
272	8	vv	vv	vv	vv
272	9	vv	vv	bv	vv
272	10	bv	vv		bv
272	11	vv	vv	vv	vv
273	1	vv	bv	vv	vv
273	2	vv	vv	bv	vv
273	3	vv	vv	vv	bv
273	4	vv	vv	vv	vv
273	5	vv	vv	vv	vv
273	6	vv	vv	vv	bv
274	1	vv	vv	bv	vv
274	2	bv	bv		vv
274	3	vv	vv	vv	vv
274	4	vv	vv	vv	vv
274	5	vv	vv	vv	vv
274	6	vv	bv	vv	vv
274	7	vv	vv	vv	vv
274	8	vv	bv	vv	bv
274	9	vv	vv	bv	vv
274	10	vv	vv	vv	vv
274	11	vv	bb	vv	vv
274	12	vv	vv	bv	bv
275	1	vv	vv		vv
275	2	vv	vv	vv	vv
275	3	vv	bv	vv	vv
275	4	bv	bv	vv	vv
275	5	vv	vv	vv	vv
275	6	bv	bv	vv	vv
275	7	vv	vv	vv	vv
275	8	vv	vv	vv	vv
275	9	vv	bv	vv	vv
275	10	vv	vv	vv	vv
275	11	vv	vv	bv	vv
275	12	vv	vv	vv	vv
276	1	bv	bb	bv	vv
276	2	bv	bv	bv	bv
276	3	bv	vv	bb	bb
276	4	vv	vv	bv	vv
276	5	bv	bv	bb	bv
276	6	vv	vv	bv	bv
276	7	vv	vv	vv	bv
276	8	vv	bb	vv	bv
276	9	bv	bb	bb	bv
276	10	bv	bb	vv	bb
276	11	bv	vv	vv	bv
277	1	vv	vv	vv	bv
277	2	bb	bv	bv	bb
277	3	vv	bv	bv	bv
277	4	vv	vv	bv	vv
277	5	bv	bb	vv	vv
277	6	vv	vv	bv	vv
277	7		bv	vv	vv
279	1	bv	vv	vv	bv
279	3	vv	vv	vv	vv
279	4	vv	bv	bv	bv
279	5	vv	vv		bv
279	6	bv	vv		bv
280	1	bb	bv	bb	vv
280	2		vv	vv	vv
280	3	vv	vv		vv
280	4	bv	vv	vv	vv
280	5	vv	vv	vv	vv
280	6	vv	vv	vv	vv
280	7	vv	vv	vv	vv
280	8	vv	vv	bv	vv
280	9	vv	bv	vv	vv
280	10	bv	bv	bv	vv
280	11	vv	bv	vv	vv
281	1	bv	bv	bv	vv
281	2	vv	bb	bv	vv
281	3	vv		vv	vv
281	4	bv	bv		vv
281	5		vv	vv	vv
281	6	bv	vv	vv	vv
281	7	vv	vv	vv	vv
281	8		vv	vv	bv
281	9	bv	bv	vv	vv
281	10		bv	vv	vv
281	11	vv	vv	vv	vv
281	12	vv	bv	vv	vv
282	1	bb	bb	bb	bb
282	2	bb	bb	bb	bb
282	3	bb	bv	bv	vv
282	7	bv	bb	bv	vv
282	8	bb	bb	bb	bb

Sites	ind	7.4	24.11	12.19	24.12
282	9	bv	bv	bb	vv
282	10	vv	vv	bv	bv
282	11	bb	bv	bv	vv
282	12	bb	bb	bv	bv
282	1	bb	bb		bv
282	2	bb	bb	bv	bv
282	3	bb	bv	bv	bv
282	4	bb	bb	bv	bb
282	5	bv	vv	bv	bv
282	6	bv	bv	bv	bv
282	7	bb	vv	bv	bv
282	8	bb		bv	bv
282	9	bv	bv	bv	bv
282	10	bb	bv	bv	bv
282	11	bv	vv	bb	bv
282	12	bb	bb	bb	bv
283	1	bb	bv	vv	vv
283	2	bb	bv	vv	bb
283	3	bv	bb	vv	bv
283	4	bb	bv	vv	bb
283	5	vv	bv	bv	bv
283	6	bb	bv	vv	bv
283	7	bb	bv	bb	bv
284	1	bb	vv	vv	vv
284	2	bb	bb	bv	vv
284	3	bv	bv	vv	vv
284	4	vv	vv	vv	vv
284	5	bb	bv	vv	vv
284	6	vv	bb	vv	bb
284	7	vv	bv	bb	
285	1	vv	bb	vv	bv
285	2	bb	bv	vv	vv
285	3	bv	bv	vv	bv
285	4	bv	bv	bb	bb
285	5	bb	bv	vv	vv
285	6	vv	vv	bv	vv
285	7	bb	bv	vv	vv
285	8	vv	bv	vv	vv
285	9	vv	bv	vv	bv
285	10	bv	bv	vv	vv
285	11	vv	bv	vv	bv
285	12	vv	bv	vv	vv
285	13	bv	bv	vv	vv
285	14	vv	bv		bv
285	15	vv	vv	vv	bv
285	16	vv	bv	vv	vv
285	17	vv	bv	vv	bv
285	18	vv	bv	bv	vv
285	19	bv	vv	vv	vv
285	20	bv	vv	bv	
285	21	vv	bv	vv	vv
285	22	bv	vv	vv	vv
285	23	bb	vv	bv	bv
285	24	vv	bv	vv	vv
285	25	vv	bb	bv	bb
285	26	vv	vv	vv	vv
285	27	vv	vv	vv	vv
285	28	vv	bv	bb	bv
285	29	bb	bv	vv	bv
285	30	vv	bv	vv	vv

Sites	ind	7.4	24.11	12.19	24.12
285	31	bv	bv	bv	vv
285	32	vv	bv	bv	vv
285	33	vv	vv	bv	vv
285	34	bv	vv	vv	vv
286	1	bb	bv	bb	vv
286	2	bb	bb	vv	bv
286	3	bb	bv	vv	
286	4	vv	bb	vv	bv
286	5	vv	bv	vv	vv
286	6	bv	bv	vv	bv
286	7	vv	bb	vv	bv
286	8	vv	bv	vv	bv
286	9	bv	bv	vv	bv
286	10	bb	bb	bv	vv
286	11	vv	bv	vv	bb
286	12	vv	vv	bv	bv
286	13		bv	bv	vv
286	14	vv	vv	bv	bv
286	15	bb	bb	vv	vv
286	16	vv	bv	vv	vv
286	17	vv	bv	bv	bv
286	18	vv	bv	vv	bv
287	1	bv	bb	bv	vv
287	2	vv	bv	vv	bv
287	3	bv	vv	bv	vv
287	4	vv	bv	bv	bv
287	5	vv	bv	vv	vv
287	6	bb	bv	bv	bv
287	7	vv	bv	bb	bb
287	8	bv	bv	vv	bv
287	9	bv	bv	bv	vv
288	1	bv	vv	bv	bv
288	2	bv	vv	vv	vv
288	3	bv	vv	vv	vv
288	4	vv	vv	vv	bb
288	5	bv	vv	bb	vv
288	6	bv	vv	vv	bv
288	7	vv	bv	vv	bv
288	8	bv	vv	vv	vv
288	9	bv	bv	bb	vv
289	1	bv	bb	vv	bv
289	2	bb	bv	vv	bb
289	3	bv	bv	bv	vv
289	4	bv	vv	bv	bv
289	5	bv	bv	vv	vv
289	6	bv	bb	bv	bv
289	7	vv	bv	vv	bb
289	8	bb	bv	bv	vv
289	9	bv	vv	vv	vv
290	1	bv	vv	vv	bv
290	2	bb	vv	bv	bv
290	3	bv	bv	bb	bv
290	4	bb	bb	bb	bb
290	5	bb	bb	bv	vv
290	6	vv	vv	vv	bv
290	7	bb	bv	bv	vv
290	8	vv	bb	vv	bv
290	9	bv	bv	bv	vv
290	1	vv	bv	bv	bv
290	2	bb	vv	bb	vv

Sites	ind	7.4	24.11	12.19	24.12
290	3	bv	bb	bb	bb
290	4	vv	vv	vv	vv
290	5	bv	bb	bb	bv
290	6	vv		bv	vv
290	7	bv	bb	bb	vv
290	8	vv		bv	vv
290	9	bb	bb	bb	bv
290	10	vv	bv	bb	bv
290	11	bv	bv		bv
290	12	bv	vv	bb	vv
291	1	vv	bv	bv	bv
291	2	bv	bv	vv	bv
291	3	vv	bv	bv	vv
291	4	vv	bv	vv	vv
291	5	vv	bv	bv	vv
291	6	bv	vv	vv	vv
291	7	bv	bv	bv	vv
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291	9	bv	bv	vv	bb
292	1	bv	vv	bb	vv
292	2	vv	bv	bv	bv
292	3	bv	vv	bv	bv
292	5	vv	bv	bv	vv
292	6	bb	bb	bb	bb
292	7	bv	bv	vv	vv
292	8	vv	bb	bv	vv
292	9	vv	bb	bv	bv
292	10	bb	bb	bb	bb
292	11	vv	bv	bb	bv
292	12	vv	vv	bv	bv
292	13	bb	bb	bb	bb
292	14	bb	bb	bb	bb
292	15	bb	bb	bb	bb
293	1	bb	bb	bb	bb
293	2	bb	bb	bb	bb
293	3	bb	bb	bb	bb
293	4	bv	bb	bb	bb
293	5	bb	bb	bb	bb
293	7	bb	bb	bb	bb
293	8	vv	bb	bb	bb
293	9	bv	bb	bv	bb
293	10	bb	bb	bb	bb
293	11	bb	bb	bb	bb
293	12	bb	bb	bb	bb
293	13	bb	bb	bb	bb
293	14	bb	bb	bb	bv
293	15	bv	bb	bb	bb
294	1	bv	bv	bv	bv
294	2	bb	bv	bb	bb
294	3	bv	vv	bv	bv
294	4	vv	vv	bv	bv
294	5	bv	vv	vv	vv
295	1	bb	bv	bv	bv
295	2	bb	bv	bv	vv
295	3	bb	bb	bv	bv
295	4	vv	vv	bv	vv
295	5	bv	vv	vv	vv
295	6	bv	vv	bv	bv
295	7	bv	vv	vv	bv
295	8	vv	bb	vv	vv

Sites	ind	7.4	24.11	12.19	24.12
295	9	bv	vv	vv	vv
295	10	vv	vv	vv	bv
295	11	bb	vv	vv	vv
295	12	bb	vv	vv	vv
296	1	bv	vv	bb	vv
296	2	bb	vv	bv	vv
296	3	bb	vv	bv	bv
296	5	bb	vv	vv	vv
296	6	bv	bv	vv	bv
296	7	bv	bv	bb	vv
296	8	bv	vv	vv	vv
296	9	bv	vv	vv	bv
296	10	vv	vv	vv	vv
296	11	vv	vv	bb	bv
296	12	vv	vv	bv	bv
297	1	vv	bb	bv	vv
297	2	vv	vv	bv	vv
297	3	bb	vv	bb	vv
297	4	bb	vv	bb	vv
297	5	bb	bv	bv	bv
297	6	vv	vv	vv	bv
297	7	vv	vv	bv	vv
297	8	bv	vv	bv	bv
297	9	bv	bb	vv	bv
297	10	bb	bb	bb	bv
297	11	bv	vv	vv	bv
297	12	bv	bb	vv	vv
297	13	vv	bb	vv	bv
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298	2	bv	bv	vv	bv
298	3	vv	bv	bb	bv
298	4	bb	vv	vv	vv
298	5	bv	vv	vv	vv
298	6	bv	bb	vv	bv
298	7	bv	bv	bv	bv
298	8	bv	vv	bv	bv
298	9	vv	vv	vv	bv
298	10	vv	bb	vv	bv
298	11	vv	bv	bv	bv
298	12	vv	vv	vv	vv
298	13	bv	bv	vv	bb
298	14	bb	bb	bv	bv
298	15	bb	bv	vv	bv
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299	2	vv	bv	bb	bv
299	3	bv	bv	bv	bv
299	4	bv	bv	bv	vv
299	5	bv	vv	bv	bb
299	6	bv	bb	bv	bb
299	7	vv	bb	vv	vv
299	8	bv	bv	bv	bv
299	9	vv	bv	bv	vv
299	10	bb	bb	vv	bb
299	11	vv	bv	vv	bb
299	12	vv	bv	bv	bb
299	13	vv	vv	bb	bb
300	1	vv	vv	bv	vv
300	2	bv	bv	bv	bb
300	3	bv	vv	bv	bb
300	4	bv	vv	bv	bv

Sites	ind	7.4	24.11	12.19	24.12
300	5	vv	vv	vv	vv
300	6	vv	bv	bb	bv
300	7	bv	bv	bv	bv
300	8	vv	vv	bv	bv
300	9	vv	vv	vv	bv
300	10	vv	vv	bv	bb
300	11	bb	vv	vv	vv
300	12	vv	vv	vv	bb
300	13	vv	vv	bv	vv
300	14	bv	vv	vv	vv
300	15	bb	vv	bv	bv
301	1	bv	vv	vv	vv
301	2	bv	vv	vv	vv
301	3	vv	bv	bb	bv
301	4	bv	bb	bv	vv
301	5	vv	vv	vv	bv
301	6	bv	vv	vv	vv
301	7	bv	bv	bb	vv
301	8	bv	vv	bb	vv
301	9	bv	bv	bv	vv
301	10	bv	vv	bv	bv
301	11	bv	vv	bv	vv
301	12	bv	vv	bv	bv
301	13	vv	vv	vv	bv
301	14	vv	vv	bv	vv
301	15	bv	vv	bb	vv
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302	2	bv	bv	vv	vv
302	3	vv	vv	bv	vv
302	4	bv	bb	vv	bv
302	5	vv	bv	bv	bv
302	6	bv	vv	vv	bb
302	7	bv	bv	vv	bb
302	8	bv	bv	vv	bv
302	9	bv	bv	bv	bv
302	10	bv	bv	vv	bb
302	11	vv	bb	vv	vv
302	12	bb	bv	vv	bv
302	13	bb	bv	bv	vv
302	14	vv	vv	bb	bb
302	15	bb	vv	bb	vv
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303	2	vv	bv	vv	bv
303	3	vv	vv	vv	bv
303	4	vv	bv	bv	vv
303	5	bb	vv	vv	vv
303	6	vv	bb	bv	vv
303	7	vv	bv	bv	vv
303	8	vv	bb	vv	vv
303	9	vv	bv		vv
304	1	vv	bv	bv	vv
304	2	bv	bv		vv
304	3	vv	vv	bv	bv
304	4	vv	vv	bb	bv
304	5	bv	bv	bv	vv
304	6	vv	bb	bb	bb
304	7	vv	bv	bb	vv
304	8	vv	vv		bv
304	9	vv	bv	bb	vv
304	10	bv	bv	vv	bv

Sites	ind	7.4	24.11	12.19	24.12
305	1	vv	vv	vv	bv
305	2	bv	bv	bb	bv
305	3	bv	bv	bv	bv
305	4	vv	vv	bv	bv
305	5	vv	vv	bb	vv
305	6	vv	vv	bb	vv
305	7	bv	bv	vv	bv
305	8	vv	bv	vv	vv
305	9	bv	bv	vv	vv
306	1	vv	vv	vv	vv
306	2	vv	vv	vv	bv
306	3	bv	vv	vv	vv
306	4	vv	bv	bb	vv
306	5	vv	bv	vv	vv
306	6	vv	bv	bv	bv
306	7	vv	vv	vv	vv
306	8	bv	bb	bv	bv
306	9	bv	bv	vv	vv
306	10	vv	bb	vv	vv
306	11	vv	vv	vv	vv
306	12	bb	vv	vv	vv
307	1	bb	bb		vv
307	2	vv	bb	vv	bv
307	3	bb	bb	bb	bv
307	4	bb	bb	vv	bv
307	5	bv	bv	bv	bv
307	6	bb	bv	vv	vv
307	7	bv	vv	vv	bv
307	8	vv	bb	vv	vv
315	1	bb	bb	bv	bv
315	2	bv	bb	vv	bb
315	3		bv	vv	bv
315	4	vv	bb	vv	bb
315	5	bv	vv	vv	vv
315	6	vv	bv	vv	bb
315	7	vv	vv	vv	vv
315	8	vv		vv	vv
315	9	vv	bv	vv	vv
315	10	bb	bv	vv	bv
317	1	vv	bv	vv	vv
317	2	vv	vv	vv	bv
317	3	vv	vv	vv	vv
317	4	bb	vv	vv	bb
317	5	bv	vv	bv	bv
317	6	bv	vv	vv	vv
317	7	vv	vv	bv	vv
317	8	bv	vv	bv	vv
317	9	bv	vv	bb	bv
317	10	bv	vv	bv	vv
317	11	vv	vv	bb	vv
317	12	vv	vv	vv	vv
318	1	bv	vv	bv	vv
318	2	vv	bv	bv	vv
318	3	vv	vv		bv
318	4	bv	bv		bv
318	5	vv	vv	vv	vv
318	6	vv	vv	vv	vv
318	7	bv	vv	bv	vv
318	8	vv	vv	bv	vv
318	9	vv	vv	bv	vv

Sites	ind	7.4	24.11	12.19	24.12
318	10	bv	bv	bv	vv
318	11	vv	vv	vv	vv
321	1	vv	bb	bv	vv
321	2	bb	bb	vv	vv
321	3	bv		bb	bv
321	4	bb	bb	vv	bv
321	5	bv		bv	bv
321	6	vv	vv	bv	bv
321	7	bb	vv	bv	bv
321	8	vv	vv	bb	bv
321	9		vv	vv	vv
321	10	bb	vv	vv	bv
327	1	bv	vv	vv	vv
327	2	bv	bv	vv	vv
327	3	vv	vv	vv	vv
327	4	vv	vv	vv	bb
327	5	vv	bv	vv	bv
327	6	vv	vv	vv	vv
330	1	vv	bv	vv	bv
330	2	bv	vv	bb	vv
330	3	vv	bv	vv	vv
330	4	bb	bv	bb	bb
330	5			vv	bb
330	6	bv	vv	vv	bb
330	7	vv	bv	vv	bb
330	8	vv	vv	bv	bv
330	9	vv		vv	bv
330	10	vv	bb	bv	vv
330	11	bb	bv	bv	bb
330	12	bb	bv	vv	bv
333	1			vv	bv
333	2	vv	bb	bv	vv
333	3	bb	bb	vv	bv
333	4	bb	bb	bv	vv
333	5	bb	bb	bb	bb
333	6	bb	bb	bb	bb
333	7	bb	bb	bv	bb
334	1	bv	vv	bb	vv
334	2	vv	vv	bv	vv
334	3	vv	vv	bb	vv
334	4	vv	bv	bb	bv
334	5	vv	vv	bb	vv
334	6	bv	bv	bb	vv
334	7	vv	vv	bb	vv
334	8	bv	bb	bb	vv
334	9	vv	vv	bb	vv
334	10	vv	vv	bb	vv
335	1	bv	bb	bb	vv
335	2	bv	bv	vv	bb
335	3	bv	bv		vv
335	4	bv	bb	bv	bv
335	5	vv	bv	bv	bv
335	6	vv	bb	vv	vv
335	7	bv	bv	vv	bv
335	8	bv	bv	vv	bv
335	9	bv	bb	vv	bv
335	10		bv	vv	bv
342	1	bb	vv	bv	bv
342	2	vv	vv	bv	vv
342	3	vv		vv	vv

Sites	ind	7.4	24.11	12.19	24.12
342	4	bv	vv	bv	bv
342	5	vv		bv	vv
342	6	bv	vv	vv	vv
342	7	bv	vv	bv	bv
342	8	vv	vv	bv	bv
342	9	vv	vv	vv	bv
342	10	vv	bv	vv	vv
344	1	vv	vv	bv	bb
344	2	vv		vv	vv
344	3	bb	vv	vv	vv
344	4	vv	vv	bv	vv
344	5	bv	bb	bv	bv
344	6	bv	bv	vv	vv
344	7	vv	bv	vv	vv
344	8	bv		vv	vv
345	1	bb	bv	vv	vv
345	2	bv	bv	vv	vv
345	3	bv	vv	vv	vv
345	4	vv	bv	vv	vv
345	5			vv	bv
345	6	vv		bv	bv
345	8	bb	bv		bv
347	1	bv	bv	vv	bb
347	2	bv	bv	vv	bb
372	1	bv	bv	vv	vv
372	2	bb	bv	bv	vv
372	3	bv	bv	bb	vv
372	4	bv	vv	vv	vv
372	5	vv	bv	vv	vv
372	6	vv	vv	vv	bv
372	7	vv	vv	bv	vv
372	8	vv	vv	bv	vv
372	9	vv	vv	vv	vv
372	10	bv	bb	bv	bv
374	1	bv	bv	bv	bv
374	2	bv	bb	vv	bv
374	3	vv	vv	vv	vv
374	4	vv	vv	vv	vv
374	5	bb	vv	bv	vv
374	6	vv	vv	vv	vv
374	7	vv	vv	vv	vv
374	8	bv	vv	bv	bv
377	1	vv	bv	vv	bv
377	2	bv	bv	vv	bv
377	3		vv		vv
377	4	vv	vv		vv
377	5		bv	vv	vv
377	6	bv	bb	bv	vv
377	7	vv	vv	bb	vv
377	8	bv	bv		bv
379	1	bb	bv	bv	bv
379	2	vv	bv		vv
379	3	vv	vv	bb	vv
379	4	bv	vv	vv	vv
379	5	vv	vv		vv
379	7	bv	vv	bv	vv
379	8	bv	bb	bv	vv
379	9	bv	vv		bv
380	1	vv	vv	bv	bv
380	2	bv	vv	bb	bv

Sites	ind	7.4	24.11	12.19	24.12
380	3		vv	bb	vv
380	4	vv	vv	vv	vv
380	5	vv	vv	bv	bv
380	6	bv	bv	vv	vv
385	1	vv	vv	bb	bv
385	2	bv	vv	bb	bv
385	3	vv	vv		vv
385	4	vv	vv		bb
385	5	bb	bv	vv	bv
388	1	vv	vv	vv	vv
388	2	vv	bv	bv	vv
388	3	vv	vv	bv	vv
388	4	bv	vv	vv	bv
388	5			bv	bv
388	6				vv
389	1			bv	vv
389	2		vv		vv
389	3	vv	bv	bb	vv
389	4	vv		bb	vv
389	5	bb	bv	bb	bv
389	6	bb	bv		bb
389	7	vv	vv	bb	vv
396	1		bb	bv	
396	2	vv	vv	bv	vv
396	3	vv	vv	vv	vv
396	4	bb	vv	vv	bv
396	5	bv	bv		bv
396	6	bv		vv	bv
396	7	bv		bv	bv
396	8	bv	vv	bv	bv
397	1	vv	vv	vv	vv
397	2	bv	vv	bv	vv
397	3	vv	bv	bb	vv
397	4	bv	bv	bb	vv
397	5	bv	bb	bb	bb
397	6	vv	bv	bb	bv
397	7	vv	vv	bv	vv
397	8	vv	bv	bb	bv

Appendix 4.1

The egg genotypes per site ('Si'), batch ('Ba'), individual ('ind') and locus. For *Bb7.4*, *Bv24.11* and *Bv12.19*, the 'v' and 'x' alleles are both *B. variegata*, and the 'b' alleles are *B. bombina*. In *Bv24.12* the 'v', 'd', 'f' and 'n' alleles are assigned to *B. variegata* and 'b' to *B. bombina*, while 'a' is not assigned to either taxon.

Si	Ba	ind	7.4	24.11	12.19	24.12
20401		1	vx	vx	vv	vv
20401		2	vv	vx	vx	vv
20401		3	vx	xx	vx	vv
20401		4	vv	vx	vv	vv
20401		5	vx	xx	vv	vv
20401		6	vx	xx	vb	vv
20401		7	vx	xx	vx	vv
20401		8	vv	xx	vv	vv
20401		9	vv	vx	vb	vv
20401		10	vv	vx	vb	vv
20402		1	bb	vx	vx	vv
20402		2	vb	vx	xx	df
20402		3	bb	xx	xx	vd
20402		4	vb	xx	vv	vd
20402		5	vb	vx	vv	df
20402		6	vb	xx	vx	vf
20402		7	vb	xx	vx	vd
20402		8	vb	xx	vv	vf
20402		9	bb	xx	vv	vv
20402		10	vb	xx	vx	df
20402		11	vb	xx	vv	df
20402		12	vb	vx	xx	df
20402		13	bb	xx	vx	vv
20402		14	bb	vx	vx	vv
20402		15	vb	vx	vx	df
20402		16	vb	xx	vx	vv
20402		17	bb	xx	vx	vf
20402		18	vb	xx	vv	vf
20404		1	vb	xx	vv	vv
20404		2	vb	vx		vv
20404		3	bb	xx	vv	vd
20404		4	vv	vx	xx	vd
20404		6	vv	vx	xx	vd
20404		7	vb	vx	xx	vd
20404		9	vv	xb	vv	vd
20404		10	vv	xb	vv	vb
20404		11	bb	xx	vx	vb
20404		12	vv	xb	vv	vb
20404		13	bb	vx	vv	vd
20404		14	vb	xx	vv	vb
20404		15	vv	xx	vx	vb
20404		16	vv	xb	vv	vd
20405		1	vx	vx	vx	vv
20405		2	vx	vx	vx	vv
20405		3	vb	vb	vv	vv
20405		4	vv	vb	vx	vv
20405		5	xb	xb	xx	vv
20405		6	vx	xx	vx	vv
20405		7	vx	vx	xx	vv
20405		8	xb	xx	xx	vv
20405		9	vb	vx	vv	vv
20405		10	vx	vx	vv	vv
20405		11	vv	xb	xx	vv
20405		12	xb	vx	vv	vv
20405		13	vx	xb	vx	vv
20405		14	vb	xb	xx	vv
20405		15	vv		xx	vv
20406		1	vv			vv
20406		2	vx		vx	vv
20406		3	vx	xx	vx	vv
20406		4	vv	vv	vx	vv
20407		1	vv	xb	vx	vv
20407		2	vb	bb	vv	vv
20407		3	vb	xb	vx	vv
20407		5	vv	bb	vv	vv
20407		6	vv	vb	vx	vv
20407		7	vv	vb	vx	vv
20407		8	vv	bb	vv	vv
20407		9	vv	xb	vx	vv
20407		10	vb	xb	vx	vv
20408		1	vb	bb	vx	vb
20408		2	vx	xb	xx	vb
20408		3	vx	bb	vx	vb
20408		4	vb	xb	xx	vb
20408		5	vb	xb	xx	vv
20409		1	vv	vx	vx	vf
20409		2	vb	vx	vx	vf
20409		3	vv	xx	vx	vv
25601		3	vb	xb		
25601		4	vv	xb		
25601		5	vv	xb		
25601		6	vv	xb	vx	bf
25601		7	vb	xb	vx	bf
25601		8	vb	vx	vx	vv
25601		9	vb	vx	vv	vf
25601		10	vb	vx	vv	vv
25601		11	vv	xb	vx	vv
25601		12	vb	vx	vv	bf
25601		13	vb	xx	vv	vv
25602		1	vv	vx		
25602		2	vb	vx		
25602		3	vb	vx		
25602		4	vb	vv	vv	vb
25603		5	vv	xx	vv	vv
25603		8	vb	vx	vx	vv
25603		9	vb	vx	vx	vv
25603		10	vb	vx	vx	vv
25603		11	vb	vx	vx	vv
25602		12	vv	vx	vv	vv
25603		13	vb	vx	vv	vv
25604		1		xb		
25604		2		xb		
25604		3		xb		
25604		4	vv	vb	vv	vb
25604		5	vb	xx	vv	vb
25604		6	vv	vx	vv	vb
25604		7	vb	xx	vv	vf
25604		8	vv	xb	vx	vb
25604		9	vv	vx	vv	vf
25605		10	vb	xb	vx	vf
25604		11	vb	vx	vv	vb

Si Ba	ind	7.4	24.11	12.19	24.12
25604	12	vb	xx	vx	vf
25606	1		vb		
25606	2		vx		
25606	3		vb		
25606	5		vb		
25606	6		vx		
25606	7	vv	xb		
25606	8	vv	xb	vv	vv
25606	9	vv	vb	vb	vb
25606	10	vv	vb	vb	vb
25606	11	vv	vb	vv	vb
25606	12	vv	vb	vv	vb
25605	1	vv	xb	vv	vf
25604	2	vv	xb	vv	vf
25604	3	vv	vb	vv	vf
25604	4	vv	xb	vx	vv
25604	7	vv	xb	vx	vv
25604	8	vv	vb	vx	vf
25604	9	vv	xb	vx	vv
25604	10	vv	xb	vx	vf
25605	11	vv	vb	vx	vv
25606	1	vv	bb		vv
25606	2	vv	vb	vv	vb
25606	3	vv	vx	vv	vb
25606	4		xb		vb
25606	5	vv	vb	vv	
25606	6	vv	vb		vb
25606	7		bb		vb
25606	8	vv	xb	vv	vv
25606	10	vv	vb		vv
25701	1	vv	xx	vb	vf
25701	2	vb	xb	vb	df
25701	3	vb	xb	vv	vf
25701	4	vv	xx	vb	vv
25701	5	vb	xb	vv	vd
25701	6	vv	xx	vv	vv
25701	7	vv	xx	vv	vv
25701	8	vx	xb	vb	vf
25701	9	vx	xx	vb	vf
25701	10	vb	xb	vb	vf
25701	11	vv	xx	vv	vd
25701	12	vb	xx	vv	
25701	13	vb	xb	vb	vd
25701	14	vv	xb	vb	vv
25701	15	vv	xx	vv	vv
25702	1	vx	vv	vv	vb
25702	2	vx	vv	vb	bf
25702	3	vx	vv	vv	bb
25702	4	vx	vv	bb	bb
25702	5	vx	vv	vb	bb
25702	6	vx	vv	vb	vf
25702	7	vx	vv	vb	vb
25702	8	vx	vv	vv	bb
25702	9	vx	vv	vb	bb
25702	10	vx	vv	vb	bf
25702	11	vx	vv	bb	vf
25702	12	vx	vv	vb	bb
25702	13	vx	vv	vb	bb
25703	1	vb	vv		vb
25703	2	vb	vv	vv	vd

Si Ba	ind	7.4	24.11	12.19	24.12
25703	3	vb	vv	vv	bd
25704	1	vx	xb	vb	vb
25704	2	vv	xb	vb	vv
25704	3	vv	xx	vb	vv
25704	5	vv	xx		vb
25704	7	vx	xx	vb	vb
25704	8	vv	xb	vx	vb
25704	13	vv	xb	vx	vb
25704	14	vx	xb	vx	vv
25704	15	vx	xx	vx	vv
25704	17	vx	xb	vx	vv
25705	1	vb	xb	bb	vf
25705	2	vb	vx	bb	vf
25705	3	vv	xx	bb	vf
25705	4	vb	xx	vb	vf
25705	5	vv	vb	vv	vv
25705	6	vb	vb	vv	vv
25705	7	vv	vb	bb	vv
25706	1	vv	vb	xb	vv
25706	2	vv	vb	xx	bf
25706	3	vv	xb	xx	vf
25706	4	vv	xb	xb	vb
25706	5	vv	vb	xx	vv
25706	6	vv	vb	xx	vv
25706	7	vv	xb	xb	vb
25707	1	vv	vb	vx	vv
25707	2	vv	vx	vx	vv
25707	3	vv	vx	vx	vv
25707	4	vv	xb	vv	vv
25708	1	vb	vx	vb	vv
25708	2	bb	vx	vx	vv
25708	3	bb	vx	vx	vv
25708	4	bb	xb	vx	vv
25708	5	bb	vb	xb	vv
25708	6	vv	vb	vv	vv
25708	7	vb	xb	vv	vv
25708	8	vb	xb	vx	vv
25708	9	vb	vx	vv	vv
25708	10	vb	vx	vb	vv
25708	11	vb	xx		vv
25709	1	bb	xb	vx	vv
25709	2	vv	vx	vx	vv
25709	3	vv	vx	vx	vf
25709	4	vv	vb	vx	vv
25709	5	vv	vb	vx	vv
25709	6	vv	xx	vv	vv
25709	7	vv	xx	vv	vv
25709	8	vb	xb	vv	vv
25709	9	vv	vx	vx	vv
25709	10	vv	xx	vv	vf
25709	11	vv	vb	vx	vv
25710	1	xx	xx	vb	vf
25710	2	xx	xb	vb	vf
25710	3	xx	bb	vb	vv
25710	4	vx	xb	vb	vv
25710	5	vx	xb		vf
25710	6	xx	xb	vb	vf
25710	7	vx	xb	vb	vv
25710	8	vx	xb	vb	vf
25710	9	vv	xx	vb	vv

Si Ba	ind	7.4	24.11	12.19	24.12
25710	10	vx	xb	vb	vf
25710	11	vx	xb	vb	vv
25711	1	vv	vb	vb	vv
25711	2	vv	bb	vb	vv
25711	3	vv	bb	vb	vv
25711	4	vv	vb	vb	vv
25711	5	vv	vb	vb	vv
25711	6	vv	vb	vb	vv
25711	7	vv	vb	vb	vv
25711	8	vv	vv	vb	vv
25711	9	vv	vb	vb	vv
25711	10	vv	vb	vb	vv
25711	11	vv	vb	vb	vv
25712	1	vv	vb	vv	vv
25712	2	vv		vv	vv
25712	3	vv	vb	vv	vv
25712	4	vv	vx	vv	vv
25712	5	vb	xb	vv	vv
25712	6	vv	vv	vv	vv
25712	7	vv	vb	vv	vv
25712	8	vb	vb	vv	vv
25713	1	vb	xb	vv	bb
25713	2	vb	xb	vx	bb
25713	3	vb	xb	vx	vb
25713	4	vb	xb	xx	bb
25713	5	vv	xb	vv	vb
25713	6	vv	xx	vx	vb
25713	7	vb	bb	vx	bb
25713	9	vb	xx	vx	vb
25713	10	vv	bb	vx	bb
25713	11	vb	bb		vb
25714	1	vv	xb	vx	vb
25714	2	vv	vb	vx	vb
25714	3	vv	vb	xx	vv
25714	4	vv	xb	vv	vv
25714	5	vv	xb	vx	vv
25714	6	vv	vb	vv	vv
25714	7	vv	vb	vx	vb
25714	8	vv	vb	vv	vb
25714	9	vv	vb	xx	vv
25714	10	vv	xb	vx	vv
25714	11	vv	xb	vx	vv
25714	12	vv	vb	vx	vb
25714	13	vv	xb	vx	vb
25715	1	vx	vv	vb	vd
25715	2	vx	vv	vb	vv
25715	3	vx	vv	vb	vv
25715	4	vv	vv	vb	vd
25715	5	vx	vv	vb	vd
25715	6	vx	vv	vv	vv
25715	7	vv	vv	vb	vv
25715	8	vx	vv	vb	vv
25715	9	vx	vv	vv	vd
25715	10	vv	vv	vb	vv
25715	11	vx	vv	vv	vd
25715	12	vv	vv	vv	vd
25716	1	vb	vb	vv	vv
25716	2	vb	vb	vv	vb
25716	3	vb	vb	vb	vv
25716	4	vb	vb	vb	vb

Si Ba	ind	7.4	24.11	12.19	24.12
25716	5	vb	vb	vb	vb
25716	6	vb	vb	vb	vv
25716	7	vb	vb	vv	vb
25716	8	vb	vb	vb	vb
25716	9	vb	vb	vv	vv
25716	10	vb	vb	vb	vb
25716	11	vb	vb	bb	vb
25716	12	vb	vb	vb	vb
25716	13	vb	vb	vv	vb
25717	7	vv	vb	vx	vb
25717	8	vv	vb	vx	vb
25717	9	vv	vb	vx	vb
25718	1	vv	vx	vb	vb
25718	2	vv	vx	vb	vb
25718	3	vv	bb	bb	vb
25718	4	vb	vx	vv	bb
25718	5	vv	xb	vv	vb
25718	6	vv	xb	bb	vb
25718	7	vv	xb	vb	vb
25718	8	vv	vx	vb	vb
25718	9	vb	vb	vb	vb
25718	10	vv	vx	vb	vb
25718	11	vv	bb	vb	vb
25718	12	vb	vb	vb	vv
25718	13	vv	xb	vv	vb
25718	14	vv	xb	vb	vb
25719	1	vx	vx	vx	vf
25719	2	vv	bb	vx	bf
25719	3	vx	vb	vv	bf
25719	4	vv	vb	vx	vb
25719	5	vv	vb	vv	bf
25719	6	vx	xb	vv	bb
25719	8	vv	vx	vx	vf
25719	9	vv	xb	vv	bb
25719	10	vv	vx		
25720	1	vv	vb	vv	vf
25720	2	vx	vx	vb	vf
25720	3	vx	vb	vb	vv
25720	4	vx	xx	vb	vf
25720	5	vv	vb	vb	vv
25720	7	vx	vx	vb	vv
25720	8	vx	xb	vv	vv
25720	9	vv	xb	vv	vf
25720	10	vv	xx	vb	vf
25720	11	vx	xx	vv	vv
25720	12	vx	xb	vv	vf
25721	1	vv	xb	vx	vf
25721	2	vx	xx	vv	vf
25721	3	vx	vx	xx	vb
25721	4	vx	xx	vx	vb
25721	5	vv	xb	xx	vb
25721	6	vv	xx	vv	vb
25721	7	vv	xb	vx	vb
25721	8	vv	vb	vx	vv
25721	9	vv	vx	vx	vv
25721	10	vx	xx	xx	vf
25721	11	vx	vx	xx	vb
25721	12	vv	xx	xx	vf
25721	13	vv	vb	vv	vv
25722	1	vb	vb	bb	vb

Si Ba	ind	7.4	24.11	12.19	24.12
25722	2	vb	vb	vb	bb
25722	3	vb	vb	vb	vb
25722	4	vb	vb	vv	vb
25722	5	vb	vb	vb	vv
25722	6	vb	vb	vv	vb
25722	7	vb	vb	bb	vb
25722	8	vb	vb	vb	bb
25723	1	vv		vx	bb
25723	2	vv	xb	xx	bb
25723	3	vv	bb	vx	bb
25723	4	vv	xb	xx	vb
25723	5	vv	xb	xx	vb
25723	6	vv	xb	vx	vb
25723	7	vv	bb	xx	vb
25723	8	vv	bb	vx	vb
25723	9	vv	xb	vx	bb
25723	10	vv	xb	vx	vb
25723	11	vv	bb	vx	bb
25723	12	vv	xb	vx	vb
25723	13	vv	xb	xx	bb
25723	14	vv	xb	xx	vb
25723	15	vv	xb	vx	bb
25723	16	vv	xb		
25724	1	vv	xx	vv	vv
25724	2	vx	xx	vb	vv
25724	3	vv	vb	vv	vf
25724	4	vv	xb		
25724	5	vv	xx	vb	vv
25724	6	vx	xb	vv	vv
25724	8	vx	xx	vv	vf
25724	9	vv	vx	vv	vv
25724	10	vv	vx	vv	bb
25725	1	vv	xb	vb	bf
25725	2	vx	bb	vb	bf
25725	3	vx	bb	vb	bf
25725	4	vv	vx	vb	bf
25726	1	vb	vx	vv	bb
25726	2	vv	xb	vx	vv
25726	3	vb	xx	xx	vv
25726	4	vb	vx	vx	bb
25726	5	vb	vb	xx	vv
25726	6	vv	vb	vx	vv
25726	7	vb	vb	vv	vv
25726	9	vb	vb	vx	bb
25726	10	vv	vb	vv	vb
25726	11	vb	vb	xx	vb
25726	12	vb	vb	vx	vb
25801	1	vb	bb	vb	vb
25801	2	vv	bb	vb	vb
25801	3	bb	bb	vv	bd
25801	4	vb	bb	vb	bd
25801	5	vb	bb	vv	vb
25801	6				vv
25801	7	bb		vv	vd
25801	8	bb		vb	
25801	9	vb	bb	bb	vv
25802	1	bb	xb	vv	bd
25802	2	bb	xb	vb	vd
25802	3	bb	bb	vb	vb
25802	6	xb	vb		vb

Si Ba	ind	7.4	24.11	12.19	24.12
25802	7	xb	xb	vv	bb
25802	8	xb	bb	vb	vb
25802	9	xb	vx	vv	vb
25802	10	xb	vx		
25803	1	vv	xb	vb	vv
25803	2	vv	xb	vb	vb
25803	3	vv	xb	vb	bd
25803	4	vv	xx	bb	vb
25803	6	vv	vx	vv	vb
25803	7	vv	vx	vv	vv
25802	1	bb	vx	bb	bb
25802	2		vb	bb	
25802	3	vb	vb	bb	vb
25802	4	xb	vx	vv	vb
25802	5	xb	bb	vv	bb
25802	8	xb	vx		vv
25801	2	xb	bb	vv	vb
25801	3	vb	bb	vv	vb
27101	1	vv	vx	vx	vv
27101	2	vv	vx	vx	vv
27101	3	vv	vx	vx	vv
27101	4	vv	vx	vx	vf
27101	5	vv	vx	vb	vf
27101	6	vv	vx	vb	vv
27101	7	vv	vx	vv	vv
27101	8	vv	vx	vx	vv
27101	9	vv	vx	vx	vv
27101	10	vv	vx	vb	vf
27102	1	vv	vx	vx	vb
27102	2	vv	vx	vx	vb
27102	3	vv	xx	vx	vb
27102	4	vv	vx	vx	vb
27102	5	vv	vx	vx	bb
27102	6	vv	vx	vx	vb
27102	7	vv	xx	vx	vb
27102	8	vv	vx	vx	vb
27102	9	vv	xx	vx	vv
27102	10	vv	xx	vx	vv
27102	11	vv	vx	vx	vb
27103	1	vx	vx	vb	bb
27103	2	vx	xb	vb	vb
27103	3	vb	vx	vb	bb
27103	4	bb	xb	vb	bb
27103	5	vx	vx	vb	bb
27103	6	vx	vx	vb	bb
27103	7	vb	xb	vb	bb
27103	8	vb	xb	vb	bb
27201	1	vb	vb	vx	vv
27201	2	vb	vx	vx	vd
27201	3	vv	vx	vv	vd
27201	4	vv	vx	vx	vv
27201	5	vb	vx	vx	dd
27201	6	vb	vb	vx	dd
27201	7	vb	vb	vx	dd
27201	8	vv	vx	vv	vv
27201	9	vb	vx	vx	dd
27201	10	vb	vb	vx	vv
27201	11	vb	vx	vx	vd
27201	12	vb	vx	vv	vd
27201	13	vv	vb	vx	vd

Si Ba	ind	7.4	24.11	12.19	24.12
27202	1			vx	vv
27202	2				vv
27202	3	vv	vv	vv	vv
27202	4	vv	vv	vv	vv
27202	5	vv	vx	vv	vv
27202	6	vv	vx	vv	vb
27202	7	vv	vx	vv	vb
27202	8	vv	vv	vv	vv
27202	9	vv	vv	vv	vb
27202	10	vv		vv	vb
27202	11	vv	vv	vv	vv
27202	12	vv	vv	vv	vb
27202	13	vv	vx	vv	vv
27401	1	vb	vx		vb
27401	2	vv	vx	vv	vb
27401	3	vb			
27401	4	vv	vx		
27402	5	vv	vx	vb	
27401	6	vb	vx		
27401	7	vv	vv		
27401	8	vv	vb		vb
27401	9	vv	vb	vb	vb
27401	10	vb	vv	vb	vv
27403	1	vv	vb	vx	vv
27403	2	vv	vb	vx	vv
27403	3	vv	vb	xx	vv
27403	4	vv	vb	vv	vv
27403	5	vv	vb	vv	vv
27403	6	vv	vb	vv	vv
27403	7	vv	vb	vv	vv
27403	8	vv	vb	vv	vv
27403	9	vv	vb	vv	vv
27403	10	vv	vb		vv
27403	11	vv	vb	vv	vv
27403	12	vv	vb		vv
27403	13	vv	vb	vv	vv
27403	1	vv	vb	vv	vv
27403	2	vv	vb	vx	vv
27403	3	vv	vb	vx	vv
27403	4	vv	vb	vv	vv
27403	5	vv	vb		vv
27403	6	vv	vb		vv
27403	7	vv	vb	vx	vv
27403	8	vv	vb	vx	vv
27403	9	vv	vb	vx	vv
27401	1	vv	vv	vv	vb
27401	2	vb	vb	vv	vb
27401	3	vb	vv	vv	vb
27401	4	vv	vb	vv	vv
27401	5	vv	vb	vv	vv
27401	6	vv	vv	vb	vv
27402	7		vb	vb	vv
27401	8	vb	vv	vv	vb
27404	1	vv	vb	vb	vv
27404	2	vv	vb	vv	vv
27404	3	vv	vb	vv	vv
27404	4	vb	vb	vv	vb
27404	5	vb	vb	vv	vv
27404	6	vv	vb		vb
27405	3	vv	vx	vv	vv

Si Ba	ind	7.4	24.11	12.19	24.12
27405	5	vv	vv	vv	vv
27405	7	vv	vv	vv	vv
27405	8	vv	vv	vv	vv
27406	2	vv		vx	vv
27407	3	vv			vv
27407	5		vx	vv	vv
27407	6	vv	vx	vv	vv
27407	10	vv		vv	vv
27601	1	vb	bb	vx	vv
27601	2	vb		vx	bb
27601	3	vb	bb	vx	bb
27601	4	bb	bb	vv	bb
27601	5	vb	vb	vb	vb
27601	6	vb	vb	vv	bb
27601	7	vb	bb	vb	vb
27601	8	bb	bb	vx	vb
27602	9	vv	bb	vx	vb
27602	10	vv	bb	vx	vb
27601	11	vv	bb	vx	vb
27601	12	vv	vb	vx	bb
27602	13	vv	bb	vv	vb
27603	1	bb	bb		vd
27603	2	bb	bb	vb	vd
27603	3	bb	bb		vd
27603	4	bb	bb	vv	vd
27603	5	bb	bb	vv	vd
27603	6	bb	bb	vv	vd
27603	7	bb	bb		vd
27603	8	bb	bb		vd
27604	5	vv	bb		vv
27604	6	vv			vv
27605	7		vb		vv
27605	8	vb	vb		vv
27605	9	bb	vb		vv
27605	10	vb	vb		vb
27605	11				vb
27605	12		vb		vb
28201	1	vb	bb	vb	bb
28201	2	vb	xx	vb	vb
28201	3	vb	bb	vb	vb
28201	4	vb	xx	vb	bb
29001	1	vx	bb	vb	vv
29001	2	vb	bb	vb	vv
29001	3	xb	bb	vv	vv
29001	4	vx	xb	vv	vv
29001	5	vx	vv	vv	vv
29001	6	vx	vx	vv	vv
29001	7	vb	vx		
29001	8	vx	xb		vv
29001	9	vx	xb	vb	vv
29001	10	vb	bb	vv	vv
29001	11	xb	xb	vv	vv
29001	12	vv	bb	vv	vv
29001	13	xb	bb	vb	vv
29002	1	vb		vv	
29002	2	vb	xb	xx	vv
29002	3	vb	vx	xx	va
29003	4	vb	xx	vv	va
29002	5	vb	xx		vb
29002	6	vb	xb	vv	va

Si Ba	ind	7.4	24.11	12.19	24.12
29004	1	vx	xb	xx	bb
29004	1	vx	vb	vv	vv
29004	2	vv	vx	vv	vb
29004	3	vx	bb	vv	vb
29016	1	bb	vx	xx	vv
29004	2	vb	vb	vx	
29004	3	vv	bb	xx	vb
29016	4	vb	xb	xx	vv
29016	5	vb	vx		
29016	6	bb			
29016	7	vv			
29016	8	bb	vx		vb
29017	1	vx	vb	vv	vb
29017	2	vv	vx	vv	vv
29017	3	vv	vx	vv	vv
29017	4	vb	vv		
29017	5	vv	vv	vv	vd
29005	1	vb	vx	vv	vb
29005	2	vb	vx	vx	vb
29004	3	vb	vx		vb
29005	4		vx		vv
29005	5		vx		vv
29005	6	vb	vb	vv	
29005	7	vv	vb	vv	vd
29005	8	vb	vv	vv	
29005	9	vv	vv	vv	vd
29005	10	vb			
29005	11	vb	vb	vv	vd
29005	12	vb	vb	vv	
29005	13	vv	vx	vb	vd
29005	14	vv		vb	vb
29005	15	vb	vx	vv	vd
29005	16	vv	vx	vb	vb
29005	17	vb	vx	vb	vb
29005	18	vb	vv	vb	
29005	19	vb	vv	vv	vd
29005	20	vb	vv	vv	
29006	1		xb	vb	bb
29006	2		vx	vv	bb
29006	3	vb	vx	vv	vb
29006	4	vb	vv	vb	bb
29006	5	vb	vv	vb	
29006	6	vb	vx	vv	vv
29006	7	vv	vv	vv	bb
29006	8	vb	vb	vv	vb
29005	1	vv	vx	vv	vb
29005	2	vv	vx	vv	vb
29005	3	vb	vx	vv	vb
29005	4	vv	vb	vv	vb
29005	5	vv	vx	vv	
29005	6	vv	vx	vb	vd
29005	7	vb	vx	vb	vd
29005	8	vb	vx	vb	vv
29005	9	vb	vx	vb	vv
29005	10	vv	vx	vv	vv
29005	11	vv	vx		vb
29005	12	vv	vx	vv	vb
29006	13	vb	vb	vv	vb
29005	14	vb	vx	vv	vb
29007	1	bb	bb	xx	vb

Si Ba	ind	7.4	24.11	12.19	24.12
29007	2	bb	vb	vx	vb
29007	3	bb	vb	vx	vb
29007	4	bb	vb	vv	vb
29007	5	bb	vb	vv	bb
29007	6	bb	bb	vx	vb
29007	7	bb	vb	vx	bb
29007	8	bb	bb	vv	vd
29007	9	bb	bb	vx	bd
29008	1	vv	vb	vb	vd
29008	2	vv	xb	vb	vv
29008	3	vv	vv	vv	bd
29008	4	vv	xb	vv	vb
29008	5	vv	vb	vv	vv
29008	6	vv	xb	vv	vd
29008	7	vv	vx	vv	bd
29008	8	vv	vv	vb	vb
29008	9	vv	vv	vv	vd
29008	10	vv	vv	vv	vv
29008	11	vv	vb		
29005	1	vb	vx	vb	vb
29005	2	vb	vx	vb	vd
29005	3	vv	vx	vb	vb
29005	4	vv	vx		vb
29005	5	vv	vx	vv	vb
29005	6	vb	vx	vv	vd
29005	7	vb	vx	vv	vd
29005	8	vb	vx	vv	vd
29005	9	vv	vx	vv	vd
29010	1	vb	xb	xx	vb
29009	2	vv	xb	xx	vb
29009	3	vb	xb	xx	vb
29010	4	vv	xb		bb
29009	5	vv	bb	bb	vb
29010	6	vv	bb	bb	vb
29010	7	vb	xb	bb	vb
29009	8	vb	xb	bb	vb
29010	9	vv	bb	bb	bb
29009	10	vb	xb	bb	vb
29009	11	vb	xb	bb	vb
29010	12	vv	xb	bb	bb
29009	13	vv	xb	bb	bb
29009	14	vb	xb	bb	bb
29011	1	vv	vb	vv	vv
29011	2	vb	vb	vv	vv
29011	3		vb	vv	vf
29011	4	vv	vb		vv
29011	5	vb	vb		vv
29011	6	vv	vb		
29011	7	vb	vb	vv	vv
29011	8	vb	vb	vv	vf
29011	9	vb	vb	vv	vf
29011	10	vb	vb	vv	vv
29011	11	vv	vb	vv	vf
29012	1	vx	xb	vv	vb
29012	2	vv	bb	vv	vb
29012	3	vv	vb	vv	vb
29012	4	vv	vb	vv	vb
29012	5	vv	bb	vv	vd
29012	6	vx	xb	vv	vd
29012	7	vx	xb	vv	vb

Si Ba	ind	7.4	24.11	12.19	24.12
29012	8	vx	xb	vv	vb
29012	9	vv	bb	vv	vb
29012	10	vv	vb	vv	vb
29012	11	vv	bb	vv	vd
29012	12	vv	bb	vv	vd
29012	13	vv	bb	vv	vd
29013	1	vb	vx	vv	bf
29013	2	vb	bb	vv	bb
29013	3	vb	bb	vb	vv
29013	5	bb	vb	vv	vd
29013	6	bb	xb	vb	bd
29013	7	bb	bb	vb	vv
29013	8	bb	vb	vb	bd
29013	9	vb	xb	vb	vv
29013	10	vb	vb	vv	bd
29009	1	vb	bb		vb
29009	2	vb	bb		bb
29010	3	bb	xb		bb
29010	4	vb	xb	vx	bb
29009	5	vb	xb	xx	bb
29009	6	vv	xb	xx	bb
29010	7	vb	bb	xx	bb
29009	8	vb	bb	xx	vb
29009	9	bb	bb	xx	vb
29010	10	bb	xb	xx	vb
29014	1	vv	vb	vv	bb
29014	2	vv	bb	vv	bb
29014	3	vv	xb	vv	vb
29014	4	vv	vx	vv	vb
29014	5	vv	bb	vv	bb
29014	6	vv	vx	vv	bb
29014	7	vv	bb	vv	vb
29014	8	vv	vx	vv	vv
29014	9	vv	vb	vv	bb
29014	10	vv	xb	vv	bb
29015	1	vv	vx	vb	bd
29015	2	vv	vb	vb	vb
29015	3	vv	vv	vv	vv
29015	4	vv		vv	
29015	5	vv	xb	vv	vd
29015	6	vv	vb	vv	vb
29015	7	vv	vb	vv	vv
29015	8	vv	vx	vv	vv
31501	1	vb	vv	bb	vb
31501	2	vv	vx	vb	vv
31501	3	vv	vx	vb	vv
31502	4	vb	vx	vx	vv
31501	5	vb	vx	vb	vv
31501	6	vv	vv	vx	vv
31503	1	vv	vx	xx	bb
31503	3	vv	vx	xx	vb
31503	4		vx		
31503	6		vx	vv	
31503	7			vv	
31504	1	vv	xb	vv	vf
31504	2	vv	xb	vv	vv
31504	3	vv	bb	vv	vv
31505	1	vv	vx	bb	vb
31505	2	vx	vx	bb	vb
31505	3	vx	xx	bb	vb

Si Ba	ind	7.4	24.11	12.19	24.12
31505	4	vv	vx	vv	vv
31505	5	vv	vx	vv	vb
31505	6	vv	vx	vb	vb
31505	7	vx	vv	vb	vb
31505	8	vx	vv	vb	vv
31507	1	vv	xb	vb	vv
31507	2	vv	vx	vb	vv
31507	3	vv	vx	vb	vv
31507	4	vv	xx	vb	vv
31507	5	vv	xx	vb	vv
31506	1	vb	vx	vb	vv
31508	2	vv	vx	vb	vb
31506	3	vv	vx	vb	vb
31508	4	vv	vx	vb	vv
31508	5	vb	vx	vv	vv
31508	6	vv	xb	vb	vv
31508	7	vb	vx	vb	vv
31508	8	vb	vx	vb	vb
31508	9	vb		vv	
31701	1	vv	vx	vv	bn
31701	2	vv	vv	vv	bf
31701	3	vv	vv	vx	bn
31701	4	vv		vv	fn
31701	5	vv	vx	vv	bn
31701	6	vv	xb	vv	ff
31701	7	vv	vx	vv	bf
31701	8	vv	vv	vv	ff
31701	9	vv	vv	vv	ff
31701	10	vv	vv	vv	bn
31701	11	vv	vx	vx	fn
31701	1	vv	vb	vx	bn
31701	2	vv	vb	vv	fn
31701	3	vv	vb	vx	bn
31701	4	vv	vx	vx	bn
31701	5	vv	xb	vv	ff
31701	6	vv	vb	vv	bn
31701	7	vv	vv	vv	ff
31701	8	vv	xb	vx	ff
31701	9	vv	vb	vx	bn
31701	10	vv	vb	vv	fn
31701	11	vv	vx		ff
31702	1	vv	vx	vb	vf
31702	2	vv	vx	vb	vf
31702	3	vv	xb	vx	vf
31705	4	vv	xb	vv	vv
31702	5	vv	bb	vx	vf
31703	1	vv	xb		vv
31703	2	vv	vx		vv
31703	3	vv	xb	vv	vv
31703	4	vv	vx	vx	vv
31703	5	vv	xb	vv	vv
31703	6	vv	xb	vx	vv
31703	7	vv	vx	vv	vv
31703	8	vv	vx	vx	vv
31703	9	vv	xb	vx	vv
31703	10	vv	xb	vx	vv
31704	1	vx	vx	vb	bb
31704	2	vx	xb	vb	vb
31704	3	vb	vx	vb	bb
31704	4	bb	xb	vb	bb

Si Ba	ind	7.4	24.11	12.19	24.12
31704	5	vx	vx	vb	bb
31704	6	vx	vx	vb	bb
31704	7	vb	xb	vb	bb
31704	8	vb	xb	vb	bb
31803	1	vv	vx	vv	vd
31803	2	vv	vx	vx	vv
31803	3	vv	vb	vb	vv
31801	1	xb	vb	vx	vv
31801	2	vb	xb	vv	vv
31801	3	xb	vb	vx	vv
31801	4	vb	vb	vv	vv
31801	5	vb	vb	vx	vv
31801	6	vv	xb	vv	vv
31801	7	vb	vb	vx	vv
31801	8	xb	xb	vv	vv
31802	1	vb	xb	vb	bb
31802	2	vb	vv	vb	vb
31802	3	vb	xb	vb	vb
31802	4	vb	vx	vb	bb
31802	6	vb	vx		
31802	7	vb	vv		
31802	9	vb	vx		
31802	10	vb	vx	vb	bb
33001	1	bb	vx	vb	vb
33001	2	bb	vv	vb	vv
33002	3	bb	vb	vb	
33002	4	bb	vx	vb	vb
33002	5	bb	vx	vb	vb
33001	6	bb	vv	vb	vb
33001	7	bb	xb	vb	vv
33002	8	bb	vv	vb	vv
33001	9	bb	vv	vb	bb
33003	1	vb	vx		
33003	2	vb	vb		bb
33003	4	vx	vb	bb	
33003	6	vx	vb	bb	vf
33003	7	vx	vb	bb	vf
33004	1	vb	vx	bb	vb
33005	2	vb	vx	vb	vb
33004	3		vx	vb	vb
33004	1	vb	xx	vb	vv
33004	2	vb	xx	vb	vb
33004	3	vb	vx	vb	vv
33004	6	xb	vx	vv	bf
33004	7	xb	xx	bb	vv
33004	8	xb	vx	vb	bf
33004	9	xb	vx	vb	vb
33004	10	bb	xx	bb	vv
33003	1	vx	vx	vb	vf
33003	2	vv	vx	bb	vf
33003	3	vx	vb	vb	vb
33003	4	vv	vx	bb	bf
33003	5	vx	vx	vv	bb
33003	6	vv	vb	vv	bb
33003	7	vb	vx	vb	vb
33003	8	vv	vb	vv	bb
33003	9	vv	vb	vb	vf
33003	10	vx	vb	vb	bb
33003	11	vv	vx	bb	vb
33003	12	vx	vb	bb	bb

Si Ba	ind	7.4	24.11	12.19	24.12
33003	13	vv	vx	bb	vf
33006	1	vb	vx	vb	vv
33006	2	vb	vb	vb	vv
33007	3	vv	vx	vv	bb
33006	4	bb	vb	vb	vb
33009	1	vv	vx	vb	vf
33009	2	vx	vb	vb	bb
33009	3	vv	vx	vb	vf
33009	4	vx	vx		vf
33007	1	vb	vx		vv
33008	2	vv	vx		vv
33008	3	vv	xx	vv	
33008	4	bb	vx	vv	vb
33008	5		xx	vv	
33008	6		vx	vv	
33008	7	bb	vx	vv	
33008	8	vb	vx	vb	
33007	9	vb		vb	vv
33008	10	bb	xx	vv	vb
33008	11	vb	vx	vb	vb
33007	12	vb	vx	vv	vv
33008	13	bb	vx	vb	vv
33011	1	vb	vx	vb	vf
33011	2		xx	vb	vf
33011	3	vb	xx	vb	bf
33011	4	vb	xx	vb	vv
33010	1	vx	vb		vv
33010	2	vx	vx	vx	vb
33010	3	vv	vx		bb
33010	4	vv	vx	vv	bb
33010	5	vv	vx	xx	vb

Appendix 4.2

Summary of data for each egg family. 'N' is the number of eggs per family, and gives the mean *B. variegata* allele frequency per family. A family's segregation at each locus is given in curly brackets by the number of individuals that are homozygous for the *B. bombina* allele, heterozygous or homozygous for *B. variegata* alleles.

Site	Family	N	$\bar{p}$	Locus			
				Bb7.4	*Bv24.11*	*Bv12.19*	*Bv24.12*
204	1	10	0,963	{0,0,10}	{0,0,10}	{0,3,7}	{0,0,10}
204	2	18	0,834	{6,12,0}	{0,0,18}	{0,0,18}	{0,0,18}
204	3	14	0,831	{3,4,7}	{0,4,10}	{0,0,13}	{0,5,9}
204	4	15	0,897	{0,6,9}	{0,6,8}	{0,0,15}	{0,0,15}
204	5	4	1,000	{0,0,4}	{0,0,2}	{0,0,3}	{0,0,4}
204	6	9	0,792	{0,3,6}	{3,6,0}	{0,0,9}	{0,0,9}
204	7	5	0,650	{0,3,2}	{2,3,0}	{0,0,5}	{0,4,1}
256	1	11	0,810	{0,7,4}	{0,6,5}	{0,0,8}	{0,3,5}
256	2	5	0,888	{0,2,3}	{0,0,5}	{0,0,2}	{0,1,1}
256	3	6	0,896	{0,5,1}	{0,0,6}	{0,0,6}	{0,0,6}
256	4	18	0,842	{0,4,11}	{0,12,6}	{0,0,15}	{0,5,10}
256	5	20	0,842	{0,0,13}	{2,3,15}	{0,2,7}	{0,9,4}
257	1	11	0,773	{0,6,5}	{0,7,4}	{0,7,4}	{0,0,10}
257	2	13	0,712	{0,0,13}	{0,0,13}	{2,8,3}	{7,4,2}
257	3	10	0,807	{0,0,10}	{0,6,4}	{0,4,5}	{0,5,5}
257	4	7	0,697	{0,4,3}	{0,4,3}	{4,1,2}	{0,0,7}
257	5	7	0,768	{0,0,7}	{0,7,0}	{0,3,4}	{0,3,4}
257	6	4	0,938	{0,0,4}	{0,2,2}	{0,0,4}	{0,0,4}
257	7	11	0,747	{4,6,1}	{0,5,6}	{0,3,7}	{0,0,11}
257	8	11	0,909	{1,1,9}	{0,5,6}	{0,0,11}	{0,0,11}
257	9	11	0,762	{0,0,11}	{1,8,2}	{0,10,0}	{0,0,11}
257	10	11	0,739	{0,0,11}	{2,8,1}	{0,11,0}	{0,0,11}
257	11	8	0,880	{0,2,6}	{0,5,2}	{0,0,8}	{0,0,8}
257	12	10	0,588	{0,7,3}	{3,5,2}	{0,0,9}	{5,5,0}
257	13	13	0,818	{0,0,13}	{0,13,0}	{0,0,13}	{0,6,7}
257	14	12	0,917	{0,0,12}	{0,0,12}	{0,8,4}	{0,0,12}
257	15	13	0,577	{0,13,0}	{0,13,0}	{1,7,5}	{0,9,4}
257	17	14	0,632	{0,3,10}	{2,7,5}	{2,9,3}	{1,12,1}
257	18	9	0,778	{0,0,9}	{1,5,3}	{0,0,8}	{2,4,2}
257	19	11	0,870	{0,0,11}	{0,6,5}	{0,6,5}	{0,0,11}
257	20	13	0,894	{0,0,13}	{0,5,8}	{0,0,13}	{0,6,7}
257	21	8	0,485	{0,8,0}	{0,8,0}	{2,4,2}	{2,5,1}
257	22	16	0,659	{0,0,15}	{4,11,0}	{0,0,14}	{7,8,0}
257	23	9	0,896	{0,0,9}	{0,3,6}	{0,2,6}	{1,0,7}
257	24	4	0,594	{0,0,4}	{2,1,1}	{0,4,0}	{0,4,0}
257	25	11	0,716	{0,8,3}	{0,8,3}	{0,0,11}	{3,3,5}
257	26	4	0,969	{0,0,4}	{0,0,4}	{0,1,3}	{0,0,4}
258	1	11	0,438	{3,6,1}	{7,0,0}	{1,4,5}	{0,7,3}
258	2	13	0,487	{4,9,0}	{3,5,5}	{2,3,5}	{4,7,1}
258	3	6	0,750	{0,0,6}	{0,3,3}	{1,3,2}	{0,4,2}
271	1	10	0,963	{0,0,10}	{0,0,10}	{0,3,7}	{0,0,10}
271	2	11	0,887	{0,0,11}	{0,0,11}	{0,0,11}	{1,8,2}
271	3	8	0,500	{1,3,4}	{0,4,4}	{0,8,0}	{7,1,0}
272	1	13	0,866	{0,9,4}	{0,5,8}	{0,0,13}	{0,0,13}
272	2	13	0,952	{0,0,11}	{0,0,10}	{0,0,12}	{0,5,8}

Site	Family	N	$\bar{p}$	Locus			
				Bb7.4	*Bv24.11*	*Bv12.19*	*Bv24.12*
274	1	16	0,783	{0,7,9}	{0,5,10}	{0,3,7}	{0,8,4}
274	2	22	0,875	{0,0,22}	{0,22,0}	{0,0,18}	{0,0,22}
274	3	6	0,767	{0,2,4}	{0,6,0}	{0,1,4}	{0,2,4}
274	4	4	1,000	{0,0,4}	{0,0,4}	{0,0,4}	{0,0,4}
274	5	4	1,000	{0,0,3}	{0,0,2}	{0,0,3}	{0,0,4}
276	1	10	0,467	{2,6,2}	{6,3,0}	{0,2,8}	{5,4,1}
276	2	8	0,468	{8,0,0}	{8,0,0}	{0,1,3}	{0,0,8}
276	3	6	0,438	{1,2,0}	{0,5,0}	-	{0,3,3}
282	1	4	0,438	{0,4,0}	{2,0,2}	{0,4,0}	{2,2,0}
290	1	13	0,743	{0,6,7}	{6,4,3}	{0,4,7}	{0,0,12}
290	2	5	0,750	{0,5,0}	{0,2,2}	{0,0,4}	{0,1,1}
290	3	7	0,715	{0,2,5}	{2,3,2}	{0,0,5}	{1,4,1}
290	4	41	0,813	{0,22,17}	{0,5,34}	{0,12,24}	{0,16,18}
290	5	9	0,657	{0,6,1}	{0,3,6}	{0,3,6}	{4,3,2}
290	6	9	0,431	{9,0,0}	{4,5,0}	{0,0,9}	{2,6,1}
290	7	11	0,845	{0,0,11}	{0,6,5}	{0,3,7}	{0,4,6}
290	8	14	0,438	{1,9,4}	{5,9,0}	{6,0,6}	{5,9,0}
290	9	10	0,413	{2,4,4}	{3,7,0}	{4,0,4}	{6,4,0}
290	10	11	0,800	{0,6,4}	{0,11,0}	{0,0,8}	{0,0,10}
290	11	13	0,741	{0,0,13}	{6,7,0}	{0,0,13}	{0,8,5}
290	12	9	0,528	{4,5,0}	{3,5,1}	{0,5,4}	{0,5,4}
290	13	10	0,604	{0,0,10}	{3,4,3}	{0,0,10}	{6,3,1}
290	14	8	0,844	{0,0,8}	{0,4,3}	{0,2,6}	{0,3,4}
290	15	6	0,761	{3,2,1}	{0,1,3}	{0,0,2}	{0,1,2}
290	16	5	0,919	{0,1,4}	{0,1,4}	{0,0,5}	{0,1,3}
315	1	5	0,800	{0,2,3}	{0,0,5}	{1,3,1}	{0,1,4}
315	2	5	0,813	{0,0,2}	{0,0,4}	{0,0,4}	{1,1,0}
315	3	8	0,766	{0,0,8}	{0,0,8}	{3,3,2}	{0,6,2}
315	4	5	0,850	{0,0,5}	{0,1,4}	{0,5,0}	{0,0,5}
315	5	7	0,777	{0,4,3}	{0,1,5}	{0,5,2}	{0,2,4}
317	1	22	0,884	{0,0,22}	{0,9,12}	{0,0,21}	{0,11,11}
317	2	4	0,884	{0,0,4}	{1,1,2}	{0,2,2}	{0,0,4}
317	3	10	0,925	{0,0,10}	{0,6,4}	{0,0,8}	{0,0,10}
317	4	8	0,500	{1,3,4}	{0,4,4}	{0,8,0}	{7,1,0}
318	2	8	0,765	{0,7,1}	{0,8,0}	{0,0,8}	{0,0,8}
318	3	8	0,519	{0,8,0}	{0,2,6}	{0,5,0}	{3,2,0}
330	1	5	0,500	{5,0,0}	{0,1,4	{0,5,0}	{1,2,2}
330	2	4	0,511	{4,0,0}	{0,1,3}	{0,4,0}	{0,2,1}
330	3	18	0,621	{0,3,15}	{0,10,8}	{8,5,3}	{6,4,6}
330	4	10	0,636	{1,8,0}	{0,0,10}	{3,6,1}	{0,6,4}
330	5	4	0,802	{0,3,1}	{0,0,3}	{0,1,2}	{1,0,3}
330	6	7	0,720	{3,2,2}	{0,0,7}	{0,3,3}	{0,3,2}
330	7	4	0,657	{0,4,0}	{0,1,3}	{0,3,0}	{1,0,3}
330	8	5	0,825	{0,0,5}	{0,1,4}	{0,0,3}	{2,2,1}
330	9	4	0,844	{0,3,0}	{0,0,4}	{0,0,4}	{0,1,3}

Appendix 5.1

The tadpole genotypes per site ('Sites'), individual ('ind') and locus. For *Bb7.4*, *Bv24.11* and *Bv12.19*, the 'v' and 'x' alleles are both *B. variegata*, and the 'b' alleles are *B. bombina*. In *Bv24.12* the 'v', 'd', 'f' and 'n' alleles are assigned to *B. variegata* and 'b' to *B. bombina*, while 'a' is not assigned to either taxon.

Sites	ind	7.4	24.11	12.19	24.12
204	1	vv	bb	vb	vv
204	2	vb	vx	vv	vv
204	3	vv	vx	bb	vv
204	4	vb	vb	vv	vv
204	5	xx	vb	vv	vb
204	6	vx	vx	vv	vb
204	7	vb	vb	vv	vv
204	8	vv	vb	vv	vv
204	9	vv	bb	vx	vb
204	10	vv	vx	vv	vb
256	1	vv	vx	vb	vb
256	2	vv	bb	bb	vb
256	3	vv	bb	vb	vd
256	4	vv	xb	vv	vd
256	5	vv	xb	vb	vb
256	6	vv	xb	vv	vb
256	7	vv	vx	xb	vv
256	8	vv	vb		vv
256	9	vv	bb	vb	vb
256	10	vv	vb	vb	vd
256	11	vv	xb		vb
256	12	vv	xb	vv	vb
256	13	vv	xb	vb	vv
256	14	vv	bb	bb	vv
256	15	vv	vb	bb	vv
256	16	vv	xx	vv	vd
256	17	vv	vb	vb	vv
256	18	vv	vx	vv	vb
256	19	vv	vx	vb	vb
256	20	vv	bb	vx	vv
256	21	vv	bb	vv	vb
256	22	vv	vx		vb
256	23	vb	vx	vv	vb
257	1	vx	bb	vv	bb
257	2	vv	vx	vv	bb
257	3	vv	vx	vv	vf
257	4	vb	xb	vb	vb
257	5	vv	vb	xx	vb
257	6				vd
257	7	vv	vx		vv
257	8				vv
257	9	vb	xb		
257	10	vv	vx		vf
257	12	vb	vb		vb
257	13	vv			
257	14	vv	vb	vv	vf
257	15	vv		vb	
257	16	vv	vb	vx	vv
257	17	vv	vb	xx	vf
257	18	vv	vb	vx	vv
257	19	vv	vx	vv	vd
257	20	vv			
257	21	vv	vx	xx	
257	22	vv			
257	23	vv			
257	24	vv	bb	vx	vb
257	25	vb	vv	vb	
257	26	vx	vb	vb	vf
257	27	vv	vb	vv	vv
257	28	vv	vx	vv	vf
257	29	vv	xb	vv	vb
257	30	vv	vb	vb	vb
257	31	vv	xb	xx	vf
257	32	vb	vv	vv	vb
257	33	vv	xb	vv	vv
257	34	vb	vb	xx	vb
257	35	vv	xb	vx	bb
257	36	vv	xx	vx	vv
257	37	vb	vx		
257	38	vv			
257	39	bb	vv	vv	vb
257	40	vv	xb		
257	41	vv	xb	vx	bb
257	42	vb	vv		
257	43	vv	xb	vb	
258	1	vv	vx		vd
258	2	vx	bb		vd
258	3	xb		vv	vb
258	4		bb	vv	
258	5	vb	bb		vb
258	6	vb	vx	bb	vf
258	7	vx	xx	vv	vv
258	8	vx	xx	vv	vv
258	9	vb	bb	bb	vf
258	10	bb	xb		vb
258	11		bb		
258	12	vb	xb		bb
258	13	vv		vx	vf
258	14	vv	xb		vf
258	15	vv	bb		vb
258	16	vb	bb	bb	vf
258	17	vb	bb	vb	vb
258	19		bb	vb	vb
258	20		vb	vb	vb
258	21	vb	bb	vb	vb
258	22	vb	bb	vx	vv
258	23	vv	vx	vx	
258	25	vb	vb	vv	bf
258	27	bb	vb	vv	vb
258	28	vv	vx		vv
258	29	vv	bb	vx	bb
258	31	bb	xx	bb	
258	34	vb		xx	
258	35	vv	bb	vx	bd
258	36		vx	vx	vv
258	37		xx	vx	vv
258	39	vb	xb	xx	vb
258	40	bb	xb	xx	vb
258	41	vb	xb	vx	vd
258	42	vb	xb	vv	vb
258	43	vb	vb	vb	vb

Sites	ind	7.4	24.11	12.19	24.12
258	44	vv	xb	vx	vv
258	45	vb	xb	xx	bb
258	46	vb	xb	xx	bf
258	47	vb	vx	vb	vd
258	48	vv	xb	vv	vb
258	49	bb	bb	bb	bb
271	1	vv	vx	xb	vv
271	2	vv	vx	vx	vv
271	3	vv	xx	vx	vb
271	4	vv	vx	vb	vf
271	5	vv	vx	vx	vv
271	6	vv	vx	vv	vf
271	7	vv	vx	vv	vv
271	8	vv	vx	vb	vv
271	9	vb	vx	vv	bb
271	10	vv	vx	vb	vf
271	11	vv	vx	vb	vv
271	12	vb	vx	vb	bb
272	1	vb			
272	2	vb	vv	vv	
272	3	vb			
272	5	vb	vv	vv	vv
272	6	vv	vx		
272	7	vv			
272	10	vb	vb	vv	vv
272	11	vb	vx		
272	12	vb	vx	vb	bb
272	14	vv			
272	15	vb			
274	1	vv	vx	vv	vv
274	3	bb	xb	vv	vv
274	4	vv	vx		
274	5		vx		
274	7	vb	vx		
274	9	xb	vb	vv	vb
274	10	vx	vx	vv	vb
274	11	vx	vx	vv	vb
274	12	vx	vb	vv	vb
274	13	vv	vx	vv	vv
274	14	vv		vb	vv
274	16	vv	vx		
274	17	vx			
274	18	vv	vx		vv
274	19	bb	xb		vv
274	20	vb	xb		vv
274	21	bb	xb	vb	vv
274	22	vv	vx	vv	vv
274	31	vb	vx		vv
274	33	vb			vv
274	37		xx		bb
274	38	vv	xx	vb	vv
274	1	vx	vb	vb	vv
274	2	vx	vx	vb	vv
274	3	vv	vb	vb	vv
274	4	vv	xb	vx	vf
274	5	xb	xb	vv	vv
274	6	vv	xb	vb	vf
274	7	vv	vx		vv
274	8	vv	vb	vx	vf
274	9	vx	vx	vb	vb

Sites	ind	7.4	24.11	12.19	24.12
274	10	vx	vx		vv
274	11	vv	xb	vx	vv
274	12	vv	xb	xx	vf
274	13	vv	vx	vb	vv
274	14	vv	vb	vb	vv
274	15				vv
274	16				bb
274	22	vv			
274	23	vv			
274	24	vv			
274	29	vx	vb	vb	vv
274	30				vv
274	31	vv	vx		vf
274	32				vv
274	34	vv	vx		
274	35	vv	vx		
274	36	vv	vx	xx	vv
274	37	vx	vx		vf
274	38	vv	vx		
276	1	vv	xx	vv	vv
276	2	vb	vb	vv	vv
276	3	vb	xb	vv	vv
276	4	vb	xb	vv	bb
276	5	bb	vb	vv	vv
276	6	vv	vb	vv	vv
276	7	vb	vb	vb	vv
276	8	vv	vb	vb	vv
276	9	xx	vb	vb	vb
276	10	bb	vx	vb	vv
276	11	vb	vb	vb	vv
276	12	vv	vb		
276	13	vb	bb	vv	bb
276	14	vv	bb	vv	bb
276	15	bb	vx	vb	bb
276	16	vb	xb		bb
276	17	vb	xb	vv	vd
276	18	vb	vb		vv
276	19	bb	vb	vb	vv
276	20	vb	vx	bb	vv
276	21	vv	xb	vv	vb
276	22	vb	vb		vb
276	23	bb	xx	bb	vv
276	24	vb	xx	vb	bb
276	25	vv	vb	vv	vb
276	26	vb	vb	bb	vv
276	27	vv	vb	bb	bb
276	28	vv	xb	vv	vv
276	29	vv	xb	vv	vb
276	30	vb	vx	vb	bb
276	31	vv	vv	vb	bb
276	32	vv	bb	vv	bb
276	33	vb	xb	vv	vd
276	34	vv	vx	vv	vd
276	35	vv	vb	vv	bb
282	2	vb	vx	bb	vb
282	3	vb		bb	
282	4	vv	xb	vv	vb
282	6	vb	bb	vv	ba
282	7	vb	xb	vv	bb
282	8	bb	bb	vb	bb

Sites	ind	7.4	24.11	12.19	24.12
282	9	vb	vv	bb	vb
282	10	vb	bb	bb	bb
282	13	vv	vv	bb	vv
282	14	bb	xx	vb	bb
282	15	bb	bb	vv	bb
282	23	bb	bb	vv	bb
290	1	vv	bb	bb	vb
290	2	xx	bb	bb	vd
290	3	xx	vx	vx	bb
290	17	vb	vx	vv	vv
290	18	vb	xb		vv
290	19	vv			
290	20	vb	vx		
290	33	vv	xb	vb	vb
290	34	vv	vb	vv	vb
290	36	xx	xb		vv
290	48	vv	vb	vx	
290	50	vv	vv	vv	vv
315	1	vv	vx	vv	vv
315	2	vb	xb	bb	vb
315	3	bb	bb	bb	bb
315	4	vb	xb	bb	vb
315	5	vb	vb		vb
315	6	vv	vx	vb	vd
315	7	vv	vx		
315	8	vb	vx	vb	bf
315	9	vb	vb	vv	vb
315	10	vv	xx		vv
315	11				vf
315	12	vv	vx		vd
315	13	bb	bb	bb	
315	14	bb	bb	vv	vb
317	1	vv	vx	vb	vv
317	2	vv	vx	vx	vv
317	3	vb	vb	vb	vv
317	4	vv	xb	vx	vv
317	5	vv	xb	vv	vv
317	6	vv	vx	vv	vv
317	7	vv	vx	vv	vv
317	8	vv	vx	vv	vv
317	9	vv	xb	vx	vv
317	10	vv	xb	vv	vv
317	11	vv	xb	vv	vv
317	12	vv	vx	vv	vv
317	13	vv	xb	vv	vv
317	14	vv	xb	vv	vv
317	15	vv	vx	vx	vv
317	16	vv	xb	vb	vv
317	17	vv	xb	vv	vv
317	18	vv	vx	vx	vv
317	19	vv	xb	vv	vv
317	20	vv	vx	vv	vv
317	21	vv	xb	vb	vv
317	22	vv	xb	vb	vv
317	23	vv	vb	vb	vv
317	24	vv	xb	vx	vv
317	25	vv	vb	vx	vv
317	26	vv	xb	vb	vv
317	27	vv	xb	vv	vv
317	28	vv	xb	vx	vv

Sites	ind	7.4	24.11	12.19	24.12
317	29	vv	xb	vx	vv
317	30	vv	vx	vv	vv
317	31	vv	vv	xx	vv
317	32	vv	xb	xx	vv
317	33	vv	xb	vx	vv
317	34	vv	xb	vb	vv
317	35	vv	vx	vx	vv
317	36	vv	xb	vx	vv
317	37	vv	vx	vv	vv
317	38	vv	vx	vx	vv
317	39	vv	xb	vx	vv
317	40	vv	xb	vv	vv
317	41	vv	vx	vb	vv
317	42	bb	vx	vv	vb
318	1	vb	xb	vb	vv
318	2	bb	xb	vb	vv
318	3	vb	vb	vv	vv
318	4	vb	xb	vv	vv
318	5	bb	xb	vv	vv
318	6	vb	vb	vv	vv
318	7	vb	vb	vb	vv
318	8	vb	vb	vb	vv
318	9	bb	xb		vv
318	10	bb	vx	vb	bb
318	11	bb	vx		vb
318	12	bb	vx	vb	bb
318	13	bb	xb	vb	bb
318	14	vb	vb		vb
318	15	bb	xb	vx	vb
318	16	bb	vx	vv	vb
318	17	bb	xb		vb
318	18	bb	xb		vb
318	19	vb	vb		bb
318	20	vv	vb		vv
318	21	vb	vx	vx	vb
318	22	bb	xb	vx	bb
318	23	vb	vx		vb
318	24	vb	xx		bb
318	25	vv	vx	vx	vv
318	26	bb	xb		bb
318	27	bb	xb		vb
318	29	vb	bb		vv
330	2	vv	vv	bb	vv
330	3	vb	vb	vb	vb
330	4	bb	vb	vb	vb
330	5	bb	xb	bb	vb
330	7	vb	vx		bd
330	8	vb	vb	vb	vb
330	9	vb	xx	bb	vv
330	10	vb	xb	bb	vb
330	12	bb	xx	vv	vb
330	13	vb	bb	bb	
330	14	bb	xx		vb
330	15	vb	vv	vv	vb
330	16	vv	vb	xx	vv
330	17	bb	xb	xx	vb
330	19	vv	vb	vv	vb
330	20	vv	vb	vv	vv
330	23	vv	bb	vb	vb
330	24	vv	xb	xx	bf

Sites	ind	7.4	24.11	12.19	24.12
330	25	vb	xb	vx	vb
330	26	vb	vx	vv	bb
330	27	vb	vb	vx	
330	28	bb	vb	vx	vv
330	29	vv	vx	vx	vv
330	30	vv	vb		vb
330	31	vb	vx	vv	vv
330	32	vv	xb	vx	vv
330	33	vx	xb	vb	vv
330	34	vv	xb	bb	vv
330	35	vx	bb	vb	vv
330	36	vv	bb	bb	vv
330	37	vb	vx		vv
330	38	vv	vx	vb	vb
330	39	vv	vx	bb	vv
330	40	xb	bb	vb	vv
330	41	bb	bb	vb	bb
330	43	vv	xx	vb	ba
330	44	bb	vx	vb	vb
330	45	vv	xx	vv	vv
330	46	bb	vx	vv	bd
330	47	vb	vx	vv	vb
330	48		vv	vv	vb
330	49	vv	vx		
330	50	vb	vv	vb	vv
330	51	bb	vx	vb	vb
330	52	vb	vx	vv	vv
330	53	vb	vx	vb	vv
330	54	bb	vv	vb	bd

Appendix 6.1

Morphological data for each tadpole. The 'stage' column refers to the developmental stage after Gosner (1960). The following columns give the morphological measurements and the *HI* refers to the mean *B. variegata* allele frequency across the four marker loci.

Sites	ind	stage	body height	body width	body length	tail height	tail muscle height	*HI*
258	1	28	5,13	5,80	7,58	4,68	1,56	1,00
258	2	28	5,35	6,02	8,47	4,24	1,56	0,50
258	3	28	5,80	7,14	8,70	6,02	1,56	0,67
258	4	27	4,24	5,13	7,36	3,79	1,56	0,50
258	5	27	4,91	5,58	7,58	5,13	1,34	0,33
258	6	28	5,13	4,01	7,81	5,58	1,56	0,50
258	7	28	5,35	6,02	7,81	5,35	1,56	1,00
258	8	28	5,35	6,47	8,47	5,58	1,56	1,00
258	9	28	5,80	6,47	8,70	6,02	1,56	0,17
258	10	27	4,46	5,35	7,81	5,13	1,56	0,33
258	11	27	5,35	6,24	8,47	5,13	1,34	0,00
258	12	28	4,24	5,13	7,36	4,24	1,34	0,33
258	13	28						1,00
258	14	30						0,75
258	15	27						0,50
258	16	27						0,17
258	17	27						0,38
258	18	30	6,24	7,36	10,5	6,02	2,01	
258	19	27	5,13	6,02	8,47	5,58	1,56	0,33
258	20	27	5,58	6,24	8,92	5,58	1,78	0,50
258	21	29	5,58	6,24	9,14	6,24	2,01	0,38
258	22	28	5,80	6,69	9,14	6,69	1,78	0,63
258	23	29	6,24	6,91	9,37	6,02	1,78	1,00
258	24	29	6,47	7,14	9,59	6,47	1,78	
258	25	27	6,02	6,69	8,92	6,47	1,78	0,67
258	26	27	5,58	5,13	8,03	4,46	1,56	
258	27	30	5,58	6,24	8,70	5,80	1,56	0,50
258	28	30	7,14	7,58	9,37	6,47	2,01	1,00
258	29	28	6,02	6,24	8,92	6,02	1,78	0,50
258	30	29	6,24	6,69	9,81	6,47	2,01	
258	31	27	6,24	5,80	8,03	5,13	1,78	0,33
258	32	28	6,02	6,47	9,37	5,80	2,01	
258	33	29	6,47	6,24	9,37	5,80	1,78	
258	34	27	5,80	6,47	8,70	6,02	2,01	0,75
258	35	27	6,02	6,47	9,14	6,47	2,01	0,67
258	36	28	6,02	6,47	9,59	6,02	1,78	1,00
258	37	28	6,02	6,47	9,37	6,24	1,78	1,00
258	38	26	4,91	6,02	8,70	4,46	1,12	
258	39	30	6,24	6,69	9,59	6,47	1,78	0,63
258	40	30	6,47	6,91	9,81	6,91	2,01	0,50
258	41	29	5,35	6,02	8,25	6,02	1,56	0,67
258	42	30	6,02	6,69	9,37	6,47	1,78	0,63
258	43	27	5,58	5,80	7,81	5,58	1,56	0,50
258	44	28	6,02	6,91	7,58	6,24	1,34	0,88
258	45	29	5,80	6,69	9,37	6,47	1,34	0,50
258	46	29	5,80	6,02	7,36	5,80	1,56	0,67
258	47	28	6,02	6,24	8,03	5,58	1,12	0,67
258	48	27	6,02	6,24	8,25	6,02	1,78	0,75
258	49	28	5,35	5,58	8,03	5,80	1,56	0,00
271	1	34	8,03	8,47	13,6	8,25	2,90	0,88
271	2	37	8,47	9,14	14,3	7,81	2,90	1,00
271	3	37	8,47	8,92	14,7	8,25	3,35	0,88

Sites	ind	stage	body height	body width	body length	tail height	tail muscle height	*HI*
271	4	32	6,24	6,69	10,3	6,47	2,01	0,83
271	5	31	6,02	6,91	9,59	5,80	2,01	1,00
271	6	31	5,80	6,47	10,3	5,58	1,78	1,00
271	7	32	6,02	6,69	10,0	6,24	2,01	1,00
271	8	32	5,80	6,69	10,3	6,24	2,23	0,88
271	9	32	5,58	6,24	9,59	5,80	2,01	0,63
271	10	31	5,35	6,24	9,37	5,58	2,01	0,83
271	11	31	5,13	5,80	9,37	5,58	1,56	0,88
271	12	31	5,13	6,02	9,14	5,58	2,01	0,50
282	2	33	6,69	7,36	10,3	7,14	2,45	0,50
282	3	32	6,24	6,69	10,3	6,69	2,23	0,25
282	4	33	6,24	6,91	9,81	6,69	2,01	0,75
282	5	29	5,35	6,02	8,25	5,58	1,56	
282	6	32	4,91	5,58	8,92	5,35	2,01	0,50
282	7	29	5,13	5,80	8,03	5,13	1,56	0,50
282	8	28	4,91	5,58	8,25	4,91	1,56	0,13
282	9	31	4,46	4,91	7,81	4,46	1,34	0,50
282	10	30	4,24	4,46	7,58	4,91	1,34	0,13
282	11	26	3,57	4,01	6,02	2,68	1,12	
282	12	26	3,35	3,57	4,46	2,01	,89	
282	13	30	4,91	5,35	8,25	5,13	1,56	0,75
282	14	30	4,24	4,91	8,03	4,68	1,34	0,38
282	15	26	4,01	4,68	6,69	4,46	,89	0,25
282	16	25	2,45	2,45	4,01	2,23	,89	
282	17	25	2,01	2,90	3,57			
282	18	26	3,35	4,01	6,02	2,23	,89	
282	19	25	2,68	3,35	5,35	1,78	,89	
282	20	25	3,79	4,24	6,02	4,01	,89	
282	21	26	4,01	4,24	6,24	4,01	1,34	
282	22	25	3,57	3,57	5,13	2,23	,67	
282	23	31	5,58	6,02	8,92	5,13	1,78	0,25
290	1	25	3,79	4,68	6,91	4,91	1,34	0,38
290	2	25	3,79	4,46	4,68	3,79	1,12	0,33
290	3	25	3,35	3,79	5,58	3,79	,89	0,75
290	4	26	4,24	4,68	6,69	4,46	1,34	
290	5	23	,89	2,45	3,79	1,56	,89	
290	6	26	3,57	4,24	6,02	4,01	1,12	
290	7	23	2,23	2,23	4,01	1,56	,89	
290	8	23	2,23	3,79	4,01	1,56	,67	
290	9	24	2,23	2,45	3,79	1,78	,89	
290	10	24	2,45	3,79	4,01	2,01	,89	
290	11	23	2,01	2,45	3,35			
290	12	23	1,56	2,23	2,45			
290	13	24	2,23	2,23	3,79	2,01	,89	
290	14	24	2,01	2,23	3,35	1,56	,89	
290	15	23	2,01	2,01	3,35	1,78	,67	
290	17	28	4,68	4,91	7,81	4,24	1,56	0,88
290	18	25	3,12	3,79	5,35	3,12	,89	0,67
290	19	26	3,57	4,24	5,58	3,57	1,00	1,00
290	20	26	3,35	4,01	4,68	3,35	,89	,75
290	21	26	3,35	4,24	5,35	3,35	,89	
290	22	26	3,79	4,24	5,80	4,01	1,12	
290	23	26	3,57	4,01	5,35	4,01	1,12	
290	24	26	3,12	3,35	5,13	3,79	,89	
290	25	26	3,35	3,79	4,68	3,12	,89	
290	26	26	3,57	3,79	5,35	3,35	,89	
290	27	27	3,79	3,79	5,58	4,01	1,00	
290	28	26	3,57	3,57	5,13	3,79	,89	

Sites	ind	stage	body height	body width	body length	tail height	tail muscle height	*HI*
290	29	26	3,57	3,57	5,35	3,79	,89	
290	30	26	3,35	3,12	5,58	3,12	,89	
290	31	26	3,35	3,35	5,13	3,35	,67	
290	32	26	3,57	3,79	5,80	3,57	,89	
290	33	26	3,57	3,57	5,35	3,79	,89	0,63
290	34	26	3,12	3,12	4,24	2,90	,67	0,75
290	35	26	3,35	3,35	5,13	3,35	,89	
290	36	26	4,01	3,79	5,58	4,01	,89	0,83
290	37	26	3,79	3,35	5,13	3,35	1,00	
290	38	27	3,35	3,35	5,35	3,35	,89	
290	39	26	3,35	3,57	5,35	3,79	,89	
290	40	26	3,35	3,12	4,68	3,35	,89	
290	41	26	3,35	3,79	5,13	3,57	1,12	
290	42	26	3,57	3,35	4,91	3,35	1,12	
290	43	26	3,57	3,57	5,35	3,35	,89	
290	44	26	3,79	3,57	5,58	3,79	1,00	
290	45	26	3,35	3,57	5,13	3,57	,89	
290	46	26	3,57	3,57	5,35	3,79	1,12	
290	47	26	3,57	3,35	5,58	3,57	,89	
290	48	26	3,57	3,57	5,58	3,57	,89	0,75
290	49	26	3,57	4,01	5,35	3,35	1,00	
290	50	26	3,57	3,35	5,13	3,35	1,12	1,00
290	51	26	3,57	3,57	5,58	4,01	1,00	
315	1	30	5,80	6,02	8,92	6,02	2,01	1,00
315	2	26	5,13	5,35	8,25	4,91	1,34	0,38
315	3	30	6,02	5,80	7,58	6,02	1,56	0,00
315	4	27	5,13	5,80	7,81	5,35	1,56	0,38
315	5	31	6,24	6,69	9,81	6,24	1,78	0,50
315	6	29	5,13	5,35	7,81	5,58	1,78	0,83
315	7	27	4,01	4,68	6,69	4,01	1,34	1,00
315	8	28	5,35	5,35	8,25	5,58	1,56	0,67
315	9	27	4,91	5,58	8,25	5,13	1,34	0,63
315	10	29	5,58	5,80	8,92	5,80	1,78	1,00
315	11	32	6,02	6,91	9,81	6,02	1,34	
315	12	27	5,13	6,02	7,81	5,35	1,78	1,00
315	13							0,00
315	14							0,38
317	1	36	7,58	9,81	15,2	6,69	2,45	0,88
317	2	33	7,14	8,03	12,7	6,24	2,01	1,00
317	3	38	9,81	10,7	16,7	9,14	3,12	0,63
317	4	36	8,25	9,37	14,5	6,91	2,23	0,88
317	5	32	6,47	7,14	10,5	5,58	2,01	0,88
317	6	34	8,25	9,37	13,6	6,91	2,45	1,00
317	7	36	8,25	9,37	13,8	7,14	2,68	1,00
317	8	35	7,81	8,70	14,0	7,14	2,45	1,00
317	9	37	9,59	10,5	16,1	7,58	2,68	0,88
317	10	38	9,59	10,7	15,8	7,58	3,12	0,88
317	11	34	7,58	8,47	14,0	7,14	2,45	0,88
317	12	31	6,69	7,81	12,0	6,24	2,01	1,00
317	13	35	8,70	9,37	13,6	6,69	2,45	0,88
317	14	32	7,36	8,70	12,7	6,24	2,23	0,88
317	15	34	8,03	8,92	12,7	6,91	2,23	1,00
317	16	33	7,36	8,70	12,9	6,47	2,45	0,75
317	17	32	7,14	8,47	11,8	5,80	1,34	0,88
317	18	32	6,47	7,14	11,2	5,58	2,23	1,00
317	19	32	6,69	7,58	11,8	5,58	2,01	0,88
317	20	32	6,91	7,81	12,0	5,80	2,01	1,00
317	21	30	5,35	6,02	9,37	4,24	1,34	0,75

Sites	ind	stage	body height	body width	body length	tail height	tail muscle height	*HI*
317	22	32	6,91	8,25	12,0	6,02	2,01	0,75
317	23	31	6,69	7,58	11,8	5,35	2,01	0,75
317	24	31	6,24	7,36	11,2	5,58	1,78	0,88
317	25	34	7,58	8,47	13,2	6,69	2,45	0,88
317	26	33	7,14	7,81	11,8	6,02	2,23	0,75
317	27	31	6,24	7,36	10,9	5,35	2,01	0,88
317	28	30	6,24	7,14	10,0	4,68	1,78	0,88
317	29	30	5,35	6,47	9,59	4,91	1,78	0,88
317	30	30	5,58	6,69	10,7	4,68	1,56	1,00
317	31	34	6,24	7,14	11,8	6,47	2,01	1,00
317	32	33	6,24	7,14	11,2	5,58	2,23	0,88
317	33	31	5,58	6,47	9,81	4,91	2,01	0,88
317	34	31	6,24	6,69	10,7	5,13	1,78	0,75
317	35	32	6,24	7,14	10,9	5,80	2,01	1,00
317	36	31	6,24	6,91	10,5	5,35	2,01	0,88
317	37	31	5,80	6,47	9,59	5,13	1,78	1,00
317	38	31	5,58	6,24	9,59	4,68	1,56	1,00
317	39	31	6,24	6,47	9,14	4,91	1,56	0,88
317	40	31	5,35	6,02	9,37	4,24	1,34	0,88
317	41	30	5,35	6,02	9,14	4,46	1,78	0,88
317	42	28	4,24	4,68	7,14	3,79	1,34	0,63
318	1	25	5,35	5,80	8,25	4,68	1,23	0,63
318	2	26	4,68	5,35	7,58	4,01	1,23	0,50
318	3	27	5,35	5,80	8,92	4,91	1,56	0,75
318	4	26	4,91	5,35	7,58	4,01	1,34	0,75
318	5	25	4,01	4,24	6,91	4,24	1,12	0,63
318	6	25	3,79	4,35	6,91	4,01	1,23	0,75
318	7	26	5,02	5,69	8,25	4,68	1,34	0,63
318	8	25	4,24	4,57	7,14	3,79	1,12	0,63
318	9	25	3,79	4,13	6,91	3,12	1,00	0,50
318	10	25	3,57	4,01	5,58	3,57	,89	0,38
318	11	26	3,20	3,82	4,84		,62	0,50
318	12	27	3,43	3,90	5,46	3,20	,94	0,38
318	13	25	3,12	3,67	5,62	2,81	,94	0,25
318	14	25	3,51	3,90	6,01	3,67	1,01	0,50
318	15	25	3,35	3,82	5,77	3,43	1,09	0,50
318	16	25	3,57	3,79	5,69	3,35	,89	0,63
318	17	25	3,68	3,90	5,69	3,79	1,00	0,33
318	18	25	3,01	3,46	5,13	3,12	1,00	0,33
318	19	25	3,79	4,01	5,91	3,79	1,12	0,33
318	20	25	3,68	3,90	5,91	3,79	1,12	0,83
318	21	25	2,56	3,12	4,46	2,90	,89	0,75
318	22	25	3,46	3,79	5,69	3,23	,89	0,38
318	23	25	3,46	4,01	5,58	3,79	1,23	0,67
318	24	25	3,12	3,57	5,35	3,23	1,00	0,50
318	25	25	3,57	3,46	5,29	3,23	1,00	1,00
318	26	25	3,68	4,35	5,91	3,57	,89	0,17
318	27	25	3,23	3,68	4,79	3,46	,89	0,33
318	28	25	2,90	3,23	5,13	2,56	1,12	0,50
321	1	26	4,01	3,57	5,58		1,34	
321	2	46	6,24	7,58	19,4			
330	1	27	5,35	5,58	7,81	5,13	1,34	
330	2	29	5,80	6,24	8,92	5,13	1,34	0,75
330	3	30	5,80	6,02	9,14	5,58	1,56	0,50
330	4	27	5,13	5,13	7,36	4,68	1,56	0,38
330	5	26	3,35	3,79	4,68	2,68	,89	0,25
330	6	27	5,35	6,24	8,70	6,02	1,34	
330	7	26	3,79	4,01	5,58	3,57	,89	0,75

Sites	ind	stage	body height	body width	body length	tail height	tail muscle height	*HI*
330	8	27	4,91	4,91	6,91	5,13	1,34	0,50
330	9	26	3,79	4,24	5,80	3,57	,89	0,63
330	10	26	3,57	3,79	5,35	3,57	,89	0,38
330	11	29	4,91	4,91	7,81	4,01	1,56	
330	12	26	2,90	3,79	5,58	2,90	,89	0,63
330	13	27	5,35	5,35	7,36	4,91	1,34	0,17
330	14	26	3,35	4,01	5,80	2,90	,89	0,50
330	15	27	4,91	6,02	7,81	5,58	1,78	0,75
330	16	26	3,79	4,24	6,02	3,12	1,12	0,88
330	17	27	4,46	4,68	6,69	4,24	1,78	0,50
330	18	26	3,35	3,79	5,13	3,57	,89	
330	19	26	4,01	4,24	6,02	4,24	1,34	0,75
330	20	26	3,57	4,01	5,58	3,79	,89	0,88
330	21	26	4,01	4,24	6,47	3,35	,89	
330	22	26	3,79	4,68	6,47	3,57	,89	
330	23	26	4,24	4,68	4,68	4,24	1,12	0,50
330	24	44	7,36	9,14	23,2			0,83
330	25	25	4,01	4,91	7,14	3,35	1,56	0,63
330	26	30	6,69	7,58	10,5	7,58	2,01	0,63
330	27	29	5,80	6,24	8,92	6,91	2,01	0,67
330	28	25	4,01	4,46	6,47	4,24	2,01	0,63
330	29	27	4,68	4,68	7,58	4,68	1,56	1,00
330	30	30	4,68	4,91	8,70	5,80	1,78	0,67
330	31	31	4,91	5,80	9,81	5,58	1,78	0,88
330	32	31	7,14	7,36	10,0	6,91	2,01	0,88
330	33	28	5,58	6,02	9,59	6,69	2,01	0,75
330	34	27	5,80	6,47	10,0	6,24	2,01	0,63
330	35	28	5,80	5,35	9,14	6,47	2,01	0,63
330	36	31	6,02	6,02	10,7	7,36	2,23	0,50
330	37	24	3,35	3,57	5,58	3,79	1,12	0,83
330	38	27	5,58	5,80	8,25	5,58	1,78	0,75
330	39	32	6,69	6,91	10,7	6,91	2,01	0,75
330	40	26	4,24	4,46	6,24	4,91	1,34	0,50
330	41	27	4,24	4,91	6,91	4,68	1,56	0,13
330	42	29	6,24	6,24	9,14	5,13	2,01	
330	43	25	4,01	4,46	5,80	4,24	1,56	0,83
330	44	25	4,01	4,24	6,47	4,24	,89	0,50
330	45	27	5,13	5,35	8,47	5,58	1,34	1,00
330	46	25	3,79	4,24	5,80	4,24	1,12	0,67
330	47	26	4,01	4,68	6,91	4,46	1,12	0,75
330	48	25	3,35	3,79	5,35	3,35	,89	0,83
330	49	25	3,57	4,01	5,35	3,79	1,12	1,00
330	50	25	3,79	4,24	5,80	3,79	1,12	0,75
330	51	25	3,35	4,01	5,58	3,35	1,12	0,50
330	52	25	3,57	4,24	5,58	3,79	1,12	0,88
330	53	26	2,90	3,35	5,35	2,68	,89	0,75
330	54	25	3,35	3,79	5,58	4,01	1,12	0,50

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Lebenslauf

Name:	Sonja Köhler
Anschrift:	Bahnhofstr. 46 82340 Feldafing
Geburtsdatum:	26.11.1973
Geburtsort:	Bocholt

Schulausbildung:

1980 – 1984	St.-Pius-Grundschule in Rhede-Krechting
1984 – 1993	St.-Georg-Gymnasium in Bocholt, Abitur (Note 1,7)

Hochschulstudium:

1993 – 1999	Studium der Biologie an der Georg-August-Universität in Göttingen, Diplom (Note: „mit Auszeichnung“)
Hauptfach:	Zoologie (Populationsgenetik)
Nebenfächer:	Naturschutz, Botanik, Humangenetik
Diplomarbeit:	„Die Bedeutung von Kernintegrationen mitochondrialer DNA für phylogenetische Fragestellungen am Beispiel der Cercopithecoidea“, AG Genetik am Deutschen Primatenzentrum

Auslandsaufenthalt:

1995 – 1996	2 Semester Erasmusstipendium in Bangor, Wales

Promotion:

2000 – 2003	Department Biologie II, LMU München
Titel:	„Mechanisms for partial reproductive isolation in a *Bombina* hybrid zone in Romania“

Berufserfahrung und Praktika:

1996 – 1999	Betreuung des Praktikums der Biologie für Mediziner
1999 – 2000	5 Monate wissenschaftliches Volontariat am Frankfurter Zoo
seit 2002	Freie Mitarbeiterin am Deutschen Museum in München zur Betreuung des molekulargenetischen Labors für Besucher

List of publications

Vines, T., Köhler, S.C., Thiel, M., Ghira, I., Sands, T.R., MacCallum, C.J., Barton, N.H., Nürnberger, B. (in press): The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. Evolution

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