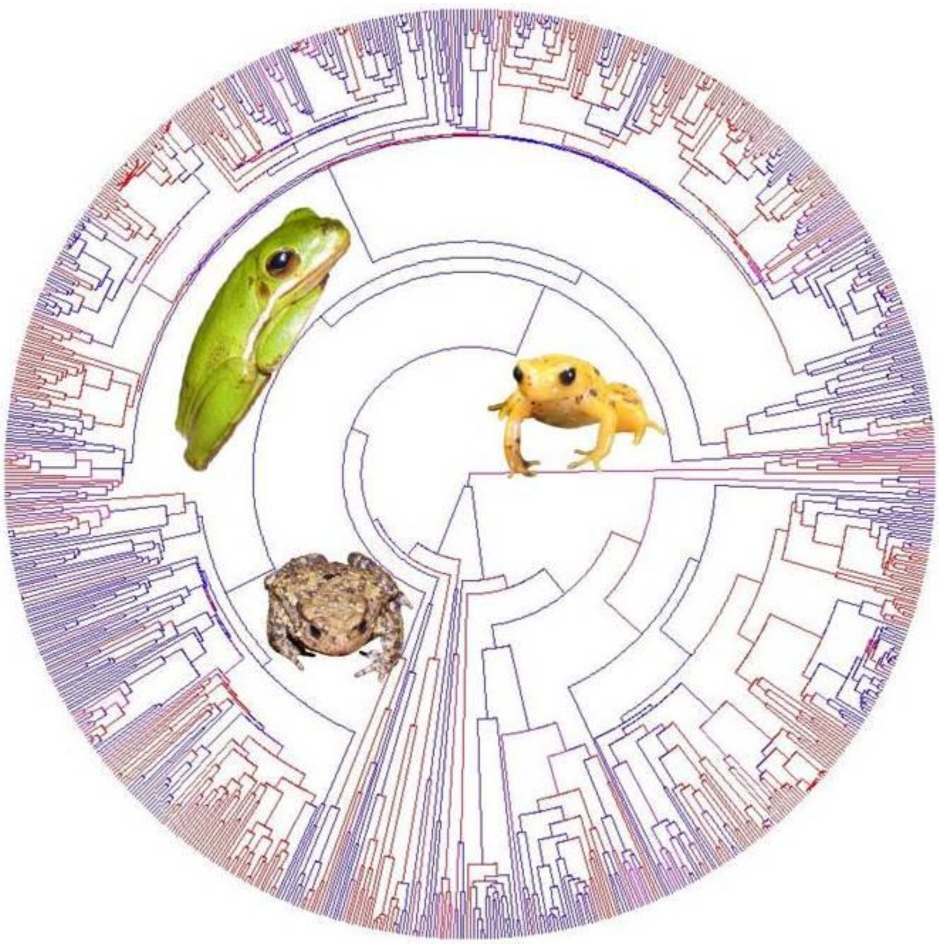
On the Macroevolution of Antipredator Defence

Thesis submitted in accordance with the requirements of the University of Liverpool
for the degree of Doctor in Philosophy by Kevin Arbuckle

June 2015



Preface

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. The text does not exceed 100,000 words and meets the formatting guidelines of the University of Liverpool. No part of this dissertation has been submitted to any other university in application of a higher degree.

1 Contents

2 Abstract

This thesis aims to improve our understanding of the macroevolutionary implications of antipredator defences, particularly with regard to how defence impacts biodiversity (including both species and trait diversity). To do this I took a phylogenetic comparative approach and used multiple study systems in an attempt to ensure the generality of my work. I begin by investigating how chemical defence and protective coloration influence ecology by testing for life history and ecological correlates of these defences (Chapter 1). Upon finding evidence for an increased niche space in chemically-defended species, and to some degree in conspicuously-patterned species, I explore whether this leads to increased diversification by increasing speciation rates and/or lowering extinction rates (Chapter 2), as also predicted by escape-and-radiate theory (a major and highly influential framework for the macroevolution of natural enemy interactions). Both conspicuous coloration and chemical defence increased speciation rates, but extinction rates were also raised in chemically-defended lineages, leading to a reduction in net diversification. Macroevolutionary extinction rates may or may not be related to contemporary extinction risk, but if they are then there may be conservation implications by allowing prediction of threat status of species with limited direct information. Consequently, in Chapter 3 I asked whether chemically-defended species are more threatened than those lacking such a defence. In accordance with the macroevolutionary results from Chapter 2, I found that chemical defence is indeed associated with a higher extinction risk even amongst contemporary species. In addition to factors that promote diversity, in this thesis I also investigated convergent evolution as a means of constraining diversity of phenotypic traits, using mimicry as a case study for antipredator defences. Many antipredator defences are convergent to some degree, with examples in the repeated evolution of chemical defences and warning coloration as well as independently derived similarity in protective mimicry. However, methods of quantifying the strength of convergent evolution are lacking, not to mention a conceptual framework to define 'strength' in this context, I began by developing a new method to do this which I called the

Wheatsheaf index (Chapter 4). Subsequently, I (in collaboration with a colleague, Amanda Minter) also designed software in the form of an R package (called 'windex') to enable user-friendly implementation of the Wheatsheaf index in a familiar statistical environment to many biologists (Chapter 5). In the final data chapter of this thesis, I apply this method in a case study to explore the patterns of phenotypic convergence that result from the evolution of Batesian and Müllerian mimicry complexes. I find that these two types of protective mimicry are generally characterised by convergence in different broad types of traits, but that the specific traits which converge in a given mimicry complex are less predictable (Chapter 6). Overall, this thesis provides novel insights into the evolutionary patterns and consequences of antipredator defences, develops a framework and methods for the analysis of convergent evolution, and suggests further avenues of research for future studies.

3 Acknowledgements

First I foremost I have to extend my absolute gratitude to my supervisor, Mike Speed. Mike has been an absolute pleasure to work with throughout my PhD and has generously given me a lot of independence to run with my ideas. I say 'work with' rather than 'work for' deliberately, because it has been far more like having a colleague and friend than a supervisor, and one who is always willing to offer advice, guidance, and support when necessary. I feel lucky to have undertaken a PhD in Mike's lab and I only hope I've done him justice in the work presented in this thesis.

I would also like to thank Mike Brockhurst and Michael Berenbrink who have been my second supervisors during my PhD and provided some helpful comments in that time. Mike started as my second supervisor until he decided to run off to the University of York to take up a professorship there (congratulations!), after which Michael stepped in to ensure I had a second supervisor still based at Liverpool. In particular, I have enjoyed the numerous conversations with Michael over the last few years on the subject of phylogenies, comparative methods, and how to teach them to undergraduates as part of his final year module.

Mike Begon enabled me to get started on my PhD and has provided support and encouragement throughout, for this I am eternally grateful. Yes, I know, another Mike, it seems like every Mike in Merseyside has had some input into my time here at Liverpool, but I'm very glad that they have. Anyway, if it were not for Mike Begon I wouldn't have got the funding to do my project, so I'll never forget the effort he made to argue my case to secure funding from NERC (who I also extend my thanks to). I feel very humbled that a veritable legend in ecology, the man who literally wrote the book on the subject, deemed me worthy of his support, so thanks once more Mike!

This thesis represents a body of work which either has been, or soon will be written up for publication. Because any real training for an academic career must involve skills in collaboration, it should not be a surprise that some of the chapters have people listed as authors other than me and my supervisors. I would like to thank all my collaborators on the work presented here for their various inputs: Cheryl Bennett, Amanda Minter, and Bob Srygley. In particular, Amanda was an incredibly easy person to work with and as such made the development and publication of the windex package for R (see Chapter 5) that much more enjoyable. I should also give Amanda an additional thank you for introducing me to creating progress bars in R, something I've used in almost every possible situation since!

In addition to my work for this thesis, I have also had the pleasure of working with other friends and collaborators on 'side-projects' which have helped to keep my work and skills diverse, and my publication record richer. In this vein, I extend my thanks to Hazel Nichols, Nick Casewell and Wolfgang Wüster (along with my other co-authors on the toxin resistance paper), Chris Mitchell, and John Lycett. I would like to additionally thank Wolfgang Wüster and David Warrell for inviting me to speak and co-chair a symposium at the upcoming World Congress of Toxinology in Oxford this September, and Anita Malhotra for inviting me to write a book chapter for an upcoming volume of the Springer Handbook in Toxinology series.

Academic interactions are one component of doing a PhD (or any other research activity), but many others have been invaluable to the logistics and organisation of my PhD, and have made life substantially easier as a result. In particular, I extend thanks to Tom Heyes, Meriel Jones, and the Accounts office team. Tom provided me with lab space and facilities in the early days when I wanted to poke some caterpillars, and was ever helpful with suggestions of caterpillar husbandry, catching butterflies, and places to keep cabbages to grow them up. Although none of this work has been included in this thesis, I remain grateful for his help. Meriel has been an absolute Godsend whenever the jungle of administrative tasks and thickets of forms needed to be transgressed, and especially so over the last run-up to the submission of this thesis. If she ever leaves I can only imagine that the corridors of the building will resemble a zombie apocalypse with wandering PhD students clutching forms and looking lost, confused, and wide-eyed. Finally, the Accounts office team have made spending money on my grant inordinately less of a chore. The smiles, conversation, and helpfulness of every member of that team have on more than one occasion prevented me from tearing my hair out over trying to book flights and accommodation.

It would be rude if I did not at least give a mention to caffeine - a most wondrous chemical that makes science happen. Whatever form it comes in is welcomed, but special mention must go to Amanda Minter and Daria Pastok for the provision of not just our office's own coffee machine, but also some of the nicest coffee I've ever had. Oh, and Daria, I promise I'll clean my coffee mug soon. Music has filled a similar role on occasion and made the days pass quicker, so in the interest of fairness I should acknowledge the part that Blondie, R.E.M., The Clash, Muse, The Divine Comedy, Nirvana, Sex Pistols, and many other bands have played in getting me through some long days.

Chemical stimulation and music is not quite enough to make the days fun, that requires people, and I'm fortunate enough to have shared this department with an excellent bunch of people that have fulfilled that role perfectly. There are so many people in this group that I'm bound to miss people out (and massive apologies to anyone I do!), but I'll try to get everyone who's been involved in the social life of the department. Therefore, my thanks go to (in no order whatsoever) Louise Reynolds, Julie Truman, Daria Pastok, Rowan Doff, Ewan Harney, Jack Thomson, Steve Parratt, Angela Sims, Susan Withenshaw, Becci Turner, Niamh Quinn, Kieran Pounder, Sam Barlow, Ewan Minter, Ellie Harrison, Kayla King, Leni Collin, Satoshi Nakayama, Crystal Frost, Annabel Rice, Tom Price, Zen Lewis, Ian Wilson, Jess Lingley, Sarah Forrester, Karen Evans, Beth Levick, Sarah Trinder, Amanda Minter, Becky Jones, Lukasz Lukomski, Chloe Heys, Georgia Drew, Amy Eacock, Steve Price, Gabriel Pedra, Rudi Verspoor, Chris Mitchell, Chris Corbin, Vinnie Keenan and Jamie Alison.

Additional mentions require me to diverge from the University of Liverpool and go to Emily Bethell at LJMU, who has not only put up with my wittering conversation on a regular basis in the office, but also looked after pets while on holiday and invited me and Hazel to some interesting activities such as a screening of a local film, an outdoor film screening in the bombed out church, a tango class, and repeated drowning...errr sorry I mean a wakeboarding lesson. There is also a number of people at LSTM who have been great fun whenever we've met up for a drink or whatever else, but special thanks goes to Paul Rowley who has given me a welcome distraction full of venomous animals on a semi-regular basis.

My heartfelt thanks go to my partner Hazel Nichols for her tireless encouragement, support, and kindness. She has been the perfect antidote to those days when things don't go to plan, and a source of many good times (including my first trips to both Africa and Southeast Asia). She also extended her helpfulness to making a very good start to the formatting of this thesis, which massively cut down the time it took me to do this tedious element of writing. Finally, Hazel's family, especially Sheila and Graham Nichols, have treated me like a member of the family and allowed us to escape out of the city most weekends to their house. For this I am grateful.

Last but not least, I thank my family for their support over the many years. In particular I want to give specific thanks to two members of my family: My dad and my gran. My dad has encouraged me every step of the way in my enthusiasm for animals and in my educational progression towards where I am now. My gran is still the person I credit with giving me the best educational start in life. From teaching me how to read, write, tell the time, and do basic maths when I was very young, to encouraging my interests by (for example) buying Dinosaur magazines for a long time when I was a kid, she has had a constant, positive, and lasting influence on my life.

Those who have provided valuable help for individual parts of this thesis are thanked in the acknowledgements of that particular chapter. If anyone feels left out of this section that should have been included, please accept both my apologies and thanks; the omission is more a fault with my memory than my gratitude!

4 Introduction

4.1 Background and Rationale

Predation is a ubiquitous risk to animals (as well as other organisms) and has serious fitness consequences; in the extreme case were an animal is predated before reproducing, (direct) fitness is immediately reduced to zero. Consequently, animals have evolved a diverse arsenal of antipredator defences to avoid being eaten (e.g. Ruxton *et al.*, 2004; Caro, 2005). Many defences, such as conspicuous warning coloration, mimicry, and toxins, have peaked the interest of scientists and laypersons alike and been widely studied since long before Darwin (1859) as a component of natural history.

The vast majority of previous work on antipredator defences has, understandably, been focussed on five main aspects: 1) diversity of defence strategies, 2) costs and benefits to defended individuals, 3) evolutionary origins, 4) maintenance of variation in particular defences, and 5) medical consequences of animal defences to humans (e.g. envenomation). As a result of this body of research, we know that animals use a vast and often ingenious range of morphological, behavioural, and chemical defences to avoid or repel predators (Ruxton *et al.*, 2004; Caro, 2005). We know that although defence provides obvious fitness benefits via reduced predator-induced mortality (Lind and Cresswell, 2005), it may be accompanied by a range of costs with respect to, for example, energetic resources (Higginson *et al.*, 2011), ecological opportunities (Stamp and Wilkens, 1993; Speed *et al.*, 2010), immune function (Smilanich *et al.*, 2009), or physiological ecology (such as when colour patterns influence both conspicuousness and thermoregulation; Lindstedt *et al.*, 2009). We know a number of ways in which different antipredator defences can originate and be maintained, largely through genomic and modelling studies (e.g. Ruxton *et al.*, 2004; Jones *et al.*, 2012). For instance, mimicry can evolve via a two-step process in which a large-effect mutation produces a phenotypic shift which brings the species near to a new (mimetic) adaptive peak in the fitness landscape, followed by more subtle ‘genetic tinkering’ of the phenotype under small-effect mutations (Balogh *et al.*, 2010). A recent review (Speed *et al*., 2012) has also highlighted many ways that variation in toxic defences can be maintained despite (presumably) selection towards an optimum level of defence, including selection for automimicry and environmental variation in sequestered toxins. Finally, we know a lot about the clinical effects, epidemiology, molecular mechanisms, and medical treatment of humans unlucky enough to suffer a defensive envenomation (e.g. Russell, 2003; Chippaux, 2006).

However, despite the universal importance of antipredator defences in the biology of animals, and its direct link to the microevolutionary process as a result of (potentially) large effects on fitness, relatively few studies have investigated macroevolutionary patterns and consequences of these defences. Most of those that have have limited themselves to estimating ancestral states of antipredator mechanisms (e.g. Dumbacher and Fleischer, 2001; Sanders *et al*., 2006), without a focus on general principles and broad hypotheses outside the study system used. Nevertheless, a growing number of exceptions exist, which reflects the general increase in the attention given to comparative biology in recent years. For example, Ceccarelli and Crozier (2007) found that in a clade of Batesian mimics of ants (*Myrmarachne* spiders), the mimics did not show evidence for cospeciation with their models as expected, but rather than the spiders represent an adaptive radiation of Batesian mimics.

The recent and rapid growth in phylogenetic comparative methods (and adequate phylogenies) now allows us to test questions about the macroevolution of antipredator defences that were difficult or impossible in the past. Aside from the necessity of accounting for phylogeny in interspecific analyses (Felsenstein, 1985), methodological advances increasingly allow us to use the information contained in phylogenies rather than simply incorporating it as a recognised source of error in statistical models (Garamszegi, 2014). In this thesis I have therefore opted to take a phylogenetic comparative approach to investigate how antipredator defences have influenced the ecology and evolution of animals.

Because of behavioural constraints on movement in animals using particular defences such as camouflage (Stamp and Wilkens, 1993; Speed *et al*., 2010), we might expect that ecological opportunities will increase if these constraints are relaxed. In other words, we should find that species with repellent chemical defences, which operate without constraints in movement, occupy a broader niche space than species relying more on camouflage or similar defences that involve ‘hiding’ from predators.

Given that we expect a broader niche space in well-defended prey, we would also predict carry-over effects on evolutionary dynamics because increased niche space is thought to lead to speciation (MacColl, 2011). In addition to this promotion of speciation, generalism (cf. specialism) is also expected to reduce the risk of extinction as generalists are less reliant on any particular resource (Kotiaho *et al.*, 2005). Furthermore, a longstanding and highly influential hypothesis proposed by Ehrlich and Raven (1964) known as ‘escape-and-radiate’ predicts that natural enemy interactions should also promote speciation. In terms of antipredator mechanisms, escape-and-radiate suggests that the evolution of effective defences should lead to a reduction in predator-imposed constraints on evolution and permit an increase in diversification rates in well-defended lineages (Hembry *et al.*, 2014). Therefore, as a result of the combined effects of increased ecological opportunity and the antagonistic coevolution inherent in predator-prey systems, we would expect well-defended lineages to experience higher diversification rates than lineages lacking such defences.

In addition to the generation of biodiversity (in terms of diversity in traits, ecology, and species), as predicted above, there are also situations where we expect that diversity should be constrained as a consequence of antipredator defence. In particular, protective mimicry is the evolution of phenotypic similarity between species in order to signal unprofitability to predators, whether honestly or deceptively (Ruxton *et al.*, 2004). Consequently, where mimicry has evolved independently it often presents a striking example of convergent evolution. Mimicry groups are usually defined by, or at least described based on, one aspect of the phenotype, most often colour pattern, but some work has found that multiple traits converge in mimicry groups. For instance, Srygley (1994) found evidence that neotropical butterflies which form mimicry groups categorised by colour patterns also display convergent flight behaviour. However, Golding *et al*. (2005) did not find substantial evidence for behavioural convergence between hoverfly mimics and their wasp models. Our understanding of how and when mimicry should involve convergence in different aspects of the phenotype or one key feature remains limited. Comparative analyses may help to answer this question, and shed light on the extent to which mimicry constrains phenotypic evolution, but have so far been underused. Such investigations have the potential to improve our understanding of antipredator mechanisms more generally, because although mimicry is a particularly good example, many defences have evolved repeatly throughout the animal kingdom. As such, convergence is a common and therefore potentially important aspect of the macroevolution of antipredator defences.

Finally, I would like to note that this introduction has aimed to give the broad background to the thesis as a whole, rather than an in depth literature review of every aspect of it. Instead, and because this thesis consists of published or soon to be published papers, each data chapter is structured as a research paper and therefore has a more detailed introduction to the literature background relevant to that part of the thesis.

4.2 Aims and Structure

This thesis aims to improve our understanding of the macroevolutionary patterns and consequences of antipredator defences in animals. In doing so, I have attempted to not only advance this particular field, but also provide conceptual and methodological tools to fill gaps that can otherwise limit avenues of research in the broad areas to which elements of this work relates (i.e. convergent evolution). Because I am interested in establishing general patterns rather than investigating what may be oddities of a particular model system, I have used a variety of animal groups throughout the thesis. As an introduction to the structure of the thesis, in this section I will give a very brief overview of what each chapter aimed to test and how they link together.

Overall, the thesis consists of two halves, each containing three chapters which link together in a progressive manner. Chapters 5-7 consider how defence may generate ecological and species diversity, whereas Chapter 8-10 consider how the convergent evolution inherent in certain defences (e.g. mimicry) can constrain and limit phenotypic diversity. Therefore the two halves of the thesis are linked in their attempts to understand the macroevolutionary impact of antipredator mechanisms on biodiversity as a whole.

Chapter 5 is the first data chapter and aims to investigate the role that repellent chemical defence (and aposematic colour patterns that warn of this defence) has played in the ecology of a group of mammals (Musteloidea). In this group (which includes skunks, otters, and badgers), the chemical defence in question is the use of repugnant anal gland secretions which are released during encounters with predators. Specifically, I tested for correlations of ecological traits with defence in order to assess whether there is indeed evidence that chemical defences lead to an increase in niche space. I was also able to test whether conspicuous colour patterns function as aposematic warning signals or if there is evidence for Batesian mimicry in the group.

Having found evidence for an increase in niche space as a result of chemical defence and conspicuous coloration, I proceeded to ask whether there is evidence of these traits increasing diversification dynamics in Chapter 6. For this, I used amphibians as a study system because they are a more diverse group than musteloid mammals, with a suitable phylogeny available for many species, and I wanted to avoid the risk of any patterns being specific to musteloids. In short, if predictions made from studies of one group also hold in studies of other groups, it is more likely that they are general patterns rather than peculiarities of a single system. In Chapter 6, I find that conspicuous coloration and chemical defence are both associated with increased speciation rates in this group, as expected by previous theory (i.e. the 'escape-and-radiate' scenario). However, I also found the more unexpected result that chemical defence (but not conspicuous coloration) lowers net diversification rates, due to an increase in extinction rates. These results therefore suggest that, by ignoring effects on extinction rates, the escape-and-radiate theory only partially explains diversification dynamics in relation to natural enemy interactions and thus requires an expansion to consider extinction.

Given the finding that chemical defences increase macroevolutionary extinction rates in amphibians (a group of vertebrates with a particularly high proportion of threatened), I decided to investigate whether this is also true for contemporary extinction risk in this group in Chapter 7. Background extinction rates may or may not be relevant to current conservation concerns because different threats are responsible for declines across the different temporal scales. However, prediction of extinction risk is an important goal for conservation biology since many species have too little information for direct assessment, and identifying traits linked to extinction risk is the first step in this process. Using IUCN Red List status as a proxy for extinction risk, I find evidence that species possessing a chemical antipredator defence face a higher level of threat. I also present more tentative (due to methodological caveats) evidence that the defence leads to the rise in extinction risk rather than a simple correlation between the two traits.

To examine the constraints imposed in mimicry groups by convergent evolution, and evaluate any patterns, we first need a way to quantify such convergence. Although many methods exist which identify cases of convergence, neither a conceptual framework nor a methodological toolbox to measure its ‘strength’ is well-developed. Consequently, in Chapter 8 I first develop a framework with which we can devise a sensible definition of a ‘strength’ of convergence, and then design a new comparative method which aims to quantify this (called the Wheatsheaf index). The method takes into account both the phenotypic similarity and the phylogenetic relatedness of a set of organisms, and measures convergence in relation to an *a priori* hypothesis of which species should converge (called a ‘focal group’).

The method developed in Chapter 8 was implemented using code written for MatLab by Mike Speed and myself, a program with which many end-users may not be familiar. Consequently, in Chapter 9 I designed an R package (called ‘windex’) in collaboration with Amanda Minter to run data analysis using the Wheatsheaf index. This implementation is has three main advantages over the MatLab code. Firstly, users of the method are likely to primarily be evolutionary ecologists, a demographic which is generally far more familiar with R than MatLab. Secondly, windex is far more flexible than the MatLab code. Thirdly, windex is far more user-friendly in that it does not require any pre-treatment of the data before analysis, data input is from commonly-used file formats, and help files are available in a standardised R format.

Finally, in Chapter 10, I use the method developed in Chapter 8 (and implemented in the windex package described in Chapter 9) to investigate patterns of phenotypic convergence in mimicry groups of neotropical butterflies. These mimicry groups were defined based on colour pattern of the wings. Specifically, the analyses explore whether the type of mimicry, Batesian or Müllerian, is related to the types of traits which converge in addition to colour pattern. I find that in general, Batesian mimics tend to converge most strongly on traits related to the appearance of butterflies in flight whereas Müllerian mimics tend to converge most strongly on a variety of defensive traits. However, beyond this general pattern the particular set of traits that show the strongest convergence vary between mimicry groups. I discuss these results in relation to mimicry theory, convergent evolution and the predictability of evolution. I also note that this chapter provides a detailed empirical case study of the use of the Wheatsheaf index to investigate evolutionary convergence.

Finally, the thesis ends with a chapter on conclusions and future work (Chapter 11), which ties together the data chapters and highlights what research would be fruitful to develop the ideas within. I would like to point out that although suggestions for future research appear in individual chapters where relevant, the ‘future work’ subsection of Chapter 11 (section 11.3) is intended to summarise what I believe to be the most important questions for future study.

In keeping with the requirements of the University of Liverpool, I explain here the role played by co-authors of papers in this thesis at the end of this introductory chapter. In addition to supervisors (who provided comments and guidance on the work), there are three co-authors listed: Cheryl Bennett (Chapter 8), Amanda Minter (Chapter 9), and Bob Srygley (Chapter 10). Cheryl was an undergraduate who collected some data and conducted preliminary analyses (re-run by me to ensure accuracy) as her undergraduate research project. Amanda is a PhD student with particular expertise and experience in writing R packages, and hence I enlisted her help in writing the package described in Chapter 9. Bob is a research ecologist with the United States Department of Agriculture and provided me with the dataset (part published, part unpublished) used in Chapter 10. Note that the contributions of all coauthors (including supervisors) are contained in slightly more detail than here at the beginning of each chapter as an ‘author contributions’ subsection.

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5 Does chemical defence increase niche space? A phylogenetic comparative analysis of the Musteloidea.

This chapter is published in *Evolutionary Ecology* (Arbuckle *et al*. 2013. *Evol. Ecol.* 27:863 - 881), and a copy of the final article is contained at the end of this thesis.

5.1 Author contributions

Kevin Arbuckle[1], Michael Brockhurst[2] & Michael P. Speed[1]

[1] Institute of Integrative Biology, Biosciences Building, University of Liverpool, Crown Street, Liverpool, Merseyside, L69 7ZB, United Kingdom.

[2] Department of Biology, University of York, Wentworth Way, York, Yorkshire, YO10 5DD, United Kingdom.

At the time of writing this chapter, Mike Brockhurst was my secondary supervisor and so, along with Mike Speed, provided comments on the manuscript and discussion of ideas. I developed the initial concept along with Mike Speed, designed the study, collected the data and conducted the analyses, and wrote the manuscript.

5.2 Abstract

Avoidance of predation can impose opportunity costs on prey species that use behavioural avoidance strategies to evade detection. An animal that spends much time hiding or remaining immobile, for example, may have less time for other important activities such as foraging or finding mates. Here we examine the idea that the evolution of chemical defence may act to release prey from these constraints, freeing defended prey to exploit their habitats more effectively, and increasing their niche space. We tested this hypothesis using comparative methods on a mammal group containing both chemically defended and non-defended species: Musteloidea. We found that defended species had a more omnivorous diet and were more likely to be active during both day and night than non-defended species. We also found that chemically defended species were less likely to be strictly diurnal or to show sexual size dimorphism, and had earlier maturing females and a shorter lifespan than non-defended species. Taken together, our results support the hypothesis that chemical defence increases the niche space available to a species. More generally, this also supports recent suggestions that strategies taken to avoid natural enemies can have important effects on diverse components of life history.

5.3 Introduction

Studying the impacts of natural enemies on the evolution and ecology of organisms is important if we are to understand how biodiversity is generated and maintained (Vamosi, 2005). Predators in particular can be a strong selective force due to the obvious high risks to prey from attacks, especially for individuals that have not yet reproduced. Consequently, antipredator mechanisms are ubiquitous in animals and come in a large diversity of forms (Endler, 1986; Witz, 1990; Ruxton *et al.*, 2004; Caro, 2005a; Stankowich, 2011). In addition to variation across species, a single individual or species can often make use of a suite of defences including behavioural (hiding or counter-attack), morphological (spines, armour or toughened integuments), and chemical mechanisms (toxins, venoms or distasteful compounds) (Pearson, 1985; Hanlon *et al.*, 1999; Caro, 2005a; Lindstedt *et al.*, 2008; Stankowich, 2011). Some of these defences may work relatively independently of one another but it is likely that prey species combine different components of their arsenal for greater effectiveness (Endler, 1986; Caro, 2005a; Cooper and Sherbrooke, 2010). An example is the use of venomous spines which combine chemical and morphological components (Bendt and Auerbach, 1991; Lakshmanan, 2004; Haddad *et al*., 2008).

Understanding the evolutionary ecology of defence is challenging in part because defences often impose varied forms of opportunity costs. Most obviously, resources allocated to anti-predator defences cannot be used for other life history components that also contribute to fitness, such as growth, development and investment in fecundity (e.g. Higginson *et al.*, 2011). There may in addition be ecological opportunity costs when an antipredator defence requires that a prey restricts its activity through cryptic behaviour. Cryptic behaviour may take the form of immobility and/or restriction of activity to periods with reduced predation risk, or restricting activity to areas that reduce risk such as shelters, burrows, under leaf litter; or backgrounds against which the animal is camouflaged. For cryptic colouration to work (crypsis, countershading, masquerade, and disruptive colouration) prey animals may often have to reduce their movements, thereby imposing considerable constraints (Stamp and Wilkens, 1993; Merilaita and Tullberg, 2005; Speed *et al*., 2010). Prey that engage in behavioural crypsis may therefore collect fewer resources, search for mates less effectively and be less good at holding territories than those that do not restrict their movements in this way (Abrahams, 1995; Santos *et al*., 2003; Ruxton *et al*., 2004; Merilaita and Tullberg, 2005; Speed and Ruxton, 2005).

In contrast, chemically defended species may be able to exploit a wider range of environmental opportunities because chemical defences, which act after detection to reduce subjugation and consumption of the prey, do not require behavioural crypsis to be effective (Wallace, 1889). It has therefore been hypothesised that chemically defended prey should be able to occupy a larger niche space, for example showing a broader diet (Bowers, 1993), and this effect should be detectable when viewed across species. Furthermore, many chemically defended prey species advertise their unprofitability with bright (aposematic) colouration that serves to warn predators away. Because aposematic display increases the ability of a predator to remember and learn to recognise defended prey as unprofitable (Speed, 2000; Ruxton *et al*., 2004) we hypothesise that aposematic colouration can reinforce and amplify any effects of chemical defence on niche space.

One limitation of many studies that have examined aposematic colouration in a variety of contexts is that conspicuousness is often used as a proxy for aposematism (e.g. Guilford, 1988; Sillén-Tullberg, 1988; Nilsson and Forsman, 2003; Merilaita and Tullberg, 2005). Conspicuous colouration can however have other functions including intraspecific communication, perhaps involved in mate choice or territoriality (Guilford, 1988; Ortolani, 1999). Batesian mimicry of an aposematic species may also confound an interpretation that bright colouration infers aposematism. Where possible, studies making the assumption that conspicuousness is aposematic should make some attempt to assess whether this holds for the group in question.

In a recent paper Stankowich *et al*. (2011) used a comparative approach to investigate the evolution of conspicuousness in the order Carnivora. They found that the level of chemical defence was positively associated with conspicuousness, indicating that aposematism is likely part of the arsenal of carnivores. They also asked whether aposematism was related to a variety of ecological variables, mostly relating to habitat but also body size and nocturnality, and found that this was the case for body size, open habitats, burrowing, and terrestrial (vs. aquatic) habits.

In this paper we adapt the approach of Stankowich *et al*. (2011) to the question of chemical defence and niche space. We focus on a particular group of carnivores, Musteloidea. Musteloids are a group of carnivores including the families Mustelidae (badgers, otters, weasels, etc.), Mephitidae (skunks), Procyonidae (raccoons, coatis, olingos etc.), and the species *Potos flavus* (kinkajou). The latter is sometimes included in Procyonidae but a recent molecular analysis has strongly supported its status as a separate lineage, probably as the sister group to all other musteloids (Agnarsson *et al*., 2010). Musteloidea is a reasonable-sized group for comparative studies, consisting of ~85 species (Wilson and Reeder, 2005), and contains a variety of species with and without chemical defences and conspicuous colouration. Therefore musteloids represent a very good study system to address our hypothesis that chemical defence increases niche space. We can also use the musteloids to investigate whether conspicuousness colouration is actually aposematic in function and, if so, whether this amplifies any effects of being defended on niche and life history traits.

We found evidence that chemical defence increases the occupied niche space at a macroevolutionary scale, most notably that defended prey had broader diets and activity periods than non-defended prey. We also report that conspicuousness most likely functions as an aposematic signal in musteloids and that it can influence relationships between ecological traits and the underlying chemical defence.

5.4 Methods

5.4.1 Phylogeny

We created a composite tree by hand in Mesquite v2.75 (Maddison and Maddison, 2011) of all musteloid species for which phylogenetic information was available. This involved starting with a star phylogeny (a completely unresolved, multifurcating tree) then moving branches around to generate the desired topology. The relationships were primarily based on Agnarsson *et al*. (2010) but this was supplemented to ensure as broad a coverage as possible with other sources (Gardezi, 1997; Bininda-Emonds *et al*., 1999; Marmi *et al*., 2004; Helgen *et al*., 2009; Del Cerro *et al*., 2010; Eizirik *et al*., 2010; Stankowich *et al*., 2011; Nyakatura and Bininda-Emonds, 2012). This method allowed us to obtain a completely bifurcating tree while specifically using clades that have received strong support in previous phylogenies. This is in contrast to many supertree methods which often return multifurcating trees that may contain clades that are poorly supported by the input trees (Wilkinson *et al*., 2005). Where different source phylogenies conflicted we chose the relationship with the highest node support (bootstrap support, Bayesian posterior probabilities etc.) and/or the study that used the more robust methods (e.g. better taxon sampling for the given relationship, more comprehensive gene sampling, congruent results from multiple construction methods such as maximum parsimony, maximum likelihood, and Bayesian inference). In total 84 species were included in the final tree.

Providing some idea of divergence times can improve many comparative analyses by allowing information on branch lengths to be incorporated. To do this for our musteloid tree we used 42 calibration points for nodes consistent with our tree from the literature (Bininda-Emonds *et al*., 1999; Marmi *et al*., 2004; Eizirik *et al*., 2010; Nyakatura and Bininda-Emonds, 2012), shown in section 5.9. These were then used to date the tree using the BLADJ function in Phylocom v4.1 (Webb *et al*., 2008). This dated tree (Fig. 5.1) was used for all subsequent analyses.

5.4.2 Trait data

Ecological information was sourced from the literature and a list of the publications used is presented in section 5.10. Where possible numerous sources were consulted for each trait for each species. This strategy also enabled a typical value for continuous traits such as body size to be extracted, rather than relying on one source. In cases where there was conflicting information between sources the values chosen were either obtained using the source with the best methods (where this was discernible) or using the majority consensus if more than two sources were available. For continuous data, discrepancies between sources (usually minor) were handled by using the mean trait value across sources.

The traits themselves consisted of 17 variables. These included 10 binary traits (conspicuous colouration, chemical defence, sociality, diurnality, nocturnality, circadianism, omnivory, sexual size dimorphism, mating system, and territoriality) and seven continuous traits (body size, diet diversity, longevity, litter size, male maturation, female maturation, and birth weight). Data were available for all species on the following traits: conspicuousness, chemical defence, sociality, diurnality, nocturnality, circadianism, omnivory, and body size. Our character coding scheme used for analyses can be found in Table 5.1, with a more detailed account in section 5.11. The number of species with available data varied for the other traits, and this was accounted for in the analyses by pruning the tree to use only those species for which data was available for both variables being tested in each case.

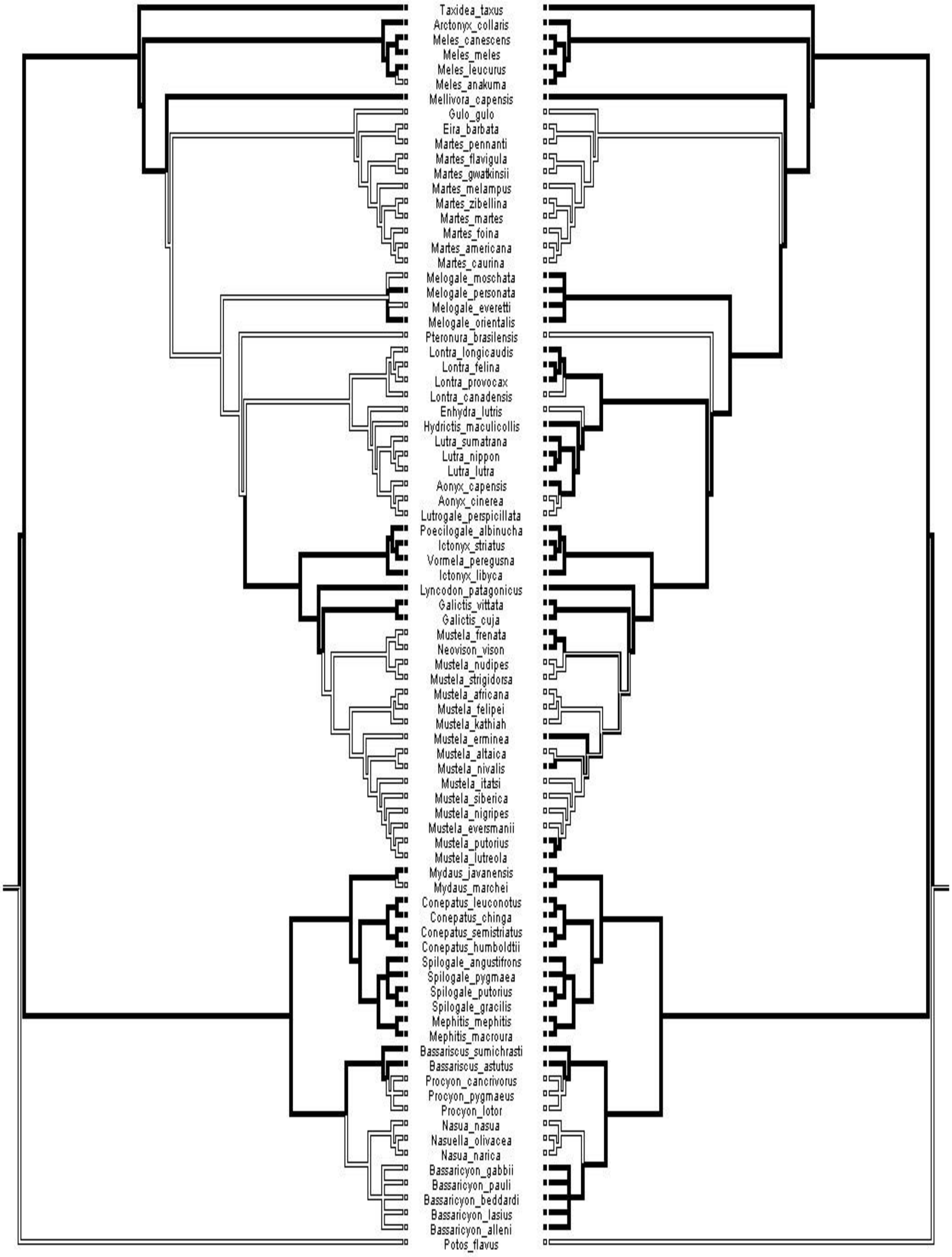

Figure 5.1 - Ancestral character state reconstruction using unordered parsimony for conspicuous colouration (on the left) and chemical defence (on the right). Traits are mapped in this way to permit easy comparison. In both cases, black represents presence and white represents absence of the trait. Branches which are half black and half white represent equivocal reconstructions.

Table 5.1. Brief description of our character coding scheme. N is the number of species for which we had data on that variable. For more details see section 5.11.

Trait	N	Description
Chemical defence	84	Anal gland secretions used in defence (1) or not (0)
Conspicuousness	84	Species shows conspicuous markings (1) or not (0)
Sociality	84	Lives in social groups (1) or solitary (0)
Diurnality	84	Usually active during the day (1) or not (0)
Nocturnality	84	Usually active at night (1) or not (0)
Circadianism	84	Usually active during *both* day and night (1) or not (0)
Sexual size dimorphism (SSD)	63	Sexually dimorphic (1) or monomorphic (0) in mass
Mating system	55	Polygamous (1) or monogamous (0)
Territoriality	30	Actively defends territories (1) or not (0)
Omnivory	84	Strongly omnivorous in main diet (1) or not (0)
Diet diversity	74	Number of higher-level taxonomic categories recorded in wild diet
Body size	84	Mean mass (kg)
Longevity	46	Maximum longevity in the wild (yrs)
Litter size	69	Mean number of young born per litter
Male maturation	35	Mean age at maturation in males (months)
Female maturation	35	Mean age at maturation in females (months)
Birth weight	37	Mean mass at birth (g)

Conspicuousness of colouration was evaluated by examination of images for evidence of conspicuous markings that may function as a warning signal, or an absence of conspicuous markings. In musteloids, crypsis is indicated by often brown colouration, often with a component of countershading; conspicuousness is indicated by stark black and white patterning (such as that of skunks, Mephitidae).

We collected multiple images of each species from specialist texts and websites (details and example photographs in Section 5.11, and classified species as conspicuous (by the presence of black and white markings) or not (by their absence). Therefore images were not standardised with regard to lighting, positioning, posture, or background, hence we could not make reliable measures of within pattern contrast and pattern-background contrasts. Rather we classified simply into presence or absence of conspicuous black and white patterning. In section 5.11 we give four example photographs which represent extremes of the range of colour patterns that we observed (two for conspicuous, two for nonconspicuous species). In our view, and as we hope the pictures in the supplementary materials show, the discrimination between species with and without conspicuous markings is clear cut, and these methods are generally similar to those used in a variety of studies looking at conspicuousness in prey (Sillen-Tullberg, 1988; Götmark and Unger, 1994; Tullberg and Hunter, 1996; Burns, 1998; Schaefer *et al*., 2002; Nilsson and Forsman, 1993; Santos *et al*., 2003; Vences *et al*., 2003; Chiari *et al*., 2004; Caro, 2005b; Inbar and Lev-Yadun, 2005; Sagegami-Oba *et al*., 2007; Bonacci *et al*., 2008; Przeczek *et al*., 2008; Pomini *et al*., 2010). To assess the consistency of this approach, where a species was included in Stankowich *et al*. (2011, which examined colour patterns of a selection of carnivorous mammals) we tested whether our assessments were independently consistent with their measure of conspicuousness (which they called 'salience'). In all cases where both species appear in both this was true, and thus we are confident that our classification of conspicuousness independently matches those used by other authors in other studies.

In considering activity periods over a 24 hours cycle we first classified individuals as activity generalists (we term 'circadian', active in parts of both day and night) or as activity specialists ('noncircadian', active in either day or night only). We next classified the times of day or night animals may be active in. When a species is known to be active in the day we class this as a diurnal component to its behaviour ("diurnal"). Similarly and animal that has activity in the night is classed as having a nocturnal component to its behaviour ("nocturnal"). Animals that are classed as "circadian" are therefore scored as both "diurnal" and "nocturnal", whereas animals classed as specialist with respect to activity period are classified as "noncircadian" and then as either diurnal or nocturnal. We thus have three categories (i. circadian/noncircadian, ii. presence or absence of a diurnal component, and iii. presence or absence of a nocturnal component in behaviour). Our choice of this coding scheme rather than a single variable with three levels (circadian, diurnal or nocturnal) was preferred for the following reasons: a) they are largely independent traits, since being coded as diurnal does not require any particular coding for nocturnality (due to circadian species being both), b) circadianism is measuring a different facet of temporal niche space, since unlike other variables, it refers to the breadth of the activity period, not merely its pattern, and c) in the context of whether an antipredator trait increases niche space it is notable that different selection pressures likely underlie nocturnal and diurnal activity (and by extension circadianism), and so we believe that looking for influences of chemical defence on each of these traits individually provides a more informative analysis.

We also used two measures of diet breadth. The first measure is “omnivory”, a binary character that records whether a species is strongly omnivorous or whether it is focused more on one type of food (vertebrates, invertebrates, or plant matter). The second measure is “diet diversity” a variable which measures the number of food categories included in each species diet, irrespective of their importance to the diet as a whole. We created nine diet categories: reptiles, amphibians, birds, mammals, fish, crustaceans, insects, other invertebrates, and plant material. These two measures of food habits capture different aspects of the biology: omnivory concerns whether the diet is typically highly variable as a whole, whilst diet diversity concerns all known food sources, including those that the animals are known to take at least in small amounts rarely.

Both the phylogeny and dataset used in this paper are available at http://dx.doi.org/10.6084/m9.figshare.1536708.

5.4.3 Analyses

In order to establish the appropriateness of phylogenetic comparative techniques we first tested the variables for phylogenetic signal. We used both Pagel's λ (Pagel, 1999) as implemented in the R (v2.14.1) package 'phytools' (R Development Core Team, 2011; Revell, 2012) and Blomberg *et al.*'s K as implemented in the MatLab program PhySig (Blomberg *et al.,* 2003). The finding of strong phylogenetic signal in all our variables justifies the use of comparative methods for analysing our data.

We tested for correlated evolution between chemical defence and all other binary variables using Pagel's (1994) test implemented in Mesquite v2.75 (Maddison and Maddison, 2011). We also tested for correlations between conspicuous colouration and all other binary traits. Pagel's test fits two models to the data, a simple (4-parameter) model assuming the traits evolve independently and a more complex (8-parameter) model assuming the traits evolve in a correlated manner. By analysing the likelihoods of the models we can test whether the more complex model is favoured over the simpler one. All of these tests were run with 25 iterations and a P-value was obtained from 2000 simulations. The directionality (positive or negative correlation) was investigated by use of a flow diagram showing the transition rate coefficients as described in Pagel and Meade (2006).

We used phylogenetic generalised least squares regression (PGLS) to test whether chemical defence (or conspicuousness) is correlated with our continuous variables. All pGLS analyses were carried out in the MatLab program REGRESSIONv2 (Lavin *et al.,* 2008).

Because regression analyses, in contrast to correlations, test for the effect of one variable on another rather than simply a correlation between two variables, we also ran phylogenetic logistic regression analyses (Ives and Garland, 2010) to test whether our continuous variables affect chemical defence or conspicuousness. These were run in the MatLab program pLogReg (Ives and Garland, 2010) with P-values determined by 2000 bootstrap replicates.

It was deemed unnecessary to correct for multiple comparisons since we did not analyse every possible combination of traits but rather focussed our analysis on a relatively few planned comparisons directly aimed at particular questions of interest (as per Nakagawa, 2004). This approach both limits the chance of spurious relationships being found and avoids potentially missing truly significant results as a consequence of lowering α (the cut-off level for accepting statistical significance). The latter is a particular problem with correction for multiple comparisons and often substantially reduces the statistical power of each test and increases the Type II error rate to an unacceptably high level (Nakagawa, 2004). Furthermore, procedures for correcting for multiple comparisons have been criticised by other authors on several grounds (Perneger, 1998). Moran (2003) highlighted that it is unclear how such procedures should be implemented: there is no logical way to decide whether such correction applies to each table, or the whole paper, or an even wider scale - therefore the application of the method becomes highly subjective and unreliable. This same author also argued that methods such as Bonferroni corrections effectively penalise detailed and/or comprehensive studies over more superficial work since as the number of questions asked increases it becomes less likely to find a significant result for any of them. In short, correcting for multiple comparison in our results would be both unnecessary and hamper clear inference.

5.5 Results

We observed significant correlations between both chemical defence and conspicuous colouration on three traits: diurnality (negative), omnivory (positive), and sexual size dimorphism (negative) (Table 5.2). There was also an extremely strong association between chemical defence and conspicuous colouration and a positive correlation between chemical defence (but not conspicuousness) and circadianism (Table 5.2). In contrast, there was no correlation between either chemical defence or conspicuousness and sociality, nocturnality, mating system, or territoriality (Table 5.2).

Chemical defence, but not conspicuous colouration, had a significant positive effect on diet diversity and a significant negative effect on longevity (Table

5.3). No effect was found of defence or conspicuous colouration on body size, litter size, maturation, or birth weight (Table 5.3).

Chemical defence, but not conspicuous colouration, was influenced by female maturation in that early female maturation favours the presence of chemical defence (Table 5.4). Neither chemical defence nor conspicuousness were affected by body size, diet diversity, longevity, litter size, or male maturation (Table 5.4).

Table 5.2. Results from Pagel's test of correlated evolution. The log-likelihood (logL) values are given for the simple model (assuming independent evolution) and for the more complex model (assuming correlated evolution). The difference between these values is given by ΔlogL. Significant P-values are highlighted in bold and their directionality is shown as being either a positive (+) or a negative (-) correlation. N is the number of species included in each analysis.

Traits	**N**	**logL Independent**	**logL Correlated**	**ΔlogL**	**P-value**	**Direction**
Chemical defence						
Conspicuousness	84	-72.500	-62.315	10.185	**<0.001**	+
Sociality	84	-81.366	-79.008	2.359	0.362	
Diurnality	84	-75.277	-68.067	7.210	**<0.001**	-
Nocturnality	84	-65.512	-62.699	2.814	0.065	
Circadianism	84	-67.066	-63.070	3.996	**0.028**	+
Omnivory	84	-64.512	-59.591	4.920	**0.016**	+
SSD	63	-64.459	-59.070	5.389	**0.018**	-
Mating system	55	-51.512	-50.784	0.727	0.730	
Territoriality	30	-34.734	-32.582	2.153	0.179	
Conspicuousness						
Sociality	84	-71.711	-70.287	1.424	0.122	
Diurnality	84	-67.009	-62.148	4.861	**0.025**	-
Nocturnality	84	-55.875	-54.597	1.278	0.128	
Circadianism	84	-58.798	-56.609	2.190	0.269	
Omnivory	84	-56.244	-50.567	5.676	**0.011**	+
SSD	63	-53.568	-48.930	4.638	**0.048**	-
Mating system	55	-38.937	-38.053	0.883	0.603	
Territoriality	30	-26.665	-24.624	2.041	0.054	

Table 5.3. Results from PGLS regression analyses. Significant P-values are highlighted in bold and their directionality is shown as being either a positive (+) or a negative (-) relationship. N is the number of species included in each analysis.

Explanatory variable	Response variable	N	r^2	F	P-value	Direction
Chemical defence	Body size	84	0.014	1.136	0.290	
	Diet diversity	74	0.360	40.521	**<0.001**	+
	Longevity	46	0.086	4.155	**0.048**	-
	Litter size	69	0.003	0.652	0.652	
	Male maturation	35	0.019	0.624	0.435	
	Female maturation	35	0.011	0.368	0.548	
	Birth weight	37	0.004	0.131	0.720	
Conspicuousness	Body size	84	0.003	0.613	0.613	
	Diet diversity	74	0.007	0.508	0.478	
	Longevity	46	0.001	0.029	0.865	
	Litter size	69	0.001	0.061	0.805	
	Male maturation	35	0.004	0.137	0.714	
	Female maturation	35	0.043	1.490	0.231	
	Birth weight	37	0.006	0.220	0.642	

Table 5.4. Results from phylogenetic logistic regression analyses. The regression coefficient (β) is given along with the standard error (SE) of this parameter. Significant P-values are highlighted in bold. N is the number of species included in each analysis. Direction is indicated by the sign of β.

Response variable	**Explanatory variable**	**N**	**β**	**SE**	**P-value**
Chemical defence	Body size	84	-0.088	0.056	0.111
	Diet diversity	74	0.098	0.077	0.236
	Longevity	46	-0.116	0.070	0.115
	Litter size	69	0.077	0.188	0.711
	Male maturation	35	-0.108	0.157	0.064
	Female maturation	35	-0.115	0.055	**0.029**
Conspicuousness	Body size	84	-0.062	0.053	0.243
	Diet diversity	74	0.058	0.057	0.517
	Longevity	46	-0.010	0.037	0.880
	Litter size	69	-0.032	0.169	0.860
	Male maturation	35	-0.059	0.055	0.264
	Female maturation	35	-0.093	0.050	0.056

5.6 Discussion

We first discuss our results related to niche space and then consider the relationship between chemical defence and particular traits.

5.6.1 Chemical defence and niche space

We found relationships, both positive and negative, between chemical defence and a number of key variables: diurnality, circadianism, omnivory, diet diversity, sexual size dimorphism, longevity and female maturation. Our most direct measures of niche space (omnivory, diet diversity, and circadianism) show an increase in frequency or magnitude in chemically defended species compared to non-chemically defended species. These effects on diet and activity periods are consistent with the idea that there are dual benefits from chemical defence: first a reduction in predation risk and second expansion of ecological opportunity. Well defended animals do not have to "hide" from potential predators, hence they can make use of a wider foraging niche (Merilaita and Tullberg, 2005; Speed *et al.,* 2010).

Chemical defence in the musteloids is then associated with a less restricted activity period (i.e. a higher likelihood of circadianism), but when defended prey do restrict themselves it is more often towards being strictly nocturnal (i.e. coded 0 for diurnality in our analysis) than diurnal. This follows from our finding that chemical defence is associated with both more circadian and less diurnal behaviour (Table 5.2). We note that reduced frequency of diurnality is more strongly significant than the increase in circadianism (Table 5.2). However, closer inspection of the transition rate parameters driving the changes (Arbuckle, unpublished data) shows that strength of the relationship itself is higher for the positive association between chemical defence and circadianism than between defence and loss of diurnality. The stronger statistical support for a relationship with diurnality may be simply due to there being more variation in this trait in musteloids since relatively few are strongly circadian, resulting in the higher observed level of significance. It is important to note that P-values themselves demonstrate our confidence in the presence of the relationship, but do not necessarily allow a good comparison of the strength of that relationship across different tests.

5.6.2 Aposematic display, chemical defence and niche space

We found that in musteloids conspicuousness almost certainly fulfils an aposematic function since it is only found in species that are chemically defended (Fig. 5.1). Hence there is no evidence of Batesian mimicry in which species which lack chemical defence copy the warning colouration of chemically defended prey (we now therefore refer to conspicuousness in musteloids as aposematic display). Notably aposematic display does not always show the same associations with our traits as chemical defence. There are three particular examples of this in our results (Table 5.2). First, a stronger positive relationship is found between omnivory and aposematism (defence plus bright colouration) than between omnivory and chemical defence alone. Although chemical defence itself can increase the diet breadth of a species, the effect is apparently enhanced if the defence is advertised by aposematic coloration. Second, we observe a weaker negative relationship between diurnality and aposematism than between diurnality and chemical defence. This suggests that the shift towards strict nocturnality is less influenced by aposematic signalling than it is by chemical defence *per se*. Finally, whereas a significant correlation exists between defence and circadianism, no such association is found between aposematism and circadianism. Therefore, although chemical defence facilitates circadian activity patterns this outcome is achieved irrespective of whether the defence is advertised with conspicuous colour patterns.

5.6.3 Predation pressure and strict nocturnality

When musteloid prey do restrict their activity period, why should they favour strict nocturnality over strict diurnality? Two obvious explanations are: 1) a lowered risk of predation at night and/or 2) a higher availability of resources at night either because there is more potential food per unit area, or less competition for the food. Although predators of musteloids are taxonomically diverse, it appears that most of those recorded are primarily nocturnal hunters including big cats, foxes, crocodiles, owls, snakes, and nocturnal musteloids (Walker, 1964; Long, 1973; Burton, 1976; Corbet and Southern, 1977; Kingdon, 1977; Lotze and Anderson, 1979; Hillman and Clark 1980; Powell 1981; Wade-Smith and Verts 1982; Chanin 1985; Clark *et al.,* 1987; Ford and Hoffmann, 1988; Poglayen-Neuwall and Toweill, 1988; Youngman, 1990; Estes, 1991; Dunstone, 1993; Sheffield and King, 1994; Gompper, 1995; Pasitschniak-Arts and Larivière, 1995; Neal and Cheeseman, 1996; Sheffield and Thomas, 1997; Gompper and Decker, 1998; Larivière and Walton, 1998; Larivière, 1998, 1999a,b, 2002a,b; Verts *et al.,* 2001; Hwang and Larivière, 2001, 2003, 2004; Dragoo and Sheffield, 2009; Prange and Prange, 2009). Interestingly, circadian species also have more nocturnal predators recorded than diurnal ones, again suggesting that predation pressure may be higher at night. Nevertheless this remains to be formally examined.

The main diurnal predators appear to be birds-of-prey, and these may be an important source of mortality (Stankowich *et al.,* 2014). For instance, Corbet and Southern (1977) suggested that *Martes martes*, an undefended species, is nocturnal due to high predation by golden eagles during the day. Hence it does not readily appear that musteloids shift away from strictly diurnal to circadian and nocturnal foraging when they acquire chemical defence because there are fewer predators. Of course this remains to be rigorously examined before it can be discounted as a reason for our result. Reduced competition or higher availability of food resources *per se* may then be a good explanation for this shift in activity patterns.

In this situation, evolution of chemical defence enables prey to exploit a predator-rich, but resource-rich, temporal environment that is relatively unavailable to non-chemically defended prey. By allowing prey access to more resources with less competition, chemical defence could allow increased fitness in nocturnal environments. In line with this idea, Bowers (1993) highlighted that cryptic caterpillars are more constrained to nocturnal activity (the period when predation is typically lower on these animals) than are chemically defended caterpillars. However we stress that to properly test this hypothesis quantitative data on diurnal vs nocturnal resource availability and competition are needed.

5.6.4 Lack of sexual size dimorphism

The presence of chemical defence and aposematic colouration was associated with a lack of sexual size dimorphism (SSD, though the effect was less strongly significant with aposematic colouration, Table 5.2). In mustelids where SSD is present (here more often animals without chemical defence), the dimorphism has been considered a result of selection favouring smaller, energetically cheaper females during reproductive periods and larger males which are able to exploit a greater variety of prey (Moors, 1980). Smaller females with lower energy requirements could then have a proportionally greater investment in reproduction.

It is not readily apparent why a negative relationship with chemical defence should be present. We can propose two hypotheses for this, one adaptive and one indirect. First, it may be that because defence can decrease constraints on activity and diet then the females of these species are under lower energetic pressures and can therefore evolve larger body size alongside males. Our data do not allow us to evaluate this hypothesis. Second, both chemical defence and SSD may both be influenced by another variable but in opposite directions. In line with this interpretation we found a strong positive correlation between SSD and diurnality (Pagel's test: N=63, ΔlogLik=5.548, P=0.009), and so it may be that being strictly nocturnal is unfavourable for a species with SSD but if activity is restricted then it is the better option for defended species.

5.6.5 Longevity

Because antipredator mechanisms exist to increase survival via reduced mortality from predators (Stewart *et al.,* 1999; Bosher *et al.,* 2006), the negative, albeit relatively weak, relationship between chemical defence and longevity is counterintuitive. Indeed, Blanco and Sherman (2005) found a positive relationship between chemical defence and an increased lifespan in fishes, reptiles, and amphibians. However, these authors did not explicitly take phylogeny into account in their analyses, they instead combined information for congeneric species together in an attempt to control for evolutionary history. As such it is unclear whether the positive correlation between chemical defence and longevity found by Blanco and Sherman (2005) will remain after full phylogenetic correction.

With regards to our own results we can propose three alternative explanations. First, some of the cost of the chemical defence may be paid for via physiological mechanisms which lead to a reduced lifespan (Faulkner and Ghiselin, 1983; Higginson *et al.,* 2011). Generation and storage of chemical defence may for example cause oxidative stress (Blount *et al.,* 2009) and hence cause nontrivial levels of tissue damage which shorten lifespan.

Second, with the acquisition of a chemical defence, prey may change their behaviour, taking more risks but gaining a higher net reward from foraging, for example switching to circadian or nocturnal foraging. A change to riskier behaviour can mean that the extrinsic rate of death for chemically defended species could be higher than for non-defended species and hence their lifetimes typically shorter, but this would be compensated by a raised rate of resource collection, perhaps by reduced competition. Overall the lifetime reproductive potential of chemically defended and non-defended musteloids may be similar, but the typical extrinsic rate of mortality quite different.

Third, an alternative explanation for the negative relationship between chemical defence and longevity may be third variable effects, whereby another variable affecting both defence and longevity causes the latter two to appear correlated in the absence of a direct relationship. We tested for life history correlations with longevity and found that it was strongly and positively related to female maturation - a trait that also negatively influences chemical defence (Table 5.4). This creates the possibility that later maturation acts to both increase longevity and decrease the probability of evolving chemical defence, causing an artefactual relationship between longevity and defence.

5.6.6 Traits not influenced by defence

In contrast to those above, the following ecological traits showed no relationship to chemical defence: sociality, strict nocturnality, mating system, territoriality, body size, litter size, maturation, and birth weight. It is interesting that many of our life history variables occur here. Life history traits are often involved in trade-offs with each other (Stearns, 1989) and this may result in a situation where each trait is unable to evolve independently, perhaps weakening the effects of a defensive trait such as chemical defence on any single life history variable. Nevertheless, traits that did and did not show a relationship with defence do not fall neatly into non-life-history and life history traits and so this cannot be a complete explanation. We note a marginally nonsignificant effect of aposematic colouration on territoriality (Table 5.2).

What comes first, broader niche use or chemical defence? - An important question is the order of change of traits within species. For example, strong competition may favour niche expansion, which in turn drives the evolution of chemical defence to protect animals that are now more exposed to predation. In an attempt to answer this question, we reran our Pagel's tests while constraining certain transition rate parameters to be equal, in line with the hypothesis tests outlined in Pagel (1994). These show a tendency to support the “defence to niche space” hypothesis over the “niche space to defence” hypothesis since the majority of the rate parameters were higher for the former scenario (Tables 5 and 6). Nevertheless only omnivory gave a significant result such that the evolution of chemical defence appears to lead to the evolution of omnivory (Table 5.5). On balance this leads us to favour the “defence to niche space” interpretation for many traits here, albeit with some caution. In particular our strongest support for this relates to dietary niche breadth, one of our most direct measures of niche space.

5.6.7 Comparison with previous work

Our study is complementary to (and in part inspired by) that of Stankowich *et al.* (2011) which examined ecological correlates of colour patterns in carnivores. But ours is different in its approach in four major ways: 1) Our focus is on how chemical defence influences the niche space that a species can occupy (Stamp and Wilkens, 1993; Merilaita and Tullberg, 2005; Speed *et al.,* 2010), not on which broad aspects of the habitat (e.g. density of vegetation) are related to aposematism. 2) We selected our traits with this aim in mind and include a number of life history traits (only two of our ecological traits overlap with Stankowich *et al.* (2011): body size and nocturnality). In addition 3) We used methods not included in the previous study; and 4) We used a different taxonomic sampling strategy: almost complete sampling of a subgroup of the taxa covered in Stankowich *et al.* (2011).

Nevertheless, it is useful to compare our results to Stankowich *et al.* (2011) for the few areas where our analyses overlapped. We found similar results with regards to a strong, positive relationship between chemical defence and conspicuousness. Contrary to our study, Stankowich *et al.* (2011) found some support for a relationship between body mass and conspicuousness, though this was relatively weak. This may result from our different taxonomic sample or a lower sample size (fewer species) causing lack of power in our analyses. However, our sample size was sufficient to detect strongly significant effects for other traits, so we do not think this is a suitable explanation. Despite recording nocturnality, Stankowich *et al.* (2011) tested for correlates of specific colour patterns and so we cannot compare our results directly.

In studies of chemical defence and niche in the dendrobatids Santos *et al.* (2003; also Santos and Cannatella, 2011) report an association between aposematism and diet specialisation, rather than the converse which we find here with the Musteloidea. One explanation is that dendrobatid frogs depend on specific dietary components for the acquisition of their toxicity (ants, termites, or mites) (Darst *et al.,* 2005), hence it is presumably not possible to both widen the feeding niche and acquire defensive toxins. Aposematism does appear to be associated with widening of other components of niche in the dendrobatids, such as a change from nocturnal to diurnal activity (similar to moths, Merilaita and Tullberg, 2005), leading to higher body mass and up regulation of metabolic rates.

5.6.8 Conclusions

We have provided evidence that chemical defence is associated with a variety of ecological traits, including measures of niche breadth, on a macroevolutionary scale in musteloids. Furthermore there is some evidence that chemical defence is driving this change in some traits (particularly omnivory) such that the defence opens up previously unavailable ecological opportunities. Finally, we found that conspicuous coloration almost certainly functions as an aposematic display in musteloids, and that aposematism can influence the relationships between chemical defence and other ecological traits.

5.7 Acknowledgements

We thank Ted Garland for kindly providing the MatLab programs used herein. M.P.S. thanks Andrew Higginson (University of Bristol) for preliminary help with comparative methods. We also thank Tim Caro for valuable discussions and two anonymous reviewers for valuable comments on the manuscript. This work was funded by a NERC Doctoral Training Grant to K.A.

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5.9 Supplementary Materials: Callibration points for phylogeny

Node	Age (mya)	Reference(s)
Musteloidea	32	Marmi *et al.*, 2004; Eizirik *et al.*, 2010
Mustelidae+(Mephitidae+Procyonidae)	30	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004
Procyonidae	22.1	Bininda-Emonds *et al.*, 1999; Eizirik *et al.*, 2010; Nyakatura and Bininda-Emonds, 2012
Bassariscus+Procyon	9.5	Eizirik *et al.*, 2010
Procyon	1.2	Bininda-Emonds *et al.*, 1999
Nasua+Nasuella	3.7	Bininda-Emonds *et al.*, 1999
Bassaricyon	17.1	Bininda-Emonds *et al.*, 1999; Nyakatura and Bininda-Emonds, 2012
Mephitidae	20	Eizirik *et al.*, 2010; Nyakatura and Bininda-Emonds, 2012
Mydaus	3.5	Bininda-Emonds *et al.*, 1999
Conepatus+(*Spilogale+Mephitis*)	17.5	Bininda-Emonds *et al.*, 1999; Nyakatura and Bininda-Emonds, 2012
Conepatus	3.3	Bininda-Emonds *et al.*, 1999
C.semistriatus+C.humboldtii	1.1	Bininda-Emonds *et al.*, 1999
Spilogale+Mephitis	11.6	Bininda-Emonds *et al.*, 1999; Nyakatura and Bininda-Emonds,

		2012
Spilogale	2.1	Bininda-Emonds *et al.*, 1999
Mephitis	5.2	Bininda-Emonds *et al.*, 1999
Mustelidae	18.4	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004; Eizirik *et al.*, 2010
Meles+*Arctonyx*	6.8	Bininda-Emonds *et al.*, 1999
Mustela+*Martes*+[other genera contained between these two]	12.5	Marmi *et al.*, 2004
Gulo+(*Martes*+*Eira*)	7.7	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004
Martes+*Eira*	7.1	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004; Eizirik *et al.*, 2010
Martes (except *M.pennanti*)	5.3	Marmi *et al.*, 2004
M.flavigula+*M.gwatkinsii*	0.9	Bininda-Emonds *et al.*, 1999
Martes (except *M.pennanti*, *M.flavigula*,*M.gwatkinsii*)	1	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004
Melogale	6.9	Bininda-Emonds *et al.*, 1999; Nyakatura and Bininda-Emonds, 2012
Mustela+*Lontra*+[other genera contained between these two]	11.5	Marmi *et al.*, 2004
Lontra+(*Enhydra*+(*Hydrictis*+(*Lutra*+(*Aonyx*+*Lutrogale*))))	9	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004
Lontra	1.7	Bininda-Emonds *et al.*, 1999; Nyakatura and Bininda-Emonds, 2012
L.longicaudis+(*L.felina*+*L.provocax*)	1.1	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004; Nyakatura and Bininda-Emonds, 2012
L.felina+*L.provocax*	0.6	Bininda-Emonds *et al.*, 1999; Nyakatura and Bininda-Emonds, 2012

Enhydra+(*Hydrictis*+(*Lutra*+ (*Aonyx*+*Lutrogale*)))	8.8	Marmi *et al.*, 2004; Nyakatura and Bininda-Emonds, 2012
Hydrictis+(*Lutra*+ (*Aonyx*+*Lutrogale*))	5.9	Marmi *et al.*, 2004
Lutra+ (*Aonyx*+*Lutrogale*)	5.1	Marmi *et al.*, 2004
Lutra	0.2	Bininda-Emonds *et al.*, 1999
Aonyx+*Lutrogale*	3.9	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004; Nyakatura and Bininda-Emonds, 2012
Poecilogale+*Ictonyx*+*Vormela*	4.2	Bininda-Emonds *et al.*, 1999
Galictis	1.8	Bininda-Emonds *et al.*, 1999; Nyakatura and Bininda-Emonds, 2012
Mustela	9.2	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004
M.africana+*M.felipei*	1.1	Bininda-Emonds *et al.*, 1999
M.erminea+*M.lutreola*+[other species contained between these two]	3.9	Marmi *et al.*, 2004
M.lutreola+(*M.altaica*+*M.nivalis*)+[other species contained between these two]	3.7	Marmi *et al.*, 2004
M.itatsi+*M.lutreola*+[other species contained between these two]	0.6	Marmi *et al.*, 2004
M.eversmanii+(*M.putorius*+*M.lutreola*)	0.2	Bininda-Emonds *et al.*, 1999; Marmi *et al.*, 2004

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5.11 Supplementary Materials: Details of character coding traits

Conspicuousness of colouration was evaluated by examination of images for conspicuous markings that may function as a warning signal. These images were obtained from internet searches and the literature. This meant that images were not standardised with regard to lighting, positioning, posture, or background, but the use of as many sources as possible for each species enabled us to get a complete picture of the colour patterns. We inspected the images for contrasting markings that appeared conspicuous and noted whether they were present or not. It should be noted that in musteloids the patterns are most often either shades of brown or contrasting black and white markings, therefore there were no cases where substantial uncertainty exists on whether or not a species was conspicuous. We have included some representative images in this appendix to illustrate the difference between conspicuous and cryptic musteloids (Fig. S5.1). While this is somewhat subjective it seems to provide a reasonable measure and has been used regularly in a variety of studies looking at conspicuousness (Sillen-Tullberg, 1988; Götmark and Unger, 1994; Tullberg and Hunter, 1996; Burns, 1998; Schaefer *et al.,* 2002; Nilsson and Forsman, 1993; Santos *et al.,* 2003; Vences *et al.,* 2003; Chiari *et al.,* 2004; Caro, 2005b; Inbar and Lev-Yadun, 2005; Sagegami-Oba *et al.,* 2007; Bonacci *et al.,* 2008; Przeczek *et al.,* 2008; Pomini *et al.,* 2010). Nevertheless, in order to assess the consistency of this approach where a species was included in Stankowich *et al.* (2011) we ensured that our judgment was independently consistent with their measure of conspicuousness (salience). In all cases this was true and thus we are confident that our classification of conspicuousness is a reliable indication of the appearance of the patterns. Although strictly speaking it was conspicuousness that was noted, we tested whether aposematism was present by looking at the association of this trait and chemical defence. This was coded simply as presence or absence.

Chemical defence was recorded as presence or absence based on whether anal gland secretions were used in defence. We did not subdivide this trait into categories based on how the secretions are used, as in Stankowich *et al.* (2011), because we were interested in whether chemical defence as a whole influences other traits, not the specific form of the defence. As such those species coded here as defended range from being able to spray the secretion over a distance and control the direction to those who simply dribbled the substance when threatened.

Sociality was recorded as whether the species is typically social or solitary. Mention of occasional groups forming in an otherwise solitary species were attributed to some unusual factor such as a rare feeding aggregation or chance encounters while moving around and were coded as solitary. Similarly where mothers with young pups where the only groups documented in an otherwise solitary species it was regarded as solitary. In other words, this variable represents the typical situation in the species ignoring any short-term contradictions since it is the common scenario that would be expected to drive any selection acting on sociality.

Activity patterns were coded in three ways: diurnality, nocturnality, and circadianism. This was done because (in circadian species) being diurnal and being nocturnal are not mutually exclusive, and so each measure captures a different facet of activity that may be under different selection pressure. Each of these traits were coded as 'yes' or 'no', diurnal if normally active during the day, nocturnal if normally active at night, and circadian if normally active during both periods. Note that for the sake of interpretation, strict nocturnality is the case when diurnality is absent (coded as 'no') and vice versa - being coded as diurnal or nocturnal does not in itself imply that they are strictly of that type due to circadian species. Since activity patterns will almost always present some exceptions from time to time, species were recorded as circadian if they spent a large amount of time active during both periods and not if they mostly limited their activity to either day or night. For our purposes, we have considered 'night' and 'nocturnal' to include crepuscular activity because diminishing light levels should present a selective environment more similar to night than day hours.

Similarly we used two measures of the dietary habits of a species. Firstly, we recorded diet as 'mostly invertebrate feeding', 'mostly vertebrate feeding', 'strongly omnivorous', or 'herbivorous' (the latter includes all plant material including fruits). Because most species were found to consume small amounts of one category but mostly another, e.g. a few invertebrates but mostly mammals, we concentrated on the food category that comprised the majority of the diet. Where a species was truly generalist and included a large proportion of more than one category (e.g. both vertebrates and invertebrates) it was coded as strongly omnivorous. We then simplified this classification to form our variable 'omnivory', which was simply coded as yes or no depending on whether the species was strongly omnivorous in the above scheme or whether it concentrated more on one category of food. Finally, to include all recorded dietary items including rare components we created the variable 'diet diversity'. This consisted of breaking up recorded diet items into nine categories: reptiles, amphibians, birds, mammals, fish, crustaceans, insects, other invertebrates, and plant material. We then recorded how many categories have been recorded in the diet of a given species, regardless of how important they are to the diet as a whole. This resulted in a relatively independent measure of diet breadth compared to our 'omnivory' variable. Firstly, a species coded with a diet of 'mostly vertebrates' can be inspected at a finer level, for example does it feed only on mammals or does it also prey on reptiles, amphibians, birds and perhaps rarely on some insects? Similarly, even those species coded as not omnivorous above maybe in fact consume a wide variety of foods, though most of them only rarely and so not be considered strongly omnivorous. Thus, our two measures of food habits capture different aspects of the biology: omnivory concerns whether the diet is highly variable as a whole whilst diet diversity concerns what foods the animal will take at least in small amounts rarely.

Sexual size dimorphism (SSD) was coded as presence or absence for each species. SSD in musteloids takes the form of bigger males, and where possible this was based on the distribution of adult body mass. If the distributions of male and female body mass showed little overlap then SSD was recorded as present, if the distributions of the sexes greatly overlapped then it was taken to be absent. In many cases such size distribution data were not available and so coding had to be based on reported sizes of males and females. Where this applied, we regarded males that were consistently 10% larger than females to be dimorphic.

Mating system was coded as monogamous or polygamous. Rarely has genetic monogamy been tested for in musteloids and so monogamy as used here must be considered to be social monogamy.

Territoriality was considered to be present when there was evidence of active defence of territories, and absent when there was evidence of tolerating intruders in the home range of an individual. We conservatively excluded cases where a species simply produces scent marks at the boundary of its home range but no fending off intruders has been noted.

Body size was recorded as the mean adult body mass of the species, or the midpoint of a given range if the mean was not presented. Where more than one source was available we extracted the value from each source and took the mean of these. Where more than one value or range was given for a species (e.g. geographic variation or SSD was present) we effectively treated each variant as another source and extracted the final value as above.

Longevity was recorded as the maximum lifespan in the wild where data on wild individuals existed. In some cases only captive longevity was provided and so to avoid upwardly biasing the data (captive lifespans are typically longer than wild lifespans for a given species) we took the mean captive longevity. We note here that in the few cases where only captive longevity was available the mean value of this was in the range of what might be expected for maximum wild longevity of the species in question. Furthermore when analyses were rerun excluding species for which captive longevities were used the results were qualitatively similar in that significant results remained significant except in one case (PGLS regression of longevity on chemical defence) where the significant result became marginally non-significant (likely as a sole result of the slightly reduced sample size). As such, the full dataset was used in the analyses presented herein.

Litter size was recorded as the mean litter size. Where different sources were available we treated the data as for body size, taking the mean value from the different sources to obtain a typical litter size for the species.

The minimum age at maturation for males and females in months was recorded where data was provided separately for each sex. If a maturation age was only available for the species as a whole then the same value was assigned to both males and females.

Birth weight was recorded in grams and where different sources were available we treated the data as for body size, taking the mean value from the different sources to obtain a typical birth weight for the species.

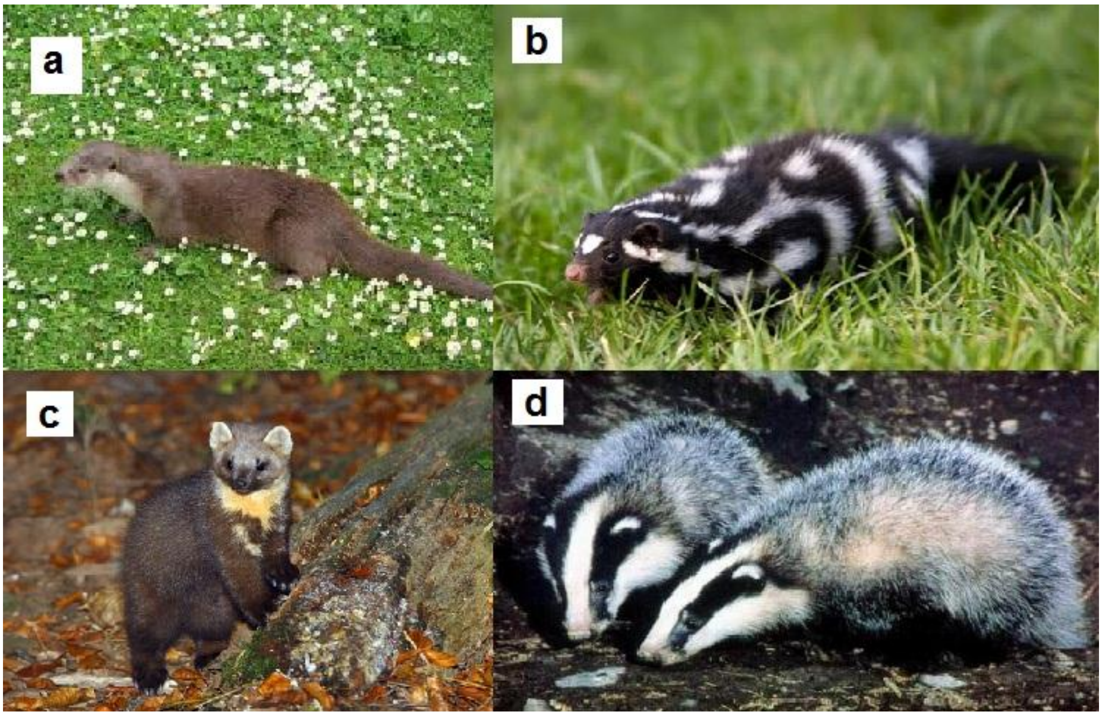

Figure S5.1 - Examples of musteloid coloration to illustrate differences between cryptic and conspicuous species. a) *Lutra lutra* - cryptic; b) Spilogale putorius - conspicuous; c) *Martes gwatkinsii* - cryptic but less clear cut end of range; d) *Meles meles* - conspicuous but less clear cut end of range.

6 Antipredator defences predict diversification rates

This chapter has recently been accepted for publication in *PNAS*.

6.1 Author Contributions

Kevin Arbuckle and Michael P. Speed

Institute of Integrative Biology, University of Liverpool, Liverpool, L69 7ZB, UK

Mike Speed provided comments on the manuscript and discussion of ideas. I developed the initial concept, designed the study, collected the data and conducted the analyses, and wrote the manuscript.

6.2 Abstract

The 'escape-and-radiate' hypothesis predicts that antipredator defences facilitate adaptive radiations by enabling escape from constraints of predation, diversified habitat usage and subsequently speciation. Animals have evolved diverse strategies to reduce the direct costs of predation, including cryptic colouration and behaviour, chemical defences, mimicry, and advertisement of unprofitability (conspicuous warning colouration). Whilst the survival consequences of these alternative defences for individuals are well studied little attention has been given to the macroevolutionary consequences of alternative forms of defence. Here we show, using amphibians as the first large-scale empirical test, that there are important macroevolutionary consequences of alternative defences, but that the “escape and radiate” theory, which focuses exclusively on speciation, does not adequately describe them. We examined how rates of speciation and extinction vary across defensive traits throughout amphibians. Lineages that use chemical defences show higher rates of speciation as predicted by 'escape-and-radiate', but also show higher rates of extinction compared to those without chemical defence. The effect of chemical defence is a net reduction in diversification compared to lineages without chemical defence. In contrast, acquisition of conspicuous colouration (often used as warning signals or in mimicry) is associated with heightened speciation rates but unchanged extinction rates. We conclude that predictions based on the 'escape-and-radiate' theory must incorporate the effect of traits on both speciation and extinction, which is rarely considered in such studies. Our results also suggest that knowledge of defensive traits could have bearing on the predictability of extinction, perhaps especially important in globally threatened taxa such as amphibians.

6.3 Introduction

The idea that defensive traits determine macroevolutionary patterns was originally suggested in the plant literature (Ehrlich and Raven, 1964) to explain heightened diversity. In this theory the presence of repellent chemical defenses was proposed to open up an 'adaptive zone' of diverse ecological opportunities and hence promote speciation by adaptive radiation. This became known as the 'escape-and-radiate' hypothesis (Schluter, 2000). Similarly it has been suggested that bright coloration can reduce constraints of hiding, enabling niche expansion and promoting diversification in animal prey (Merilaita and Tullberg, 2005; Speed *et al*., 2010). The 'escape-and-radiate' hypothesis is influential in the adaptive radiation literature (Schluter, 2000; Vamosi, 2005; Agrawal *et al*., 2009) but has been tested surprisingly rarely (Farrell *et al*., 1991; Agrawal *et al*., 2009; Weber and Agrawal, 2014). The very few macroevolutionary studies on animal defenses focus only on coloration, are small scale, and often only consider net diversification using comparisons of species richness in relation to the defensive trait of interest (Vamosi, 2005; Przeczek *et al*., 2008; Santos *et al*., 2014). However, diversification consists of two processes – speciation and extinction – and recent methodological developments (e.g. Maddison *et al*., 2007; FitzJohn, 2012) enable us to investigate each of these in relation to the evolution of a phenotypic trait. By explicitly modeling the effects of chemical defense and color variation on speciation and extinction rates separately, we are able to test the 'escape-and-radiate' prediction that chemical defense leads to greater diversity and to examine whether 'escape-and-radiate' is able to explain the macroevolutionary effects of defense variation.

Amphibians provide an exceptionally good case study with which to test the effects of defense variation on macroevolutionary trends. The phylogenetic history of this species-rich group is now relatively well resolved (Pyron and Wiens, 2013), they inhabit ecologically diverse habitats and include species that use a wide range of antipredator strategies, including chemical defense, camouflage, and conspicuous (aposematic) coloration (Duellman and Trueb, 1994). Importantly, chemical defense and coloration strategies are not strongly dependent on each other since some conspicuous species may not be chemically defended but use mimicry (or with conspicuousness favored by sexual selection instead) and chemical defense may be effective without advertisement, since many such species are cryptic (e.g. many toads). This therefore allows us to tease apart the effect of these two types of defense on diversification. Furthermore, because amphibians are currently facing severe threats from many different sources (Stuart *et al*., 2004), information on predictors of susceptibility to extinction is timely.

6.4 Methods

We used the most comprehensive and well-resolved phylogeny of amphibians to date (Pyron and Wiens, 2013) as the basis for this study. We then assembled a large dataset by searching published literature for information on the presence or absence of chemical antipredator defenses and for images from which to assess coloration for every species contained in the phylogeny (consisting of over 40% of currently known amphibian diversity, or 2871 species spanning all major clades). Chemical defense data were available for 857 species and coloration data were available for 2683, providing a large and well-sampled dataset for all analyses contained herein.

6.4.1 Data collection

We used both searchable and 'non-searchable' (e.g. books) literature to obtain data on chemical antipredator defense for all amphibians species for which we had phylogenetic data (i.e. that were included in the tree). For searchable literature, we used the following search term on a range of online literature databases and search engines, especially Web of Science and Google Scholar (where 'species' was substituted for the name of each species in turn):-

```
    ("species" OR "synonyms") AND ("chemical defense" OR toxin
OR venom OR poison OR "skin secretion")
```

Since amphibian taxonomy has been revised a great deal, we included all nomenclatural synonyms (obtained from the Amphibian Species of the World database) in the first part of the search term, replacing 'synonyms' in the search term above. Our search term was designed to generate a broad search so as not to exclude any literature. Every hit was then inspected manually to extract any information on the presence or absence of chemical defenses contained in each publication. In other words, we recorded data from literature where species were found to be toxic or where (after investigation) no toxins were found. Of the 857 species for which we found data on chemical defense, 35% were not found to possess chemical antipredator defense whereas 65% did. The defenses themselves can be either biosynthesized by the amphibians, sequestered from the diet, or both. A full list of all publications from which we obtained data is available in section 6.8 (Supplementary Materials).

Judgments of coloration are always dependent on the observer's visual system and other biological and situational factors, and so are unavoidably subjective to some degree, but such classification by human observers is a commonly used method to study animal color patterns in the literature. Perhaps most notably, coding of coloration based on the human visual system cannot account for ultraviolet (UV) colors, although we acknowledge that UV may also contribute to diversification dynamics. However, such detailed spectrographic data as would be needed to investigate this fully was neither available nor feasible to collect on this scale, and we feel that useful information can be obtained from visible (to the human eye) colors. Nevertheless, we took steps to ensure our coloration scores were as robust as possible. Firstly, we began with an extensive *a priori* definition of our basic coloration categories (cryptic or conspicuous), which was as follows:-

"A species was deemed to have conspicuous coloration if it possesses bright or contrasting patterns that creates a distinctive appearance which draws the attention of the observer, at least at close range. This often involves a combination of black with bright yellow, red, green, blue, or white, though single bright colors may also be judged conspicuous if they appear to make the animal stand out against its typical environment. In contrast, species were rated as cryptic when their color pattern renders them subjectively camouflaged, often consisting of brown, green (in arboreal species), or mottled patterns. Since some bright patterns may function as disruptive coloration and thus provide camouflage, where this is suspected coloration is recorded as 'uncertain' in order to remain conservative. Similarly, polymorphic species with both cryptic and conspicuous morphs will be recorded as such."

We then randomly selected 50 amphibian species using a random number generator and obtained photos of each of these. This set of 50 photos was given to three observers to score independently as either cryptic or conspicuous, along with the definition above. Note that only these two codings were allowed (not 'uncertain'), and so this initial assessment of inter-observer variability is less conservative than the actual procedure used during data collection. Nevertheless, full agreement was found for ~95% of species, and so coding of coloration was at least consistent amongst human observers and therefore not overly subjective. Consequently, data collection for both chemical defense and coloration was undertaken by one observer. Photos were obtained from various literature and online sources as well as directly from live animals, as available, for each species. As many photos as possible were obtained for each species in order to assess the variability within a species (to ensure we were able to detect polymorphism - which we recorded as such if some color forms were conspicuous and others were cryptic). Although we used a diverse search strategy, major online sources of images included AmphibiaWeb, Arkive, www.iucnredlist.org, and Google Images. Both the R scripts and data used in the analyses in this paper are available as a csv file from http://dx.doi.org/10.6084/m9.figshare.1248939.

6.4.2 Diversification models

We used a model-based inference approach (*sensu* Anderson, 2008) based on extensions and modifications of BiSSE (binary state speciation and extinction) models (Maddison *et al.*, 2007) that allow analyses of characters with more than two states (MuSSE, 'multistate speciation and extinction', used for coloration data), interactive effects of different traits on diversification, and that account for incomplete sampling (FitzJohn, 2012). All diversification models were fit in the R package diversitree version 0.9-7 (FitzJohn, 2012).

BiSSE models fit speciation (λ) and extinction (μ) rates for each state of a binary trait, giving four diversification parameters in total: λ_0, λ_1, μ_0, μ_1. Transition rate parameters are also estimated for the rate of shift from state 0 to state 1 (q_{01}) and from state 1 to state 0 (q_{10}). Therefore, the full BiSSE model contains 6 parameters, which can then be constrained to represent particular hypotheses. MuSSE models are simple extensions to BiSSE whereby speciation and extinction rates are estimated for more than two states and for transition rates for shifts between all states. They therefore contain a greater number, but essentially the same set of parameters as described for BiSSE above.

MuSSE multitrait models, which we use here to test for interactive effects of chemical defense and coloration on diversification use a different parameterization that is more akin to a general linear model framework. A 'background' rate (equivalent to the intercept in a linear model) for speciation and extinction is estimated, then coefficients representing changes attributable to trait 1 (i.e. chemical defense), coefficients representing changes attributable to trait 2 (i.e. coloration), coefficients representing changes attributable to the interaction of traits 1 and 2, and transition rates between each combination of states.

There has been a call for caution when estimating extinction rates from molecular phylogenies (Rabosky, 2010). However, all the models we implemented have been shown to give accurate estimates in simulations providing sample sizes are sufficiently large (over ca. 400 species) (Maddison *et al.*, 2007; Davis *et al*. 2013). Here, sample sizes for all analyses in this paper were far in excess of those required to derive accurate estimates (2871 species in the phylogeny in total, 2683 with data for coloration and 857 with data on chemical defense).

For both chemical defense (BiSSE) and coloration (MuSSE) data, we fit a set of five models designed to test whether and how these traits influence diversification of amphibian lineages: 1) a 'full' model including all parameters (no constraints); 2) a 'null' model assuming no influence of the trait and equal transition rates (all speciation, extinction, and transition rates constrained to be equal); 3) an 'equal diversification' model assuming no influence on speciation or extinction but allowing unequal transition rates (all speciation and extinction rates constrained to be equal); 4) an 'equal speciation' model assuming an influence on extinction but not speciation (all speciation rates constrained to be equal); and 5) an 'equal extinction' model assuming an influence on speciation but not extinction (all extinction rates constrained to be equal). In all cases, we accounted for missing species by including information on the proportion of amphibian species sampled, and for missing data by assuming that the proportion of species in each state for a given trait was equal to the observed frequencies. This approach is commonly used in such analyses, but assumes random sampling of species. To assess whether this assumption is reasonable, we tested for a correlation between total species richness and sampled species richness across the 74 currently recognised families of amphibians. In line with this assumption, we find strong correlations of these measures in both our chemical defence (r=0.76, t=9.78, P=7.6e-15) and coloration (r=0.94, t=23.19, P<2.2e-16) datasets.

Each of the above models were fit with maximum likelihood (ML) and comparisons were made within each model set using an information theory approach. The evidence for each model was quantified using ΔAIC scores (difference in Akaike Information Criterion between each model and the 'best' one as defined as that with the lowest AIC score), model probabilities (or 'Akaike weights'), and evidence ratios (the ratio of model probabilities for the best model compared to each other model in turn). This approach provides an explicit and ready means of comparing the strength of support for the models within our model sets.

Since a large phylogeny will inevitably have high heterogeneity in diversification rates, it is possible that any 'multi-rate' model (such as BiSSE or MuSSE) will be favored over a single-rate model (such as the null models described above) even for an arbitrary trait. To rule out this possibility and provide additional evidence that our results are indeed a consequence of the traits in question, we used a randomization approach. Specifically, we randomly distributed a trait with the same properties (i.e. frequency distribution and number of states) as our observed data over the tips of the phylogeny 100 times. For each of these 100 randomly distributed traits we fit the best model (as identified above) and list the minimum and maximum values obtained for our model comparison statistics. Such an approach makes use of an identical trait in all senses other than its association with diversification rates (as a result of decoupling the trait from the tree structure) to separate arbitrary preference of multi-rate models from the preferred (multi-rate) model for our observed data.

To ensure our coding of coloration was robust, given the subjective element of judging conspicuousness, we recoded all ambiguous species firstly as if they were cryptic and then as if they were conspicuous and followed the same analytical procedure as above using BiSSE models. This enabled us to investigate both extremes of bias (either towards cryptic or conspicuous judgments). The results from these analyses were qualitatively identical to the MuSSE models using our original coding scheme and are presented in section 6.8 (Table S6.3).

In order to incorporate uncertainty in parameter estimates and therefore allow a more robust inference from our models, we also fit the full models using Markov chain Monte Carlo (MCMC). Our MCMC analyses essentially followed the guidelines in the diversitree manual and help files (http://cran.r-project.org/web/packages/diversitree) and used an unbounded prior but with informative starting parameters (the ML estimates). We initially ran a Markov chain for 1000 steps in order to optimize the step size (w), and subsequently used this optimized value in the final MCMC run of 15000 steps. We conservatively discarded the first 5000 posterior samples for further analysis (although qualitatively identical results were achieved using the entire posterior sample in both the BiSSE and MuSSE models) and so used the posterior distributions of the last 10000 MCMC samples for inference. These distributions were then visualized along with their 95% confidence intervals to allow intuitive and robust interpretation of the results.

For our MuSSE multitrait models, we adopted a different model set to reflect our different aim - to assess evidence for an interactive effect between chemical defense and coloration on diversification rather than to infer each trait's influence. We fit a set of four models using ML wherein constraints were only imposed on the interaction coefficients: 1) a model including all interactions (no constraints); 2) a model assuming no interactive effect on diversification (interaction coefficients for speciation and extinction rates constrained to equal zero); 3) a model assuming no interactive effect on speciation, but allowing for one on extinction (interaction coefficient for speciation constrained to equal zero); 4) a model assuming no interactive effect on extinction, but allowing for one on speciation (interaction coefficient for extinction constrained to equal zero). Models were compared using the same information theoretic approach as for our BiSSE and MuSSE models, described earlier.

We note that a recent paper has highlighted that significant results can be obtained with the BiSSE class of models when the trait has only one of few origins in the phylogeny, even when no significant effect of the trait on diversification exists (Maddison and Fitzjohn, 2015). However, chemical defense has originated many times independently across the phylogeny (see section 6.8, fig. S6.3) and coloration similarly has many origins. Such a distribution, combined with our sister group analyses which are not susceptible to this issue, suggest that our analyses are robust to the problems highlighted here.

6.5 Results and Discussion

To carry out the first large scale, empirical test of the 'escape-and-radiate' hypothesis, we assembled a dataset of presence/absence of chemical defense in amphibians from the literature (of the 2871 species investigated, 857 had available data on the presence/absence of chemical defense). We then fit a range of trait-dependent models of diversification (BiSSE models assuming different influences of the chemical defense), and compared them using Akaike's information criterion (AIC).

In the best supported model from our analyses both speciation and extinction rates differ between chemically defended and non-chemically-defended amphibians. The speciation rate for chemically defended lineages is approximately twice as high as that for non-chemically-defended lineages, supporting a major prediction of the 'escape-and-radiate' theory. Unexpectedly however, extinction rates are three times higher in lineages with chemical defense than without it (Fig. 6.1), such that comparing chemically defended with non-defended lineages we see a reduction in net diversification rate. This lower net diversification rate in chemically defended lineages is not anticipated by 'escape-and-radiate' theory, suggesting that it may be adequate to predict effects on speciation but inadequate to predict effects on diversification as a whole. We further note that chemical defense was gained at a rate an order of magnitude (~ten-twentyfold) higher than it was lost (Table 6.1, Fig. 6.1). We note that many species-rich amphibian families with a large proportion of threatened species also have a large number of chemically defended species (e.g. Bufonidae and Ambystomatidae) (Stuart *et al.*, 2004).

To enable an investigation of protective coloration strategies in addition to chemical defenses, we assembled a dataset of cryptic versus conspicuous coloration (incorporating polymorphism, with both states) in amphibians from the literature (of the 2871 species investigated, 2683 had available data on coloration). We fit a similar range of trait-dependent models of diversification as with chemical defense (MuSSE models assuming different influences of the coloration), and compared them using AIC. The best supported model is one in which coloration influenced only speciation rate, but not extinction rate (Table 6.2). The speciation rate for conspicuous species is 2-3 times higher than that for species with cryptic coloration (Fig. 6.2). Since extinction rates were equal between these two coloration strategies, net diversification rate was also higher in conspicuous than cryptic species (Fig. 6.2). Polymorphic species (those with both cryptic and conspicuous forms within or between populations) have speciation rates equivalent to that of cryptic species (Fig. 6.2). Hence the macroevolutionary consequences of cryptic coloration and polymorphism are approximately equal. These results strongly support the hypothesis that conspicuous coloration increases speciation and net diversification rates compared to other forms of protective coloration, in line with developments of 'escape-and-radiate' theory (Merilaita and Tullberg, 2005; Vamosi, 2005; Przeczek *et al.*, 2008; Speed *et al.*, 2010).

Table 6.1. Model selection table for the influence of chemical defence on diversification. K = number of parameters; AIC = Akaike information criteria; ΔAIC = difference in AIC from the best model; w = Akaike weights (model probabilities); ER = evidence ratios (evidence for the best model/evidence for each model); λ, μ, q = speciation, extinction, and transition rates for species where chemical defence is absent (0) or present (1). Transition rates are denoted such that q_{ij} is the transition rate from state i to state j.

Model	**K**	**AIC**	**logLik**	**ΔAIC**	**w**	**ER**	**λ_0**	**λ_1**	**μ_0**	**μ_1**	**q_{01}**	**q_{10}**
Full	6	8592.83	-4290.42	0	0.989	1	0.1412	0.2836	0.0846	0.2632	0.0227	0.0010
Null	3	8623.05	-4308.53	30.22	<0.001	3.66E+06	0.2229	-	0.1906	-	0.0061	-
Equal diversification	4	8602.95	-4297.48	10.12	0.006	157.65	0.2255	-	0.1934	-	0.0096	0.0037
Equal speciation	5	8604.93	-4297.46	12.10	0.002	423.64	0.2222	-	0.1900	0.1898	0.0095	0.0037
Equal extinction	5	8604.53	-4297.27	11.70	0.003	347.89	0.2181	0.2221	0.1876	-	0.0088	0.0038
Random trait (min; max)	6	8800.23; 8936.67	-4462.34; -4394.12	207.40; 343.84	<0.001; <0.001	1.09E+45; 4.62E+74						

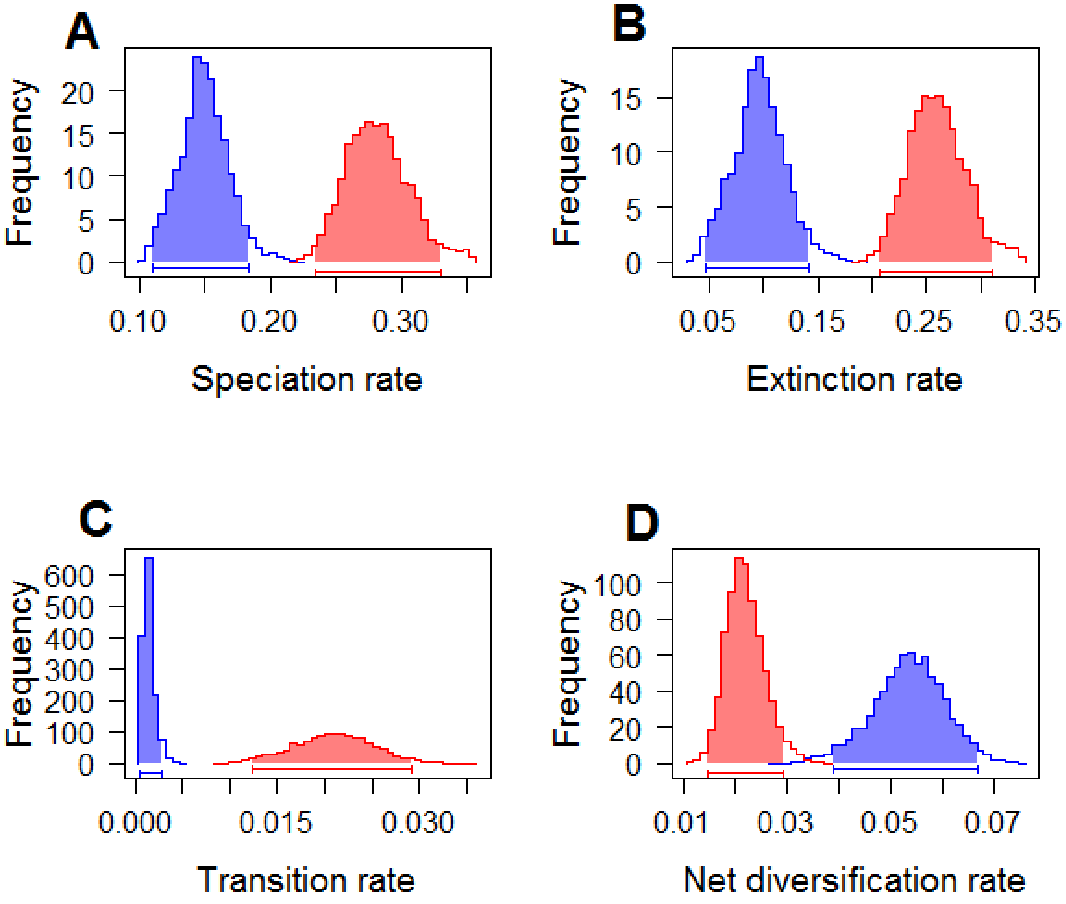

Figure 6.1. Posterior distributions of parameter estimates for speciation (a) and extinction (b) rates of chemically defended (red) and non-chemically-defended (blue) amphibians, and for transitions rates for gain (red) and loss (blue) of chemical defence in amphibians (c). Net diversification rate is shown in (d). Lines immediately beneath each distribution are 95% confidence intervals.

Table 6.2. Model selection table for the influence of colouration on diversification. K = number of parameters; AIC = Akaike information criteria; ΔAIC = difference in AIC from the best model; w = Akaike weights (model probabilities); ER = evidence ratios (evidence for the best model/evidence for each model); λ, μ = speciation and extinction rates for cryptic (0), polymorphic (1), and conspicuous (2) species. Transition rates are not shown here for clarity, see Extended Data for further details. Diversification parameters for 'uncertain' states are not shown but were estimated in the models.

Model	**K**	**AIC**	**logLik**	**ΔAIC**	**w**	**ER**	λ_0	λ_1	λ_2	μ_0	μ_1	μ_2
Full	20	24969.67	-12464.8	3.62	0.141	6.12	0.0614	0.0535	0.1472	0.0000	0.0027	0.0658
Null	3	25364.48	-12679.2	398.42	<0.001	3.29E+86	0.0675	-	-	0.0143	-	-
Equal diversification	14	25138.05	-12555.0	172.00	<0.001	2.23E+37	0.0676	-	-	0.0145	-	-
Equal speciation	17	25065.12	-12515.6	99.07	<0.001	3.26E+21	0.0636	-	-	0.0010	0.0515	0.0000
Equal extinction	17	24966.05	-12466.0	0	0.859	1	0.0614	0.0493	0.1093	0.0000	-	-
Random trait (min; max)	17	25476.80; 25774.77	-12870.4; -12721.4	510.75; 808.72	<0.001; <0.001	1.32E+110; 6.68E+174						

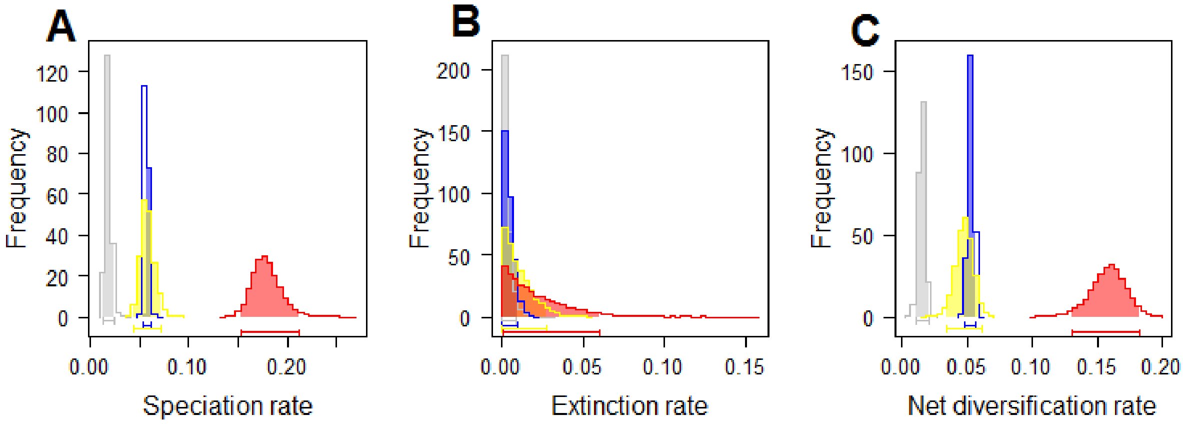

Figure 6.2. Posterior distributions of parameter estimates for diversification of amphibians using different protective colouration strategies: conspicuous species are in red, cryptic species are in blue, polymorphic species (those with both conspicuous and cryptic forms within or between populations) are in yellow, and species with uncertain strategies are in grey (these were not used for inference). (a) shows speciation rates, (b) shows extinction rates and (c) shows net diversification rates. Lines immediately beneath each distribution are 95% confidence intervals.

We note that BiSSE and MuSSE analyses may falsely find an effect of a trait if the pattern is driven by only one or few large clade(s), and can be susceptible to different diversification patterns across the tree. To ensure our results are robust to these potential issues, we corroborated our results with a relatively powerful sister group analysis, the richness Yule test (see section 6.8 for further details). Sister group analyses consider each clade as a single data point and so are not vulnerable to results dominated by a few large clades, and would require a consistent finding across multiple clades to yield a significant result. In all cases we find that our results are supported ($P<0.05$) by these analyses which strengthens our conclusions derived from BiSSE and MuSSE modeling. Specifically, sister group analyses found that chemically defended amphibians had lower diversification rates than non-chemically-defended amphibians, and conspicuous species had higher divesification rates than cryptic species.

Analyzing defensive traits separately as we have done leaves open the possibility that chemical defense and coloration have a non-additive effect on diversification rates, perhaps through synergetic benefits of aposematism (chemical defense plus conspicuous 'warning' coloration). However, when we explicitly model an interaction between chemical defense and coloration (conspicuous versus cryptic) we find that the best models support limited or no interactions on diversification (see section 6.8, Table S6.1). Specifically, there are two best models: no interactive effect on extinction rates only or no interaction term for extinction or speciation rates (see section 6.8, Table S6.1). In addition, although we found a positive correlation between the presence of chemical defense and conspicuous coloration (see section 6.8), these traits influence diversification in different ways (cf. Figs. 6.1 and 6.2). Combined, this evidence strongly indicates that our results for chemical defense are not being driven by conspicuous coloration and vice versa. Our results are consequently the first to reveal that different antipredator strategies can have independent effects on speciation and extinction.

'Escape-and-radiate' predicts that chemical defense should lead to higher diversification rates. Our results show that the theory, which is widely cited and used, requires extension because of its failure to account for effects on extinction rates. We therefore propose that 'escape-and-radiate' should be seen as one component of a more general theory of macroevolutionary effects of antipredator defense that includes both speciation and extinction. 'Escape-and-radiate' is therefore considered to be a special case when the change in extinction rate conferred by the trait is less than the change in speciation rates, or when there is either no effect on extinction rate or it is lowered. However, when a trait leads to a greater increase in extinction than speciation rates, as with our results for amphibian chemical defense, we find a lower net diversification rate in contrast to predictions from 'escape-and-radiate'. Therefore, our expanded theory encapsulates 'escape-and-radiate' predictions whilst also providing a framework for considering deviations from these predictions, accounting for all of our observations in the present study.

'Escape-and-radiate' predicts that effective chemical defense opens up 'adaptive zones', in effect more diverse niches for colonization, leading ultimately to raised speciation rates. Addition of bright coloration implies the reduced hiding from predators, and hence greater use of opportunity in a habitat, which may explain the rise in speciation rates since ecological opportunity has previously been shown to promote speciation (Burbrink and Pyron, 2010; Weber and Agrawal, 2014). In effect, conspicuousness could be an indicator of this mechanism, rather than directly influencing diversification. On the other hand, bright coloration is often associated with mate choice, so that sexual selection can act on variation in particular color patterns to drive heightened speciation in conspicuous sexual signals (Higashi *et al.*, 1999). In the polymorphic dendrobatid frog, *Dendrobates pumilio*, for example, males in conspicuously colored populations are more aggressive in male-male contests and more explorative than those in cryptically colored populations (Rudh *et al.*, 2013), facilitating divergence via intrasexual competition and intersexual mate preferences. Furthermore, a recent study on birds has shown that color polymorphic lineages have higher speciation rates, further highlighting that sexually selected color patterns can be responsible for increasing speciation rates (Hugall and Stuart-Fox, 2012).

How can defenses lead to heightened extinction rates? We can suggest three mechanisms which could account for this result. First chemical defense may impose particular kinds of cost which render prey populations vulnerable to other kinds of enemy such as infectious diseases, as illustrated in the case of a nymphalid caterpillar (Smilanich *et al.*, 2009). Second, chemically defended species may radiate by moving into habitats with low carrying capacities, making them intrinsically vulnerable to extinction. Third, recent comparative work with amphibians shows that chemical defense may cause a shift toward slower life histories, which in turn can weaken the resilience of the population to detrimental environmental change (Davidson *et al.*, 2009; Hutchings *et al.*, 2012) and raise extinction rates. At present there are no data with which to tease these potential explanations apart, but together they provide a plausible set of hypotheses to explain the (strongly supported) patterns we find here.

We suggest in addition that even though it is often transient, conspicuousness confers a benefit to chemically defended lineages on an evolutionary time scale: offsetting the increased extinction risk. Because effects of chemical defense and conspicuous coloration on diversification are independent, when a chemically defended species becomes conspicuous, the increased diversification rate conferred by the coloration can compensate for the reduced diversification rate conferred by the chemical defense. This could partly explain the positive correlation between the presence of the two traits (see section 6.8, Table S6.4, fig. S6.2), since chemically-defended lineages without such ‘diversification compensation’ are more likely to die out than those that also possess conspicuousness.

Therefore, a higher proportion of extant chemically-defended species will be conspicuous than we would expect based on the total number of chemically-defended species that have ever existed, as such species are less likely to have gone extinct, and this disproportionate distribution of conspicuousness amongst chemically-defended species should manifest itself in the positive correlation that we observe.

Our results also suggest that chemically-defended species (and perhaps especially those lacking conspicuous coloration) may justifiably be targeted by conservation programs, all else being equal, due to their higher extinction rates. When faced with a threat such as habitat loss or disease, these species may particularly struggle to recover their populations in the aftermath and so may require more conservation effort than an equivalent non-chemically-defended species.

Many traits and processes are likely to influence diversification in any animal group, including amphibians, and previous studies have found effects of factors such as latitude (Wiens, 2007; Pyron and Wiens, 2013) and the rise of angiosperm forests (Roelants *et al.*, 2007) and the new habitats and prey diversification that arose as a consequence, amongst other traits. As such, we note that our aim here was not to investigate the relative influence of antipredator defence compared with other traits, but to specifically examine a highly influential macroevolutionary theory for such defences. We therefore do not wish to claim that antipredator strategies are the only, or most important, factor influencing diversification, but rather that it is a contributing factor that sheds light on our understanding of the evolutionary consequences of natural enemy interactions.

In summary, we report the first large-scale test of the diversification predictions of 'escape-and-radiate' theory. While there is strong support for its central prediction of heightened speciation rates, we show that it is a special case which is subsumed within a more general theory of defense-driven diversification that incorporates both speciation and extinction. Finally, we stress that overall antipredator defense often consists of multiple components which have contrasting effects on diversification. Only large-scale investigations such as this study will be able to elucidate the overall impact on evolutionary diversification.

6.6 Acknowledgements

We thank R.A. Pyron for providing a copy of the dated amphibian phylogeny prior to its publication and R. Ward for giving permission to use his photo of *Dendropsophus leucophyllatus*, which appears in Figure S6.2. We also thank M. Begon, M. Berenbrink, M. Brockhurst, T.A.R. Price, and J. Rees for constructive comments on the manuscript. M. Pennell suggested improvements to the manuscript which were gratefully received and helped strengthen our conclusions further. K.A. was funded by a Doctoral Training Grant from NERC.

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6.8 Supplementary Materials

Table S6.1. Model selection table for the interactions between chemical defence and colouration (cryptic versus conspicuous) on diversification. K = number of parameters; AIC = Akaike information criteria; ΔAIC = difference in AIC from the best model; w = Akaike weights (model probabilities); ER = evidence ratios (evidence for the best model/evidence for each model).

Model	**K**	**AIC**	**logLik**	**ΔAIC**	**w**	**ER**
All interactions	15	8807.67	-4388.84	52.36	<0.001	2.35E+11
No diversification interaction	13	8757.31	-4365.65	2	0.269	2.71
No speciation interaction	14	8824.80	-4398.40	69.49	<0.001	1.23E+15
No extinction interaction	14	8755.31	-4363.66	0	0.731	1

Table S6.2. Maximum likelihood estimates of the transition rate parameters estimated in the full MuSSE model for colouration. See Figure S1 for posterior distributions of these same parameters as estimated by MCMC.

Transition from	**Transition to**	**ML estimate**
Uncertain	Cryptic	0.0055
	Polymorphic	0.0048
	Conspicuous	0.0001
Polymorphic	Uncertain	0.0036
	Cryptic	0.0209
	Conspicuous	0.0059
Cryptic	Uncertain	0.0006
	Polymorphic	0.0015
	Conspicuous	0.0003
Conspicuous	Uncertain	0.0079
	Polymorphic	0.0113
	Cryptic	0.0150

Table S6.3. Model selection table for the influence of colouration on diversification when ambiguous species are coded as cryptic (con0 model set) or conspicuous (con1 model set). K = number of parameters; AIC = Akaike information criteria; ΔAIC = difference in AIC from the best model; w = Akaike weights (model probabilities); ER = evidence ratios (evidence for the best model/evidence for each model); λ, μ, q = speciation, extinction, and transition rates for species where colouration is cryptic (0) or conspicuous (1). Transition rates are denoted such that q_{ij} is the transition rate from state i to state j.

Model (con0)	**K**	**AIC**	**logLik**	**ΔAIC**	**w**	**ER**	**λ0**	**λ1**	**μ0**	**μ1**	**q01**	**q10**
Full	6	23670.98	-11829.49	112.07	<0.001	2.17E+24	0.0594	0.1714	0.0000	0.1648	0.0013	0.0023
Null	3	23799.82	-11896.91	240.92	<0.001	2.07E+52	0.0656	-	0.0126	-	0.0007	-
Equal diversification	4	23719.27	-11855.63	160.36	<0.001	6.64E+34	0.0653	-	0.0121	-	0.0007	0.0242
Equal speciation	5	23695.63	-11842.82	136.73	<0.001	4.89E+29	0.0606	-	0.0010	0.0470	0.0010	0.0058
Equal extinction	5	23558.90	-11774.45	0	1	1	0.0592	0.1818	0.0239	-	0.0015	0.1473

Model (con1)	**K**	**AIC**	**logLik**	**ΔAIC**	**w**	**ER**	**λ0**	**λ1**	**μ0**	**μ1**	**q01**	**q10**
Full	6	24547.92	-12267.96	0	0.998	1	0.0612	0.0985	0.0000	0.0812	0.0040	0.0044
Null	3	24705.56	-12349.78	157.64	<0.001	1.70E+34	0.0658	-	0.0128	-	0.0027	-
Equal diversification	4	24602.86	-12297.43	54.94	<0.001	8.51E+11	0.0658	-	0.0128	-	0.0021	0.0169
Equal speciation	5	24560.83	-12275.41	12.90	0.002	633.80	0.0616	-	0.0000	0.0384	0.0034	0.0067
Equal extinction	5	24610.30	-12300.15	62.38	<0.001	3.51E+13	0.0518	0.1419	0.0185	-	0.0051	0.1102

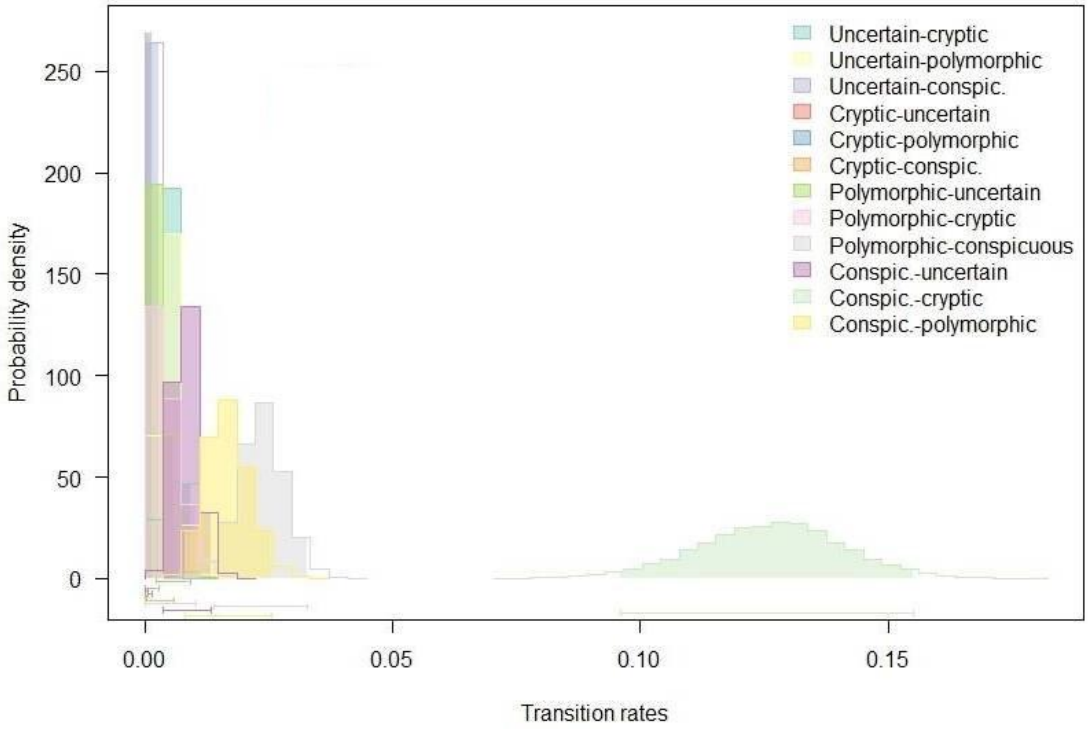

Figure S6.1. Posterior distributions of transition rates from MCMC analysis of the full MuSSE model for colouration. Lines immediately beneath each distribution are 95% confidence intervals and, as well as being colour matched, are in the same vertical order as the legend. Note in particular the relatively high rate of loss of conspicuousness by reverting straight to a cryptic state. Furthermore, the 95% confidence intervals of some transitions overlap zero (including cryptic-conspicuous and polymorphic-cryptic, although the latter has a wider range). Taken together, these transition rates imply that cryptic lineages first shift to a polymorphic state, from which they commonly change to conspicuous only. Once conspicuous, reversions to polymorphism can occur but a direct reversion to cryptic colouration is far more common.

Correlated Evolution Between Chemical Defence and Colouration

We tested for correlated evolution between our two traits of interest (chemical defence and colouration) since they are often reported to coevolve (including in poison dart frogs; Summers and Clough, 2001) and understanding the relationship between the traits may inform discussion and interpretation of analyses which use both traits. We used two alternative methods and find evidence for correlated evolution with both. Firstly, we fit Pagel's (1994) models of correlated and independent evolution in the corHMM package in R (Beaulieu *et al*., 2014), and assessed the fit of these models using AICc scores (the best model considered to be that with the lowest AICc). Since these models require binary traits, we first converted our colouration data to binary format. Because 'ambiguous' (e.g. polymorphic) lineages tend to show evolutionary patterns indistinguishable from cryptic lineages, we considered those as cryptic, and only those which we coded as such to be conspicuous in the following analyses. However, we also ran models with the alternative extreme coding ('ambiguous' colouration considered to be conspicuous) and obtained qualitatively identical results. The best model represented correlated evolution (AICc = 1057.694, cf. AICc = 1088.119 for an independent evolution model) and is visually displayed in Figure S6.2 using flow diagrams - a standard way to present such models (Pagel and Meade, 2006).

Secondly, we fit generalised estimating equation (GEE) models to account for phylogeny, with colouration as the response variable and chemical defence as the explanatory variable. A null model (intercept only) was also fit to assess whether the first model explained the data well. The models were compared using QIC - an equivalent criteria to AIC for GEE models which similarly gives a lower score for better models). GEEs were fit in the ape package in R (Paradis *et al*., 2004). Consistent with the Pagel's models, we found that chemical defence was a significant predictor of conspicuous colouration (Table S4), and that this model was far better than the null model (QIC = 440.00, cf. QIC = 492.47 for the null model).

Both of these analyses strongly support a model of correlated evolution between colouration and chemical defence wherein chemically defended species are more likely to be conspicuous and vice versa. However, there are also many exceptions and hence we were still able to recover independent effects of these two traits on diversification in amphibians (see main text).

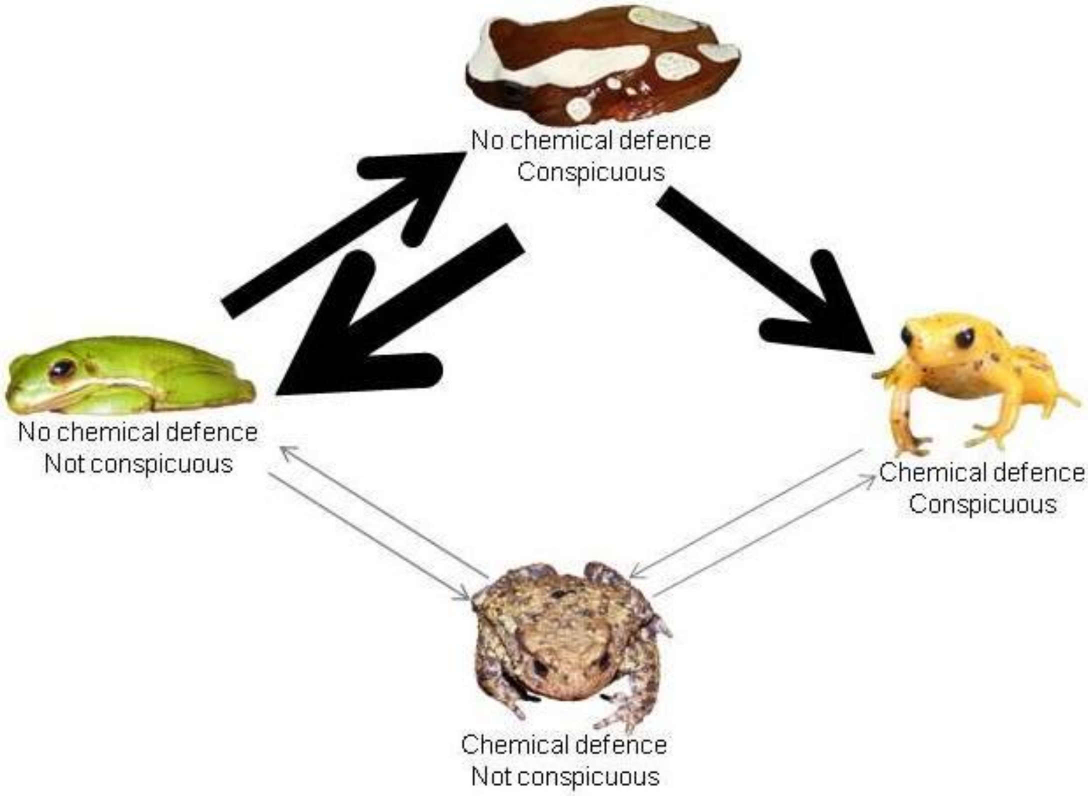

Figure S6.2. Flow diagram showing the correlated evolution of chemical defence and conspicuousness in amphibians. Arrow thickness is related to transition rate (thicker arrows, higher transition rate) and grey arrows represent very small (but non-zero) transition rates.

Table S6.4. Results from a phylogenetic GEE model with conspicuousness as the response variable and chemical defence as the explanatory variable. Model was run on all species for which we had data on both colouration and chemical defence (N = 857).

Model term	**Coefficient (Std Err)**	**t**	**P**
Intercept	-6.381 (1.003)	-6.363	4.57e-9
Chemical defence	4.234 (1.011)	4.189	5.65e-5

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Sister Group Comparisons

In order to check whether our results obtained from BiSSE and MuSSE models could be corroborated by other methods, we used sister groups analysis to compare the diversity in sister lineages which differ by trait. Paradis (2011) developed a new sister group method (the 'richness Yule test') and compared it to existing alternatives. He found the richness Yule test to be more powerful than other approaches and so we used this method to ask whether we find the same effects of chemical defence and colouration on net diversification as we did using BiSSE and MuSSE models.

Lineages with chemical defence had fewer species than sister groups that did not ($\chi^2 = 7.7269$, df = 1, P = 0.0054). The method requires binary traits, so colouration was coded as for the correlation analyses in the previous section. Nevertheless, conspicuous lineages had more species than cryptic lineages whether ambiguous species were treated as cryptic ($\chi^2 = 4.0311$, df = 1, P = 0.0447) or conspicuous ($\chi^2 = 11.9799$, df = 1, P = 0.0005). Therefore, in all cases, results were the same between the richness Yule tests and the BiSSE/MuSSE models.

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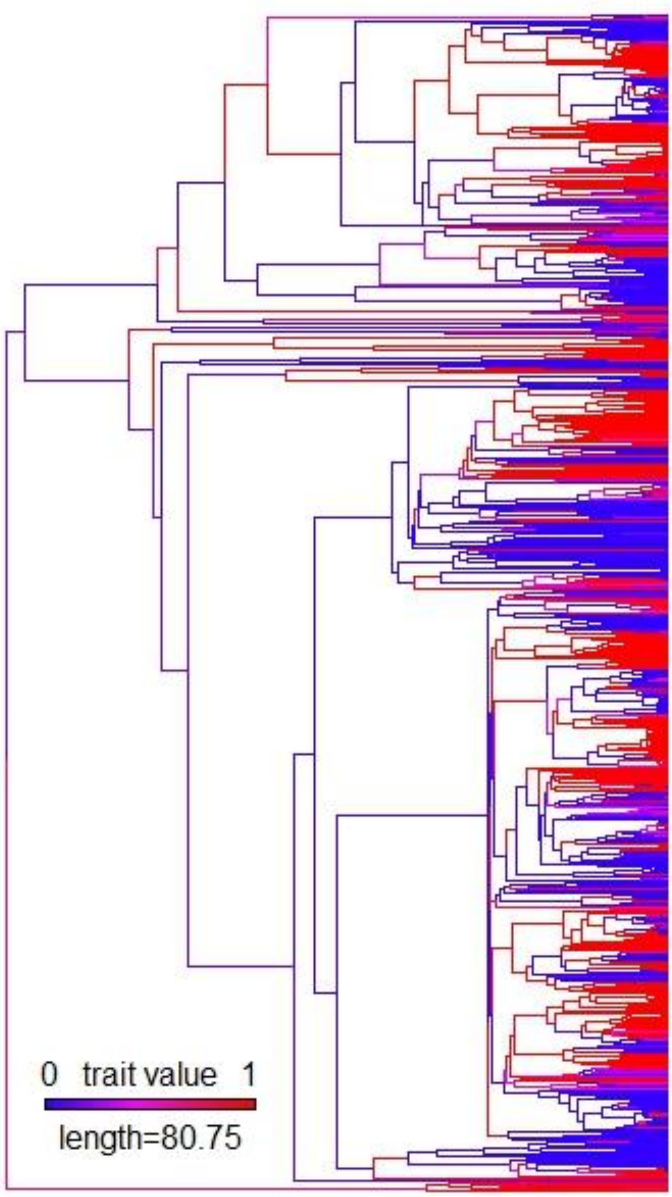

Figure S6.3. Ancestral state reconstruction for chemical defence based on the parameters from the best BiSSE model. Colours refer to the probability that chemical defence was present in the branch. Note that chemical defence has evolves many times across the phylogeny.

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7 Chemical antipredator defence is linked to higher extinction risk

This chapter is currently being updated with additional analyses following comments during viva prior to submission as a paper..

7.1 Author contributions

Kevin Arbuckle and Michael P. Speed

Institute of Integrative Biology, Biosciences Building, University of Liverpool, Crown Street, Liverpool, Merseyside, L69 7ZB, United Kingdom.

Mike Speed provided comments on the manuscript and discussion of ideas. I developed the initial concept, designed the study, collected the data and conducted the analyses, and wrote the manuscript.

7.2 Abstract

Many attributes of species have been found to impact on macroevolutionary (background) extinction rates of diverse taxa, but how such results relate to contemporary extinction risk is uncertain. Here, we test whether a trait associated with higher background extinction rates, chemical antipredator defence, is also associated with current extinction risk using amphibians as a model system - a group facing global population declines. We find that chemically-defended species are ~60% more likely to be threatened than species without chemical defence, and that species experiencing the highest levels are more likely to have chemical defence. Our results confirm that background and contemporary extinction rates can be predicted from the same traits, at least in certain cases. They also provide novel insights into predisposing factors for conservation concerns and add to a growing number of traits which may be useful for predicting threat status for allocation of limited conservation resources.

7.3 Introduction

The conservation of biodiversity remains an important application of ecology , evolution, and behaviour. Consequently, there is a growing requirement that decisions about allocation of limited resources (such as finances and personnel) to conservation efforts have a firm evidence-base (Pullin and Knight, 2009; Waldron *et al.*, 2013). Typically, such decisions are aimed to benefit those species deemed to be at higher risk of extinction, as often determined by the International Union for Conservation of Nature's (IUCN) Red List categories (Mace *et al.*, 2008). However, the IUCN currently lists 16.5% of species (12,609 of 76,199 species) as data deficient (IUCN 2014) and many species are not yet included on the list at all. Moreover, attempts to predict the conservation status of data deficient species have suggested that they are likely to have higher extinction risks than fully assessed species (Morais *et al.*, 2013; Bland *et al.*, 2015a).

As a result of this lack of information on the conservation status of many species, several studies have attempted to investigate potential correlates of extinction risk (mostly using Red List status as a proxy) from phenotypic traits (e.g.

Purvis *et al*., 2000; Reed and Shine, 2002; Fisher and Owens, 2004; Bielby *et al*., 2008; Cooper *et al.*, 2008; García *et al*., 2008; Verde Arregoitia *et al*., 2013; Jeppsson and Forslund, 2014; Bland *et al*., 2015a). This is an attractive option as we know at least some information about the biology of many species which have not yet been fully assessed by the IUCN. Furthermore, it is expected that certain traits should impact on factors such as mortality rates (e.g. Boonekamp *et al*., 2014; Healy *et al.*, 2014) or the ability of populations to recover after declines (Hutchins *et al.*, 2012; Saenz-Agudelo *et al*., 2015), and therefore such traits are expected to be justifiable predictors of extinction risk. Nevertheless, since each trait is imperfectly linked to extinction risk, a combined evidence approach is necessary which requires evaluation of many traits that may be linked to conservation status. Once we know which traits predict conservation status, and how well they do so, we can use these to inform decisions relating to resource allocation for conservation.

There has been a recent surge of interest in identifying traits that correlate with macroevolutionary ('background') diversification rates, and these studies have found that a wide range of traits influence the net diversification, speciation, and extinction rates of many groups (e.g. Przeczek *et al.*, 2008; Hugall and Stuart-Fox, 2012; Pyron and Weins, 2013; Silvestro *et al.*, 2013; Weber and Agrawal, 2014). Traits that are linked to increased background extinction rates may also lead to a greater contemporary extinction risk, however studies looking across temporal scales are lacking. It is perhaps unsurprising that this area has been neglected since different threats are likely to be in operation now than throughout evolutionary history. For instance, many threats currently facing biodiversity today are anthropogenic in nature (Dudgeon *et al.*, 2006; Jennings and Rohr, 2011; IUCN, 2014), such as rapid habitat destruction, exploitation, or pollutants. As such, different traits may be important in mediating the extinction risk of species today than would have been important in the past. Nevertheless, the effect of a trait on extinction risk may operate by general mechanisms that make it possible to carry over conclusions about background extinction rates to contemporary extinction risk. For instance, species with a slow life history may find it difficult to recover populations after declines when compared to similar species with faster life histories (Bielby *et al*., 2008; García *et al*., 2008), regardless of the cause of the decline. At the very least, studies of trait-dependent diversification can lead to testable hypotheses that may point to traits that predict present-day extinction risk.

Amphibians are currently considered the most threatened vertebrate taxon and are experiencing population declines globally for both anthropogenic and enigmatic reasons (Stuart *et al.*, 2004; Wake and Vredenburg, 2008; IUCN, 2014). Consequently, a range of traits have been evaluated as predictors of extinction risk in this group (Bielby *et al.*, 2008; Cooper *et al.*, 2008; Howard and Bickford, 2014), and also as drivers of evolutionary diversification patterns (Przeczek *et al.*, 2008; Wollenberg *et al.*, 2011; Pyron and Weins, 2013; Arbuckle and Speed, in press, see Chapter 6). A recent macroevolutionary study revealed that chemical antipredator defence is associated with higher extinction (Arbuckle and Speed, in press; see Chapter 6), a trait not previously considered in studies of contemporary extinction risk. In this paper, we test the prediction from this work that chemically-defended amphibian species also face a greater current risk of extinction, and in doing so test whether studies of background extinction rates can offer insights on a contemporary scale that is relevant to setting conservation priorities.

We demonstrate that, as predicted from macroevolutionary work, chemical defence is associated with a greater extinction risk in contemporary amphibian species. This adds a novel trait to those useful in evaluating the conservation status of species and therefore provides additional information that may be useful for setting conservation priorities (as part of a holistic approach). Furthermore our results suggest that, at least for some traits, macroevolutionary studies of trait-associated diversification rates can be relevant to present-day conservation concerns.

7.4 Methods

7.4.1 Data collection

Data on the presence or absence of chemical defence in 857 amphibian species were extracted from Arbuckle and Speed (in press; see Chapter 6). Briefly, this dataset was assembled from literature searches using a conservative approach in which data were only recorded for each species if it had been investigated and found to either possess or lack a chemical defence. If information was not available for that given species no data were recorded and, consequently, species included in this study are known to either possess or lack a chemical antipredator defence. Further details on the collation of this dataset are available in the original paper (Arbuckle and Speed, in press; see Chapter 6).

To assess extinction risk, we used IUCN Red List categories as a standard and widely-used proxy (Mace *et al*., 2008). We searched the IUCN Red List database (IUCN, 2014) for all 857 species in our chemical defence dataset and recorded the conservation status of all species for which the information was available. This resulted in a final dataset consisting of 809 species from across the amphibian tree of life for which we had data on both extinction risk and chemical defence. We coded extinction risk in two ways. Firstly as a binary trait (which we term 'threat') in which we considered Red List categories LC (least concern) and NT (near threatened) as 'non-threatened', and other categories (VU=vulnerable, EN=endangered, CR=critically endangered, EW=extinct in the wild, EX=extinct) as 'threatened', in line with recommendations by the IUCN (2014). Secondly, we coded extinction risk as an ordinal trait (which we term 'status') representing increasing levels of threat as follows: 0=LC, 1=NT, 2=VU, 3=EN, 4=CR, 5=EW&EX.

To account for the non-independence of species as data points due to shared evolutionary history, we took a comparative approach using the time-calibrated phylogeny of Pyron and Weins (2013). This was pruned to include only the 809 species for which data were available for both chemical defence and conservation status, and the resulting tree was used for all subsequent analyses. The full dataset and R scripts used for analyses in this study is available at http://dx.doi.org/10.6084/m9.figshare.1399172. All analyses were performed in R v3.1.3 (R Core Team, 2015), using ape (Paradis *et al*., 2004) for basic manipulation of the phylogeny and other packages as stated for particular methods.

7.4.2 Phylogenetic regression models

We first tested whether threat was predicted by the presence of chemical defence using phylogenetic logistic regression with Ho and Ané's (2014) method implemented in phylolm. We checked that phylogenetically informed analysis was justified over standard logistic regression in three ways, all of which provided support for this and so we report only the results from the phylogenetic logistic regressions. Firstly, we fit a standard (non-phylogenetic) logistic regression and compared these alternative models using AIC. We find a ΔAIC value of 61.8 in favour of the phylogenetic model (values greater than ~5 are often considered strong evidence to prefer one model over another). Secondly we examined the α parameter estimated in the phylogenetic model, which ranges between 0 and 1 with low values indicating greater phylogenetic signal. In this model α=0.016, again suggesting that the phylogenetic logistic regression was justified. Finally, we tested whether the residuals from the model exhibited significant phylogenetic signal (as expected if phylogenetic control is necessary) and found this to be true whether using Pagel's λ (λ=0.653, P=3e-26) or Blomberg's κ (κ=0.065, P=0.024), both estimated in phytools (Revell, 2012).

We then tested whether extinction risk was predicted by the presence of chemical defence using phylogenetic generalised estimating equations (GEEs; Paradis and Claude, 2002) with a Poisson error structure, also implemented in phylolm (Ho and Ané, 2014). GEEs are not likelihood-based and so cannot be compared with other models using information theoretical measures such as AIC, and nor do they fit an α parameter as with the phylogenetic logistic regression models. Nevertheless, we checked that the use of a phylogenetic model was justified here by evaluating the phylogenetic signal in the residuals as above. This was again found to be the case using both Pagel's λ (λ=0.701, P=2.9e-38) and Blomberg's κ (κ=0.071, P=0.009), and so we present the results from the GEE in this paper.

7.4.3 Evolutionary pathway models

The regression-based models in the previous section test whether chemical defence and extinction risk are linked, and do not assume that the traits evolve along a phylogeny (only that the residuals from the models are phylogenetically structured). This has a benefit as we acknowledge that our proxy of extinction risk, IUCN Red List status, is a human classification and so does not, in a strict sense, evolve. Nevertheless, such models are limited in their ability to provide inference about cause-effect relationships, which can be evaluated for binary traits using evolutionary pathway models (Pagel, 1994). Therefore, we attempt to use these to test more directly whether the hypothesis that chemical defence leads to an increase in extinction risk is supported, but we do so tentatively with the following three justifications for treating extinction risk as an evolving trait.

Firstly, a Pagel's (1994) test for correlated evolution conducted in phytools, which uses pathway modelling, corroborate those from our phylogenetic regression (see Results) by finding evidence for correlated evolution between threat and chemical defence ($P=0.003$). This is despite the Pagel's test assuming that both traits evolve and taking into account inferred ancestral transitions, suggesting that it is likely that assuming threat evolves is not strongly misleading.

Secondly, threat exhibits significant phylogenetic signal as measured by Fritz and Purvis' (2010) D statistic for binary traits ($D=0.495$, $P<0.0002$), estimated in caper (Orme *et al.*, 2013) based on 5000 permutations. Therefore, extinction risk as measured using IUCN Red List category as a proxy show similar properties to an evolving trait, in that it is more likely to be shared by more closely related species. Taken alongside the first point, this suggests that extinction risk behaves as an evolving trait for the purposes of comparative analyses.

Thirdly, IUCN Red List categories have been widely used as a proxy for extinction risk, which is itself a function of many attributes. Since many of these factors contributing to extinction risk are likely to be evolving traits of the species, extinction risk should be expected to evolve, in a sense, over the phylogeny via the evolution of related variables. Therefore, while Red List categories do not evolve, we consider it reasonable to expect that the underlying extinction risk (which it is intended to represent) should 'evolve' in the sense that it experiences transitions over the phylogeny, as modelled by evolutionary pathway analyses.

Consequently, we tentatively treat threat as an evolving trait for the purposes of the following analyses in order to investigate the hypothesis behind this paper in more detail. To do this, we first determined whether transition rates best fit an equal rates (ER), symmetrical rates (SYM) or 'all rates different' (ARD) model, and used the best of these (ARD according to AIC values) in subsequent pathway models. We then constructed two models and compared their fit using a likelihood ratio test. The first was a full (8-parameter) model assuming correlated evolution between threat and chemical defence without imposing constraints on the evolution. The second was a constrained (7-parameter) model incorporating a single constraint which assumes that chemical defence is gained first which then leads to the lineage becoming threatened, as per Pagel (1994). Comparison of these models allows direct evaluation of the evidence for a pattern of defence-driven extinction risk as opposed to a non-directed correlation or extinction risk-driven defence.

7.5 Results

Contemporary amphibian species which possess chemical defences were 60% more likely to be threatened than species lacking such defences, according to our phylogenetic logistic regression model (β=0.596, SE=0.196, z=3.045, P=0.002; Fig. 7.1). Our Poisson GEE model of conservation status (using all IUCN Red List categories) shows similar results when using a more fine-grained measure of threat status (β=0.049, SE=0.022, z=2.263, P=0.024). Although most amphibians are classified as LC, the proportion of chemically defended species increases with extinction risk (Fig. 7.2).

Our evolutionary pathway analyses further corroborate the connection between extinction risk and chemical defence. Firstly, a Pagel's test to test the evolutionary correlation of these two traits found strong support for such a link (likelihood ratio=16.344, P=0.003). Furthermore, when testing the directionality using constrained models, we find strong evidence for a model wherein toxicity is gained first which leads to an increase in extinction risk (likelihood ratio=11.714, P=0.0006).

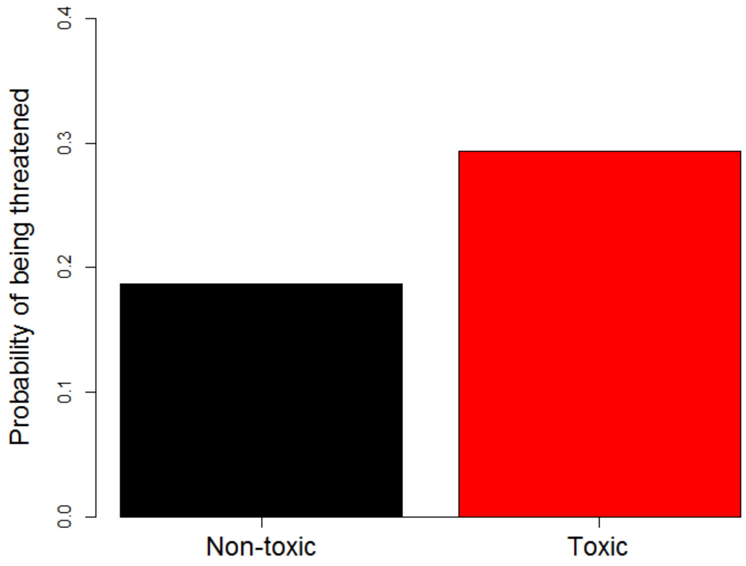

Figure 7.1. Probability of being classified as threatened for amphibians lacking (black) or possessing (red) chemical defences, based on back-transformed parameter estimates from phylogenetic logistic regression. Species with a chemical defence are 60% more likely to be threatened than those without (P=0.002).

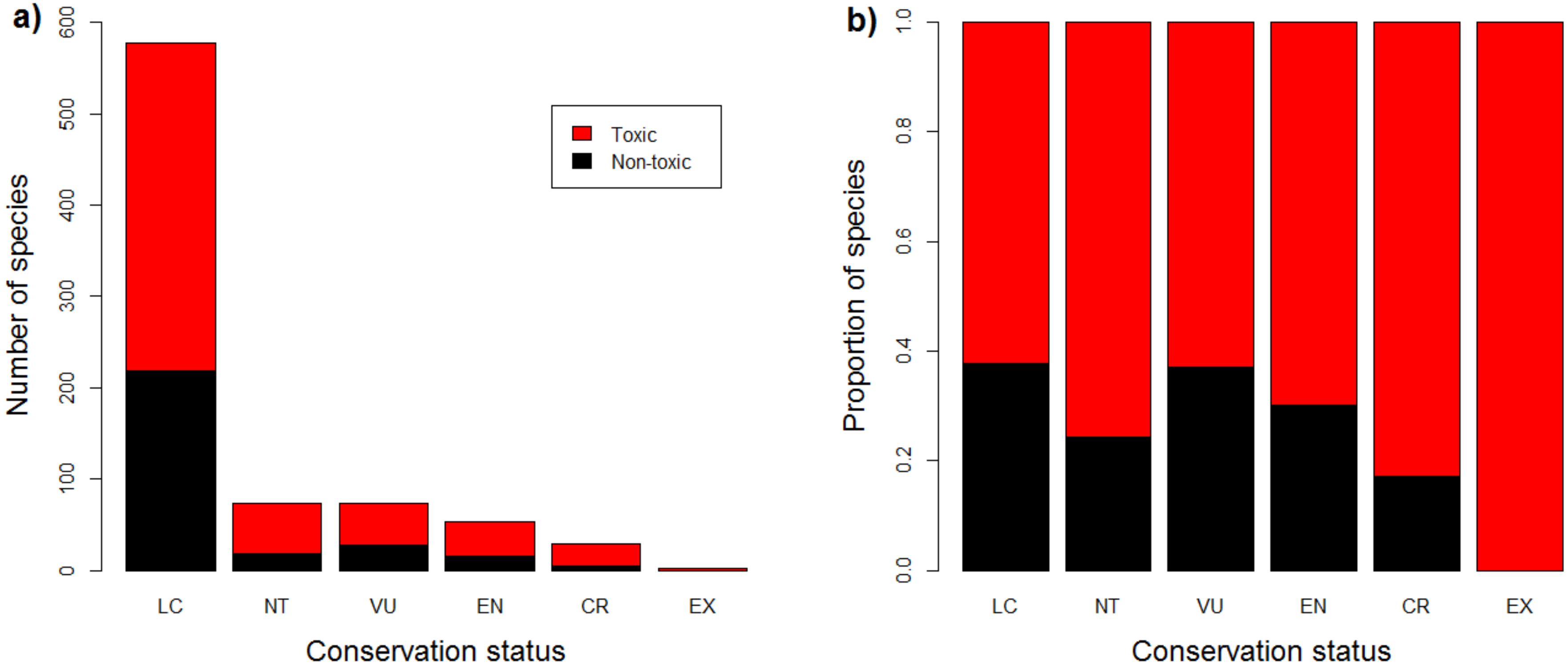

Figure 7.2. Number (a) and proportion (b) of species possessing (red) or lacking (black) chemical defence for each IUCN red list category (severity of extinction risk increases from left to right on the x-axis). Species at higher extinction risk are progressively more likely to possess a chemical defence (P=0.024).

7.6 Discussion

Our results support the hypothesis that chemical defence is linked to contemporary extinction risk and tentatively support the directional hypothesis that the evolution of chemical defence causes an increased propensity to become threatened. Using a large-scale dataset of amphibians (a taxon of global conservation concern), we demonstrate that chemically-defended species are more likely to be threatened and that as extinction risk increases so does the proportion of chemically-defended species. Such results are in line with predictions made from a recent study of background extinction rates in amphibians (Arbuckle and Speed, in press; see Chapter 6) and therefore suggest that such diversification rate estimates can potentially inform estimates of current extinction risk based on species' traits.

Due to the large number of species for which IUCN conservation status is unknown, either due to no assessment being carried out or to insufficient information (i.e. Data Deficient species), there is a need for predictive methods if we are to adequately assess the global extinction risk of many groups of organisms. Comparative approaches which investigate traits that are linked to conservation status have been used as a promising approach (Purvis *et al.*, 2000). Furthermore, a recent paper has suggested that predictive models using such traits are often reasonably reliable and could generate a cost saving of ~$220m (Bland *et al.*, 2015b) over assessing all Data Deficient species directly. Many aspects of the biology of a particular species are likely to influence the accuracy of these predictions via their effects on extinction risk, and so knowledge of additional traits that can be incorporated into such models should provide an additional benefit.

We should of course stress that we are not advocating chemical defence as the only, or even the most important, trait for predicting extinction risk - many others are also linked to conservation status. For instance, Bielby *et al.*, (2008) found that amphibians experiencing rapid declines more frequently had an aquatic life-stage even when compared to other threatened species, and that species experiencing enigmatic and rapid declines were additionally characterised by low clutch size environmental variables such as high altitude and stable climates. Cooper *et al.* (2008) also found that amphibian extinction risk was associated with low clutch sizes, and also larger body size, but concluded that these traits modulate geographic range size, which directly leads to increased extinction risk. However, if we are to predict the extinction risk of species based on trait, it is essential to use as much information as possible in a comprehensive modelling framework as inaccuracy can be detrimental to conservation efforts. As a hitherto unstudied trait in this context that predicts a 60% increase in the probability of a species being threatened, we propose that chemical antipredator defence should be included in the toolbox of useful traits.

We acknowledge that the use of IUCN Red List categories as a proxy for extinction risk does carry limitations, as with the use of any proxy. In particular, Mooers *et al.* (2008) noted that the actual extinction risk represented by the categories follow an ordinal rather than an interval scale. In other words, the difference in actual extinction risk between two adjacent categories (e.g. NT and VU) is not necessarily equal to the difference between another two adjacent categories (e.g. VU and EN). Therefore Mooers *et al.* (2008) argue that analysing such data under the implicit assumption of an interval scale (as in our analyses of our 'status' variable treated as a Poisson variable) may warrant caution. Nevertheless, the alternatives they suggest, involving transformations based on assumptions of the shape of the relationship between extinction risk and conservation status, are no less subjective and have not been widely adopted. Consequently, we follow many other studies which have used (and continue to use) Red List status as a reasonable proxy of extinction risk that conveys information on the relative severity of threat faced by the species (Purvis *et al.*, 2000; Bielby *et al.*, 2008; Cooper *et al.*, 2008; Mace *et al*., 2008; Morais *et al.*, 2013; Howard and Bickford, 2014; Jeppsson and Forslund, 2014; Bland *et al.*, 2015a). We also note that our results from our two separate codings of Red List status ('threat' and 'status') were consistent which reduces the chance that the assumption of an interval scale for our analysis of 'status' is misleading. Indeed, our analyses consistently found a link between extinction risk and chemical defence regardless of coding or modelling assumptions, suggesting that they are robust to such methodological details.

Data for contemporary extinction risk and background extinction rate estimates are independent of each other, but despite this our results confirm predictions made from a recent macroevolutionary study (Arbuckle and Speed, in press; see Chapter 6) using present-day IUCN Red List categories. This correspondence suggests that studies on evolutionary diversification can give valuable information to shed light on contemporary conservation concerns. We predict that such inferences may be common because species will vary in their susceptibility to extinction as a function of a range of biological attributes. For instance, traits associated with slower life histories are commonly found to be associated with higher extinction risk in a range of taxa facing a variety of threats (e.g. Bielby *et al.*, 2008; García *et al.*, 2008; Jeppsson and Forslund, 2014). Therefore, we expect that many traits will have a consistently detrimental influence on susceptibility to extinction when faced with a variety of specific threats that are driving the population declines, and consequently that associations between traits and extinction may often be temporally stable.

The evolutionary pathway models we implemented herein indicate that it is the gain of chemical defence that increases the species susceptibility to extinction, rather than a simple correlation between the two traits. We acknowledge that this conclusion is more tentative than our others as we assume that conservation status is an evolving trait, but nevertheless we contend that it is still meaningful as per our justifications given in the Methods section. The concordance of our results from models assuming that both traits evolve and those that do not make the assumption (along with previous analyses of background extinction rates in Arbuckle and Speed, in press; see Chapter 6) further suggest that similar underlying biological processes may be influencing susceptibility to extinction now as in the past. This is despite the proximate causes of extinction, and consequently the scale and rate of contemporary extinction, being very different.

There is a remaining uncertainty as to the mechanistic underpinnings of the influence of chemical defences on the probability of extinction, but we can offer some hypotheses that could explain this. Arbuckle and Speed (in press; see Chapter 6) suggested three possibilities to explain higher background extinction rates in chemically-defended amphibians: 1) chemical defence is energetically costly, 2) chemical defence allows shifts to 'marginal' (low carrying capacity) habitats, which are intrinsically more vulnerable, and 3) chemical defence is associated with slow life histories, which damages the recovery potential of populations after declines. The first two of these (the ‘costly chemical’ and ‘marginal habitats’ hypotheses) are perhaps poorer explanations for increased extinction risk at both evolutionary and contemporary scales. In essence, if these patterns hold across vastly different reasons for population decline, then the underlying mechanism is likely to be a general factor that makes population recovery difficult after declines. In contrast, the reduced competition after decline should increase the resources available to individuals and therefore ease the energetic trade-offs at the heart of the costly chemical hypothesis. Similarly, the marginal habitats relies on the intrinsic vulnerability of low carrying capacity environments, but a substantial decline in the population should again allow a relatively fast recovery until the carrying capacity is once again reached (Gilpin and Ayala, 1973), all else being equal. However, the ‘slow life-history’ hypothesis predicts that chemical defence should lead to the evolution of slower life histories as a result of reduced extrinsic mortality (Stearns *et al*., 2000). Because such traits are also characterised by a slow rate of population growth (Hutchins *et al*., 2012), this is a prime candidate mechanism for a general relationship of chemical defence to increased extinction risks in the face of many different threats.

Overall, we present the first evidence that an antipredator defence is associated with increased contemporary extinction risk in amphibians. We highlight that this conclusion follows a prediction deriving from work on background extinction rates and that, therefore, such studies may have relevance to the setting of evidence-based conservation priorities. Specifically, in this case, chemical antipredator defence can be incorporated into predictive models of conservation status to improve their accuracy in estimating the extinction risk of non-assessed or Data Deficient species. Finally, we provide tentative evidence that chemical defence is driving the increased susceptibility to extinction in amphibians, rather than the relationship being a product of an incidental correlation, thus shedding further light on our knowledge of the evolutionary consequences of antipredator defence.

7.7 Acknowledgements

K.A. was funded by a NERC Doctoral Training Grant.

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8 A simple measure of the strength of convergent evolution

This chapter is published in *Methods in Ecology and Evolution* (Arbuckle *et al*. 2014. *Meth. Ecol. Evol.* 5:685 - 693), and has been highly commended for the Robert May Young Investigator Prize by the British Ecological Society. A copy of the final article is contained at the end of this thesis

8.1 Author contributions

Kevin Arbuckle, Cheryl M. Bennett & Michael P. Speed

Institute of Integrative Biology, Biosciences Building, University of Liverpool, Crown Street, Liverpool, Merseyside, L69 7ZB, United Kingdom.

Mike Speed provided comments on the manuscript and discussion of ideas, and also wrote the MatLab code to implement the method. Cheryl Bennett collected the data for the empirical example and analysed this as her BSci(hons) project, though I subsequently reanalysed the data to ensure accuracy. I developed the initial concept (along with Mike Speed, who had convergently thought of a similar idea), developed the method, simulated the data, conducted the analyses, and wrote the manuscript.

8.2 Abstract

Convergent evolution, the independent occurrence of phenotypic similarity, is a widespread and common phenomenon. Methods have been developed to identify instances of convergence, but there is a lack of techniques for quantifying the strength of convergence. We therefore investigated whether convergent evolution can be quantified in a meaningful way. We have developed a simple metric (the Wheatsheaf index) that provides an index of the strength of convergent evolution incorporating both phenotypic similarity and phylogenetic relatedness. The index is comparable across any quantitative or semi-quantitative traits and thus will enable the testing of various hypotheses relating to convergence. The index performs well over a range of conditions. We apply it to an empirical example using *Anolis* lizard ecomorphs to demonstrate how it can be used. The Wheatsheaf index provides an additional tool that complements methods aimed at identifying cases of convergent evolution. It will enable cases of convergence to be analysed in more detail, test hypotheses about its mechanics as an evolutionary process and, more generally, the predictability of evolution (how often do we see strong convergence and does this mean evolutionary solutions are limited?).

8.3 Introduction

The independent evolution of similar phenotypic traits in multiple organisms, or convergence, has been recognised as a key evolutionary process since Darwin (1859). Convergent evolution is often a consequence of adaptation to a similar niche (though not always, see Stayton, 2008), and has therefore been recognised and studied in cases of replicated adaptive radiations such as *Anolis* ecomorphs (Losos, 1992; Beuttell and Losos, 1999; Losos, 2009) and African cichlids (Kocher *et al*., 1993; Muschick *et al*., 2012). In addition, convergence may be seen when organs have similar uses and converge on a similar form, as in the camera eye which has evolved in both vertebrates and invertebrates. Convergence between organisms for a particular niche can promote speciation by causing divergent selection within a lineage inhabiting two niches (Rosenblum, 2006), limit the suite of phenotypic traits that will evolve as adaptations (Martin and Wainwright, 2013), and drive distantly related organisms towards the same phenotypic adaptive optima (Mahler *et al*., 2013). Notably, Conway Morris (2003) has argued that convergence of traits toward a limited number of ‘engineering optima’ is a central guiding force in phenotypic evolution. For example, there are only a small number of ways to construct an effective, functioning eye; hence engineering constraints cause convergence and limits biological diversity in this trait. If correct, Conway Morris’s view is profoundly important for our understanding of biological variation. Therefore an understanding of convergent evolution is important to understanding the generation of biodiversity, constraints on adaptation, and how natural selection optimises an organism for a particular niche. For the purposes of this paper we use ‘niche’ to refer to an aspect (or aspects) of the biotic and/or abiotic environment of an organism that is of interest for a hypothesis under study.

There have been several approaches and methods developed to identify instances of convergent evolution, and these have enabled a large number of cases to be described and recognised as such. At its simplest, convergence may be identified by carefully cataloguing traits across many species. McGhee’s recent text (2011) is an excellent example of this.

More formally, perhaps the most commonly used and simplest method for identifying convergence is ancestral state reconstruction of the (purportedly) convergent trait. For example, this method has provided support for convergent evolution of plumage colouration in *Icterus* orioles (Omland and Lanyon, 2000) and in the chemically defended *Pitohui* birds (Dumbacher and Fleischer, 2001). In such an analysis, the phenotype is reconstructed in some way over the phylogeny and independent origins (multiple shifts to the same state) are taken as evidence of convergence.

Muschick *et al*. (2012) used an alternative approach to test for convergence in cichlid fishes by considering that convergence should result in a pattern of reduced phenotypic differentiation when compared with phylogenetic distance. These authors thus calculated Euclidean distances between species in the morphological traits of interest and plotted them against the phylogenetic distances. They then used simulations to identify instances where phenotypic divergence was significantly lower than expected based on phylogenetic distance. Since this method involves a straightforward comparison of phylogenetic and phenotypic distances, Muschick *et al*. (2012) included both convergence and slower-than-expected divergence within their measure, as the two would produce the same signature.

A third approach was described by Ingram and Mahler (2013) which explicitly models trait evolution onto a phylogeny to identify convergent evolution. Their 'SURFACE' method takes a continuous trait and fits Ornstein-Uhlenbeck models with varying numbers of selective regimes and with shifts at varying points on the tree. Akaike's information criterion is then used to select the best fitting model. Convergence is identified by the independent adoption of the same selective regime at multiple points on the phylogeny.

Each of these methods represents a technique to identify when convergence has occurred. Statistical recognition of convergence is, of course, fundamental. However, once convergence is established a number of important questions can be explored. For example, we may be interested in whether there are general rules in the way convergence operates. Do some traits show stronger convergence than others? Do different types of traits converge more easily than others (e.g. morphological vs. biochemical traits), and if so, is evolution more predictable for some kinds of traits than for others? Do particular 'levels' of convergence (e.g. functional, structural, developmental, genetic) vary in their contribution to adaptive evolution? Why might such differences exist (e.g. what might drive stronger convergence in protein sequences than limb anatomy)? It is perhaps notable that most analyses of convergent evolution have focussed on morphological traits, which limits our knowledge base on how different types of traits may differ in aspects of convergence; however some exceptions do exist (e.g. Mirceta *et al*., 2013).

To answer such questions, we need a way of quantifying the strength of convergence. When we have a suitable measure of convergent evolution we can start to test hypotheses about the nature of convergence, rather than simply recognising it. Specifically, we require a metric that is comparable across many types of traits, incorporates both phylogenetic relatedness and the extent of phenotypic similarity, and is quantitative.

In this paper, we describe a simple measure of the strength of convergent evolution, which we call the 'Wheatsheaf index'. For the purposes of our method and this paper, we consider convergence to be the pattern that results from the process of convergent evolution, rather than the process itself. Furthermore, because we use a pattern-based description of convergence, parallelism is indistinguishable from 'true' convergence using our method and so comes under the concept of convergence for the purposes of this paper. The index was designed to meet the requirements outlined above and with the underlying assumption that we can define a set of species as convergent or have a working hypothesis as to the niche upon which the organisms are adapted (or adapting towards).

8.4 The Wheatsheaf Index

To calculate the Wheatsheaf index we take a set of organisms, and within that identify a subset that we treat as convergent (we call this the subset of 'focal' taxa), and the residual species as members of the 'non-focal' subset. The index measures the similarity of focal species to each other and the isolation in phenotypic space of the focal group from non-focal species, all penalised for phylogenetic relatedness. To understand this in a conceptual way, we can consider convergence to be movement in phenotypic space over a fitness landscape towards an elevated position (such as an adaptive peak) which characterises a particular environment or niche. The distance between non-focal and focal species represents the distance across such a landscape that focals have had to move to reach the peak, with movement over larger distances representing more evolution and therefore a stronger signature of convergence. In addition, the more tightly clustered the focal species are in this phenotypic space (the more similar they are to each other), the stronger are the selective forces pulling converging species towards the peak, or the narrower the peak itself, which in either case would indicate a more intense pull towards a particular point in phenotypic space.

Both of these aspects seem to be good foundations for a conceptual view of the strength of convergence providing phylogenetic relatedness is accounted for, as is the case with the Wheatsheaf index. Thus, we consider convergence to be stronger when focal species are more phenotypically similar to each other, and when the focal species are more dissimilar to the non-focal species - in other words when they have had to evolve further from the baseline of non-focal species to reach the convergent state. We note that some patterns of convergence may leave convergent species still more similar to their close relatives than each other in many phenotypic attributes (Stayton, 2006), but we view this as a manifestation of differing strengths of convergence rather than a challenge to our definition. This phenotypic aspect of the index is penalised for close phylogenetic affinities, and generates a quantitative measure which can subsequently be used to test hypotheses about the strength of convergence across traits.

Before we can apply the Wheatsheaf index, we require a clade to work with in which some members have been demonstrated to exhibit convergent evolution. In other words, we would use other methods (e.g. ancestral state reconstruction or SURFACE) which identify convergence so that we can start with a supported assumption that there is convergence in our group of interest. We then need to assign (*a priori*) species within that group as either ‘focal’ or ‘non-focal’ species. This is often related to a working hypothesis on the niche the organisms are expected to be converging on such that focals are those species occupying that niche (expected to show convergent adaptations) and non-focals are those species not occupying that niche. To give two examples, we might be interested in measuring convergence in body form for burrowing in lizards; in this case burrowing species would be assigned to the focal group. Or we might look at convergence in salinity tolerance for brackish habitats, in which case species inhabiting estuaries and other such environments would form the focal group. Alternatively, we could consider the species already identified as convergent as the focal group, which would allow us to measure how strong the convergence is in selected phenotypes of these taxa, regardless of any adaptive reason for it.

Other information required for the Wheatsheaf index is a phylogeny for the clade of interest and trait information. How we choose traits will depend on the purposes of the study. If we are interested in whether a particular set of traits are important for a given niche, then the selection of traits should be hypothesis-driven such that traits are chosen so that they may be convergent for that niche. This approach has the benefit that specific adaptive hypotheses of convergence for a given niche are examined. If, on the other hand, we are interested in an unguided investigation of which traits might be convergent for a given niche (if we have no working hypothesis with which to make *a priori* predictions) then we could use a large number of traits spanning the range of those we can measure, run the index on all of them and therefore obtain estimates of which ones are most convergent. However, an important stipulation is that the traits must be (semi-)quantitative (e.g. continuous, count, or ordinal data; see Discussion for further details).

Calculation of the Wheatsheaf index requires the data (both phylogenetic and phenotypic) to be represented in pairwise distance matrices. For the phylogeny, a matrix of proportion shared distances between species is used, such that the total tree height is scaled to one and distances are given as the proportion of the tree shared between two species. In other words, bigger distances represent more closely related species. For phenotypic traits (which are first standardised for variance by dividing by the standard error of the trait across species), a matrix of Euclidean distances between species is used, which enables any number of traits to be incorporated and bigger shared distances represent more dissimilar species for the included traits. This allows us to look at single traits individually or grouped traits as appropriate for the hypothesis being tested, e.g. we could obtain a distance matrix for a set of morphological traits and a second one for a set of physiological traits. Again, the selection of traits to include in the study as a whole and in a given distance matrix will be driven by the hypothesis in question.

To calculate the Wheatsheaf index, we first obtain a corrected (for phylogenetic relatedness) phenotypic distance matrix as follows:-

$$\dot{d}_{ij} = \frac{d_{ij}}{1 - \log(p_{ij} + 0.01)} \qquad \text{Eqn. 1}$$

Where d_{ij} is the phenotypic (Euclidean) distance between species *i* and *j*, p_{ij} is the shared proportional distance between species *i* and *j* obtained from the phylogeny, and $\dot{d}_{ij}$ is therefore the phenotypic distance between species *i* and *j* corrected for phylogeny. Note that p_{ij} is transformed by adding a small (and arbitrary) value and logging; this is so that p_{ij} scales approximately linearly with $\dot{d}_{ij}$. If a pair of species are closely related, and therefore p_{ij} is close to 1, then $\dot{d}_{ij}$ will be much larger than d_{ij}. As species become more distantly related then p_{ij} will decrease and $\dot{d}_{ij}$ will become progressively smaller and approach d_{ij}. This is an intuitive way of correcting for phylogeny since more weight (i.e. a smaller distance) is assigned to more distantly related taxa being similar, therefore penalising the phenotypic similarity of closely related species. Since p_{ij} and $\dot{d}_{ij}$ are approximately linearly related in the equation, this is in effect assuming that the phenotype diverges in proportion to time (phylogenetic history). Note that since we consider convergence to be a pattern in this paper, no model is fitted and so no parameterisation is conducted, and thus eqn. 1 should be robust to the particular evolutionary model that best fits the trait data, providing that we can expect more phenotypic divergence when species-pairs are more distantly related. Nevertheless it might be possible to extend this method in the future to incorporate specific evolutionary models in the penalising term, should this become necessary.

Using the corrected phenotypic distances (pairwise matrix of $\dot{d}_{ij}$ between each pair of species), we can now calculate the Wheatsheaf index (w) as follows:-

$$w = \frac{\overline{\dot{d}_a}}{\overline{\dot{d}_f}} \qquad \text{Eqn. 2}$$

Where $\overline{\dot{d}_a}$ is the mean $\dot{d}_{ij}$ for pairwise comparisons between all species, and $\overline{\dot{d}_f}$ is the mean $\dot{d}_{ij}$ for pairwise comparisons between focal species only. As $\overline{\dot{d}_a}$ increases and $\overline{\dot{d}_f}$ decreases then w will increase, showing stronger convergence and *vice versa*. A visual representation of this is provided in Fig 8.1. Because a greater separation in phenotypic space between the focal and non-focal groups will result in larger distances between focal taxa and non-focal taxa, $\overline{\dot{d}_a}$ will be larger and so $\overline{\dot{d}_f}$ will be relatively smaller, therefore showing stronger convergence (a larger w). Similarly a tighter clustering of focal species will decrease $\overline{\dot{d}_f}$, relatively increasing $\overline{\dot{d}_a}$ and so again showing a signature of stronger convergence. We note that our method shows some similarity to that of Stayton (2006) in that both use ratios of phenotypic and phylogenetic measures to generate a corrected phenotypic distance and compare convergence species to the set as a whole. However the Wheatsheaf index differs in a number of ways including calculating pairwise phylogenetic and phenotypic distances between all species in the phylogeny, rather than using information only from sister groups (or similar comparisons).

Since the calculation of w is not amenable to multiple, independent sampling (it uses information from the entire sample – all species in the clade), 95% confidence intervals are generated by jackknifing the dataset and using the resulting distribution of values to calculate the intervals.

Because the topology of the tree may constrain the possible values of w we used a bootstrapping approach to resample the tips of the tree along with their trait values and thus obtain a distribution of possible w indices given the phylogeny and the trait values for each species. Using this distribution and the calculated value of w, we can generate a ‘P-value’ by taking the proportion of bootstrap samples that are greater than or equal to the value of w calculated from the original dataset (see Fig 8.2). We stress that this P-value is *not* a test for the presence of convergent evolution, as described earlier we begin an analysis with the Wheatsheaf index with the knowledge that convergence has occurred in our clade of interest. Rather, it represents a test of whether convergence is significantly stronger than we would expect compared to a random distribution of trait values across the specified tree. A further advantage of this is that comparisons of the P-values provide a measure of convergence that accounts for the given tree structure and so, in effect, standardises for this. In other words we can potentially use the P-value to compare the strength of convergence across trees, which is not possible using our value of w

alone. However, we would add that since P-values are bound between zero and one, comparisons using this part of the method may be limited in extreme cases by floor and ceiling effects.

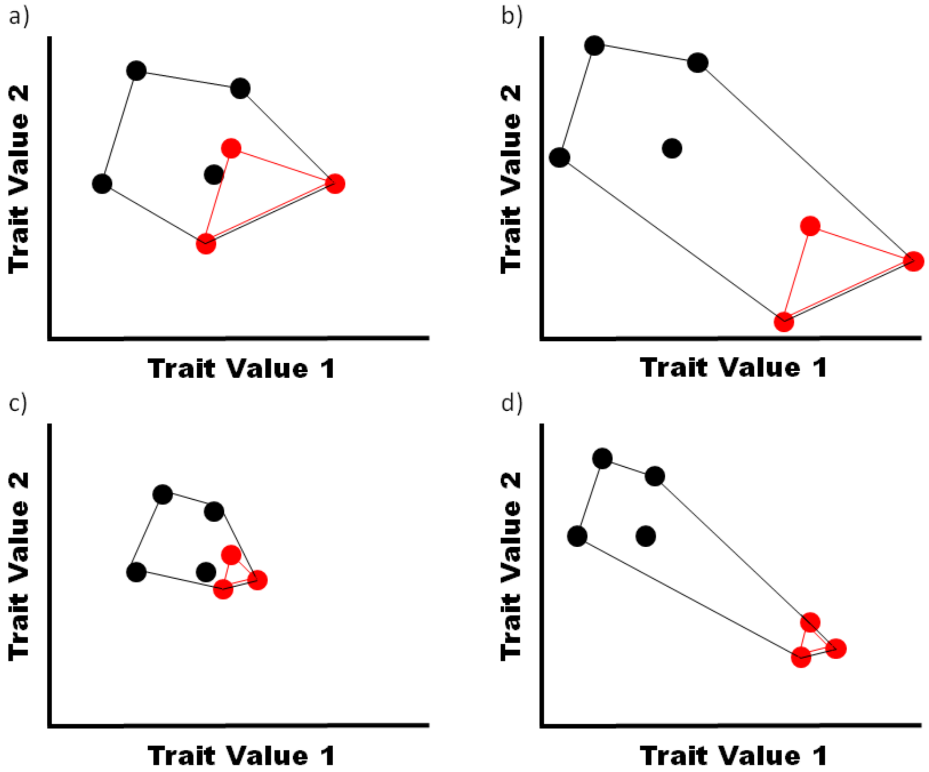

Figure 8.1. Schematic diagram showing examples where w is expected to be relatively low (a), high (d), and intermediate (b and c). The two axes represent a two-dimensional phenotypic space. Black circles represent non-focal, red circles represent focal species. The tightness of the clusters is either high (c and d) or low (a and b), and the isolation of focal taxa is relatively high (b and d) or low (a and c). The area contained in the black loop represents that within which $\overline{\dot{d}_a}$ is calculated, whereas the area contained in the red loop represents that within which $\overline{\dot{d}_f}$ is calculated. Note that this figure is intended only to provide a visual understanding of the relationship between w and phenotypic space, it is not meant as a realistic example and ignores the phylogenetic penalty of these distances for clarity.

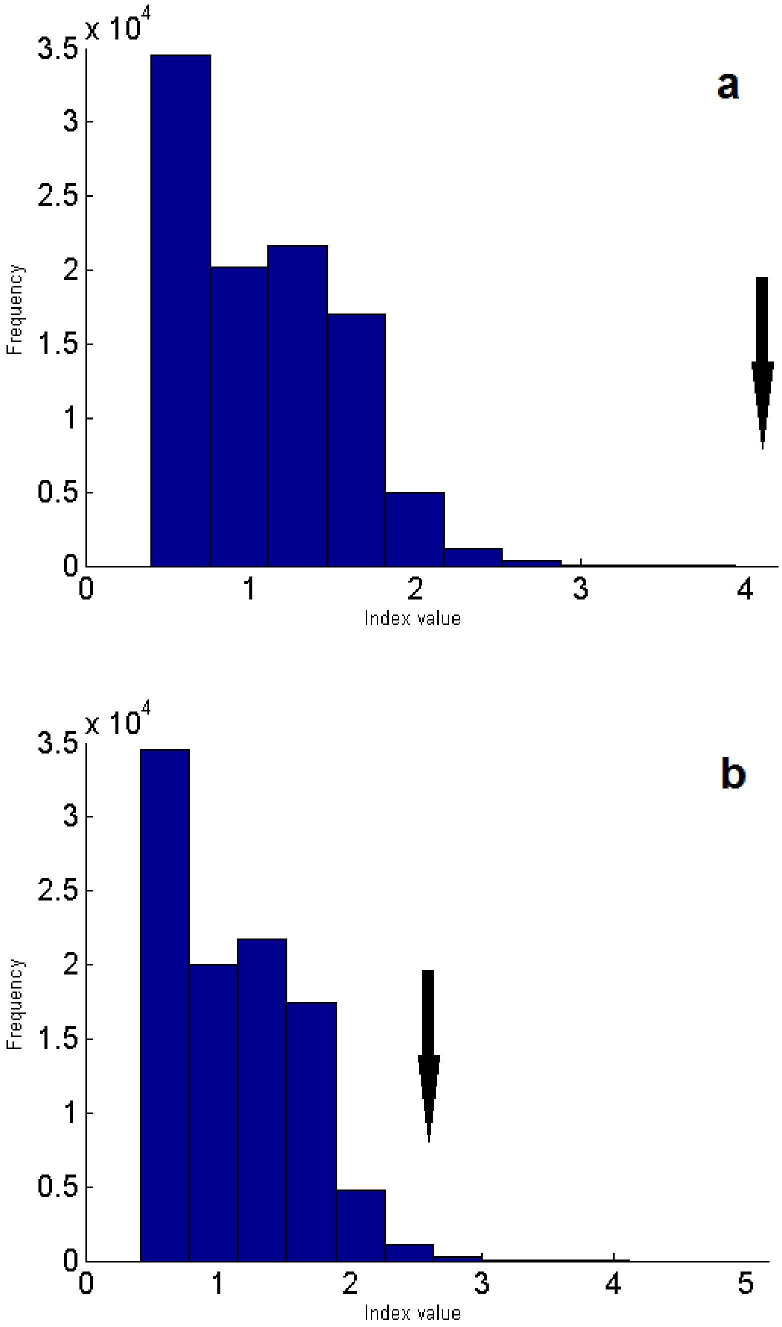

Figure 8.2. Examples of histograms showing the distributions of Wheatsheaf index values obtained from bootstrap resampling of the tips of the tree. Vertical arrows represent calculated values and the proportion of the distributions greater than or equal to the calculated value (to the right of the arrow) are used to generate the P-values obtained from the method. We can see that the example in a) is more strongly convergent than b) both in absolute terms (calculated value is higher) and with respect to the topological constraints of the tree (further to the right of the distribution).

8.5 Methods

We evaluated our index in two separate ways: simulations and empirical data. Using data simulated with specific parameters means we can investigate how particular attributes of a given dataset influence the calculation of the Wheatsheaf index and therefore whether there are any particular conditions that warrant caution. We should also ensure our method is appropriate for use on empirical data, and so we present an example to show how it can be used on an ecomorphological dataset of *Anolis* lizards.

8.5.1 Simulations

To assess the general performance of the Wheatsheaf index under various conditions, we simulated a range of phylogenies and continuous traits in R version 2.15.2 (R Development Core Team, 2012). All data manipulation, such as generating the Euclidean distance matrices, prior to calculation of the index was also conducted in R. The matrices of shared proportional distances from the phylogenies were extracted using the packages ape (Paradis *et al*., 2004) and GEIGER (Harmon *et al*., 2008).

Ten trees were simulated using a birth-death model in GEIGER with a birth rate of 0.5 and a death rate of 0.1 resulting in 100 species each (except when number of species was the parameter being varied, in which case 10 trees with each number of species were simulated). Trait data were simulated over each tree in two ways. Firstly, in order to assess type 1 error trait data was simulated under a Brownian Motion (BM) model across the tree, such that convergence would be very unlikely to occur amongst focal species (Stayton, 2008). Secondly, to assess type 2 error trait data were simulated under a BM model for non-focal species but under an Ornstein-Uhlenbeck (OU) model for focal species. In each of these simulations, focals and non-focals were present in equal numbers, except where the proportion of focals was the parameter being varied. Trait simulation was conducted in diversitree (FitzJohn, 2012), with parameters as follows (except when a particular parameter was the one being varied, as detailed below): $\sigma^2=10$ for BM models, and $\alpha=5$, $\theta=20$, $\sigma^2=10$ for OU models. All analyses were conducted on Euclidean distances over one, two, and three traits for each tree to check sensitivity to number of traits involved in the calculations.

We varied three parameters (in turn) to assess what influence they had on the performance of the Wheatsheaf index: the number of species in the tree; the proportion of focal species in the tree; and the ‘strength of selection’ (variation around the optima, or α in the OU model). We recognise that ‘strength of selection’ is perhaps an overly simplistic interpretation of α in an OU model (Hansen, 2012; Ingram and Mahler, 2013), but we use it here for ease of intuitive discussion (as in Hansen and Orzack, 2005; Beaulieu *et al*., 2012) while acknowledging that factors other than the strength of selection can influence α. The number of species in the phylogeny, reflecting sample size, was varied with the following values: 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 1000. The proportion of focal (cf. non-focal) species was varied with the following values: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9. The ‘strength of selection’ was varied by changing α in the OU model to the following values: 0.1, 0.5, 1, 2, 3, 4, 5, 10, 20, 50.

We used the P-values to assess how the Wheatsheaf index performs across these parameter values. Specifically, we expected $P>0.05$ when all traits were simulated under BM and $P\leq0.05$ when focal species were simulated under an OU process. We were also able to determine the power of our method as 1-[type 2 error rate].

8.5.2 Empirical example

In order to examine how the index performs on a real dataset, we performed analyses using an empirical example consisting of ecomorphological traits in anole lizards, a model system for studies of convergent evolution (Harmon *et al*., 2005) for which morphological data, phylogenetic information, and a good literature base to assess our results are available. Caribbean *Anolis* lizards have repeatedly and independently evolved six 'syndromes' consisting of linked morphological, behavioural, and ecological traits; these forms are termed 'ecomorphs' (Williams, 1972; Losos, 2009). The six *Anolis* ecomorphs are named after the microhabitat they inhabit as follows: crown-giant, trunk-crown, twig, trunk,

trunk-ground, and grass-bush (Losos, 2009). We therefore decided to apply the Wheatsheaf index to investigate the strength of morphological convergence in ecomorphs as an empirical demonstration of the utility of the method.

Morphological data were extracted from the literature (Losos, 1990a, 1992, 2009; Thomas *et al*., 2009). Data were obtained for six traits (snout-vent length, tail length, body mass, forelimb length, hindlimb length, and number of toe lamellae) in 28 species, and a phylogeny for *Anolis* was taken from Thomas *et al*. (2009). Species were coded for ecomorph, but the trunk ecomorph and one unique species (i.e. not falling within any of the ecomorph classes) were represented by one species each, precluding analysis of convergence in these two ecomorphs. The tree was pruned in Mesquite v2.75 (Maddison and Maddison, 2011) in order that the final tree contained only the 28 species for which we had data. These species were *A. aliniger, A. bahorucoensis, A. baleatus, A. chlorocyanus, A. christophei, A. coelestinus, A. cybotes, A. distichus, A. insolitus, A. longitibialis, A. olssoni, A. semilineatus, A. singularis, A. cristatellus, A. cuvieri, A. evermanni, A. gundlachi, A. krugi, A. occultus, A. poncensis, A. pulchellus, A. stratulus, A. garmani, A. grahami, A. lineatopus, A. opalinus, A. sagrei* and *A. valencienni*.

A datafile for analysis was created for each ecomorph, such that each file had one ecomorph coded as the focal group. The index was first calculated for each datafile using 6-dimensional phenotypic distances consisting of an aggregate of all our traits ('total morphology'). Next, the traits were analysed as functionally-related aggregates to provide a more detailed, and biologically meaningful, look at morphological convergence amongst ecomorphs. These aggregate traits were as follows: body size (snout-vent length and body mass combined), limb length (forelimb and hindlimb length combined as together they are indicative of locomotor adaptations [Losos, 1990b]), tail length (on its own due to a potentially separate role from body size in balancing ability or other adaptations to arboreal habits), and number of lamellae (on its own due to its functionally independent potential role in climbing ability). P-values were generated from 100,000 bootstrap replications.

8.6 Results

8.6.1 Simulations

Using general linear mixed models (accounting for the particular tree and parameter values as random effects) we found no effect of the number of phenotypic traits used to generate the Euclidean distance matrix on the value of w for any of our six datasets (one to assess type one and type two error each for number of species, 'strength of selection', and proportion of focal species; all $P>0.05$), bearing in mind that each of the three traits were simulated using the same parameter values. This suggests that the incorporation of distances between species in 'combined' traits does not, in itself, influence the method and that it appears to perform adequately across this variation. As such, all of the following results are given on analyses conducted on 1-dimensional Euclidean distances only.

Fig 8.3 shows the results of our simulations. When all traits were simulated under BM most of the estimated P-values were greater than 0.05, giving an overall type 1 error rate of 0.053 (across all simulations), and no obvious relationship with any of our parameters is evident. When traits were simulated under OU for focal species almost all of the estimated P-values were less than 0.05, giving an overall type 2 error rate of 0.003 (across all simulations). Although the index performed well across all parameter estimates, it did so slightly worse when the 'strength of selection' in the OU model was very low and when the total number of species in the tree was low (although even in our 10 species trees all simulations gave $P<0.05$) (Fig 8.3). The Wheatsheaf index, when used with the P-value as a test, has good statistical power (0.997) to detect the presence of particularly strong convergence.

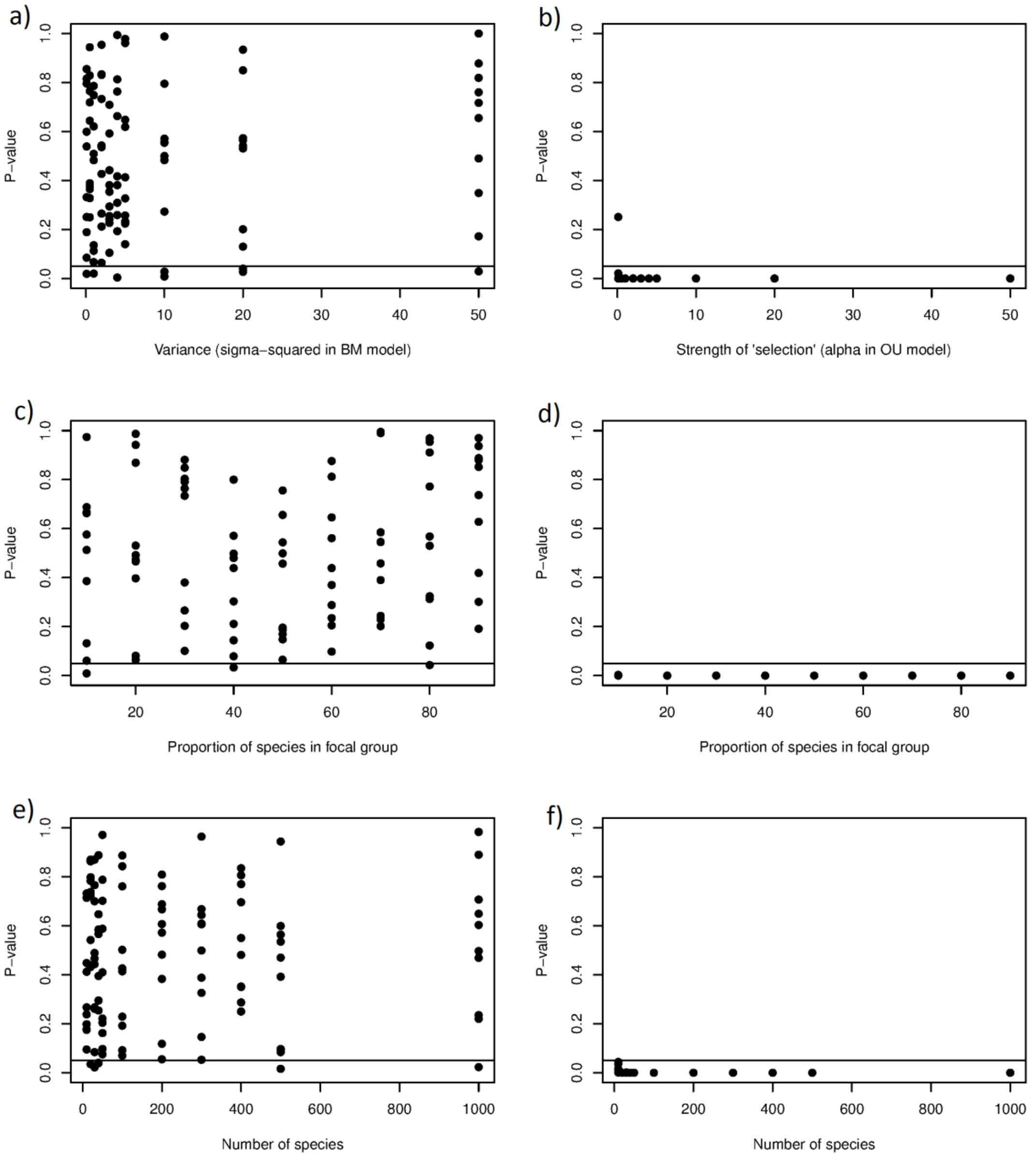

Figure 8.3. Results from our simulations. Ranges of parameter values are plotted against P-values in each graph. The horizontal line represent P=0.05 and individual data points are single simulations. The left-hand column (a, c, e) represents simulations of both focal and non-focal species under a BM model (e.g., with no convergence), and the right-hand column (b, d, f) represents simulations of non-focals under a BM model and focals under an OU process (e.g. where convergence is likely). See text for additional details of simulations.

8.6.2 Empirical example

Table 8.1 presents the calculated values of w for all analyses on the *Anolis* datasets, along with their 95% confidence intervals and P-values. The convergence within most ecomorphs (although present based on previous work) was not significantly stronger than expected given the tree. However, grass-bush anoles consistently showed very strong convergent evolution in all traits tested, as did trunk-ground anoles in overall (total) morphology and number of lamellae (Table 8.1). Furthermore, despite not being significantly stronger than expected, based on the P-values, a number of other instances of relatively strong convergence (P<0.1) were also observed (Table 8.1).

Table 8.1. Wheatsheaf indices (w) with associated 95% confidence intervals (given as lower and upper bounds as they are not necessarily symmetric) for each group of traits in each *Anolis* ecomorph. P-values from analyses are also provided, and significant (P<0.05) values are highlighted in bold. Out of the 28 species in total, the number of focal species for each ecomorph used as a focal in the analyses was as follows: twig (3), crown-giant (3), grass-bush (6), trunk-crown (8), trunk-ground (6).

Phenotype	Ecomorph	w	Lower bound	Upper bound	P-value
Total morphology	twig	2.9213	2.8609	2.983	0.494
	crown-giant	1.2232	1.2127	1.2343	0.678
	grass-bush	4.4692	4.4156	4.5188	**<0.001**
	trunk-crown	1.8444	1.8257	1.8654	0.129
	trunk-ground	2.5807	2.5533	2.6038	**0.023**
Body size	twig	2.3238	2.2748	2.3829	0.686
	crown-giant	0.72149	0.71219	0.73258	0.745
	grass-bush	9.0667	8.9076	9.2539	**<0.001**
	trunk-crown	1.9864	1.9663	2.0138	0.253
	trunk-ground	1.3605	1.3357	1.3665	0.059
Tail length	twig	3.0959	3.0426	3.1694	0.458

	crown-giant	2.0299	1.9878	2.092	0.384
	grass-bush	4.0083	3.9642	4.0651	**0.01**
	trunk-crown	1.7331	1.7158	1.7539	0.177
	trunk-ground	1.9416	1.9168	1.9514	0.063
Limb length	twig	2.6358	2.5895	2.6853	0.51
	crown-giant	1.4204	1.4085	1.4335	0.508
	grass-bush	5.5203	5.4029	5.6252	**0.002**
	trunk-crown	1.9208	1.8984	1.9416	0.089
	trunk-ground	1.4258	1.4074	1.4341	0.15
Number of lamellae	twig	1.7206	1.6943	1.7577	0.574
	crown-giant	3.5478	3.5182	3.578	0.129
	grass-bush	3.0619	3.0211	3.0976	**0.026**
	trunk-crown	1.2035	1.1957	1.2146	0.24
	trunk-ground	1.6949	1.6725	1.696	**0.01**

8.7 Discussion

An important question in evolutionary biology is whether convergence can be quantified. To begin to examine this question we have described a new method (the Wheatsheaf index) for measuring the strength of convergent evolution. The index provides a simple quantification of convergence and achieves a number of desirable qualities: comparability, intuitive interpretation, and phylogenetically informed.

The basis of the index is the relative phenotypic distances rather than absolute distances (and particularly since the traits are standardised to account for the degree of variation), and consequently is comparable between a wide variety of traits. It therefore provides a useful measure which can be compared directly between, for example, behavioural, morphology, and molecular traits, or between functional and developmental traits, for species within the same overall set. This provides a high level of flexibility in how the method can be used and opens up a range of questions which can now be explicitly tested. Because w increases as convergence becomes stronger it has an intuitive interpretation.

Although the interpretation of a particular value is made more difficult by the possible influence of topological constraints, the P-value incorporates this aspect and can also be used to compare across trees – further assisting with interpretation. The index provides a measure that incorporates both the similarity of focal species to each other, and the differentiation from non-focal species, which we regard as two key aspects of convergence. However, we must note that a high (or low) Wheatsheaf index can result from either of these aspects, e.g. from close similarity in phenotypic values or from less phenotypically similar species that are more phylogenetically distant. Therefore if we are interested in how a given value arose we must look back at the tree to further inform our interpretations of the underlying patterns. In most or all cases, it is probable that both of these elements will be responsible in part.

8.7.1 Limitations to the application of the index

As mentioned earlier, the Wheatsheaf index requires (semi-)continuous rather than discrete traits, unless there are multiple discrete traits to be included in the same analysis. This restriction is imposed on logical grounds. If a trait is either present or absent then organisms cannot be more or less convergent for that trait: they either are convergent (share the trait) or not. Therefore in the case of single discrete traits it is meaningless to give a measure of the strength of convergence and the best we can do is to identify whether or not convergence has occurred and look for correlates with any hypothesised focal niche. If, however, there are multiple discrete traits then we may sensibly ask questions about the strength of convergence providing we are concerned with a set of such traits rather than each one individually. In this case we can measure the strength of convergence in a phenotypic space defined by a set of binary traits, as this essentially creates a quantitative scale of similarity across traits (i.e. species can be more similar by sharing a larger number of discrete traits).

We have not examined the impact of taxon sampling within a clade, but given that all distances are pairwise distances, we do not expect incomplete sampling to be a problem, at least for analyses on the same tree. If incomplete sampling does not pose a problem, we could potentially take a large taxonomic group (e.g. birds, insects, animals) and sample a number of species from this group, encompassing both focal and non-focal taxa, with which we can calculate the Wheatsheaf index. However, we recommend where possible using reasonably well-sampled clades for analysis as this will reduce any concerns over selection of species for inclusion and so avoid potential confirmation bias arising from non-random choice of species to include. In particular, and given that the index works well on small trees, we would recommend that such questions are addressed by taking a number of smaller trees and comparing results across them, rather than using a very large but very poorly sampled clade.

It is important to choose the focal group based on clear, objective criteria based on an *a priori* hypothesis for two reasons. Firstly, if we assume that convergence is due to adaptation for a particular niche, then it must be considered in relation to that niche. In essence this instils a biological context to studies on convergence and encourages hypothesis-driven research. Even if we do not assume that the observed convergence is adaptive, the analysis should still be hypothesis-driven in that focals may be defined based on *a priori* identification of convergent species using other methods (e.g. SURFACE). Secondly, where we consider convergence to be adaptive it allows us to consider whether convergence has been driven by adaptation to the hypothesised niche. In the case of body shape in burrowing lizards, we might have three datasets with different classifications for the focal group: burrowing, sandy soils, and dense ground vegetation. We could then compare the strength of convergence for each of these and examine whether one shows a stronger signal than the others.

A final limitation of our method is that in the current implementation it is problematic to include fossil taxa. Because phylogenetic relatedness is penalised based on the distance from the root of the tree till the point when the pair of species diverged, it assumes that the species' continued along independent lineages until the present day. Since an extinct taxa pair may have been closely related at the time of their extinction but would be penalised based only on the time of their divergence, they would be considered by our method to be more distantly related than they actually are. Therefore the Wheatsheaf index can currently only be applied to trees of extant species, although this could potentially be addressed in a future development by using a co-phenetic phylogenetic distance to penalise phenotypic similarity when extinct species are included in the study.

8.7.2 Concordance of empirical results with previous literature

In our *Anolis* lizard dataset, perhaps the most notable finding is that ecomorphs differ in the strength of their convergence - grass-bush and trunk-ground anoles stand out as having particularly strong convergence compared to others. Furthermore, some traits are more strongly convergent within some ecomorphs but not others. Therefore patterns of convergence in particular traits are ecomorph-specific. Given the different niches inhabited by each ecomorph this is perhaps not surprising since different traits may be more or less needed for a given situation and so the divergence between ecomorphs drives the evolution of different combinations of traits. We will now discuss and highlight that many of our results are consistent with previous literature, which again indicates that the Wheatsheaf index is a useful and meaningful measure of convergent evolution.

Our analyses found the strongest convergence in limb length occurred in grass-bush anoles compared to the other ecomorphs, consistent with Losos' (1990b, 2009) finding of relationships between limb length and jumping and sprinting (perhaps particularly important for grass-bush anoles). The strong convergence of lamellae number detected in trunk-ground anoles suggests that there is a notable degree of adaptation in this trait. This could be a consequence of opposing selection pressures favouring fewer lamellae than highly arboreal ecomorphs but still enough to permit adequate climbing ability, e.g. for making quick dashes down tree trunks to capture prey (Losos, 2009). Grass-bush anoles have a small body size to facilitate movement through their structurally complex microhabitat, and have long hindlimbs, short forelimbs, and an exceptionally elongated tail (Losos, 2009).

Consistent with this we found that the Wheatsheaf index was very high for body size, limb length, and tail length in grass-bush anoles.

8.7.3 Extendibility and final comments

It should be noted that, in the current version of the index, the term used to penalise phenotypic similarity for phylogenetic relatedness includes a matrix of shared proportional distances. Consequently, penalised phenotypic distances increase with time since divergence of a given species pair. This implicitly assumes an evolutionary model similar to Brownian motion, wherein we expect greater phenotypic disparity with greater time since divergence. However, the method can be readily extended to explicitly incorporate other evolutionary models by generating the matrix of phylogenetic distances under these models, such as the various variance-covariance structures available in the R package ape (Paradis *et al*., 2004). This is a simple extension that relates to the creation of the input files before the calculations of w are conducted, but may serve to increase the flexibility of the index further.

Another useful extension would be a 'multi-focal-group' implementation of the Wheatsheaf index. By this I mean the ability to investigate many focal groups in the same analysis. For instance, having several focal groups (e.g. ecomorphs) included in the same index value to assess the extent of convergence in the clade as a whole. However, care would need to be taken to ensure that differences between focal groups would not mask convergence within each focal group.

Finally, we would like to highlight once more that the Wheatsheaf index is not designed to test for the presence of convergence. There are many good methods available for this (see introduction) and we assume that the selection of a group to use our index on is based on the presence of convergent evolution in the clade and that species contained within it have desirable characteristics for the question being asked in a given study. When convergence has been demonstrated, our method then allows the strength of this convergence to be quantified. Also, and particularly if the specific value of w is to be interpreted, the P-values must be discussed in relation to any inference in order to account for topological constraints on w.

We have developed and herein presented a novel method for the quantification of convergent evolution. The Wheatsheaf index is intended as an addition to the methodological toolkit for the analysis of convergence (used along with other methods, e.g. those for identification of convergence), and it is hoped that it will prove useful in elucidating details of this important and widespread evolutionary process.

8.8 Acknowledgements

The authors thank M. Berenbrink, S. Cornell, and J. Hodgson for helpful discussions while the method was being developed, H.J. Nichols and two anonymous reviewers for comments on the manuscript, and J.B. Losos for providing additional literature for the *Anolis* example. M.S. thanks S. Conway Morris for a highly stimulating talk on convergent evolution at the University of Liverpool (November 2009) which led the authors to consider the question of whether convergence can be quantified. K.A. was funded by a NERC Doctoral Training Grant. The Wheatsheaf index was named in honour of the pub in which K.A. first devised the method. Datafiles from the *Anolis* example are publically hosted on Figshare, accessible via the following URL: http://dx.doi.org/10.6084/m9.figshare.987096. MatLab scripts for implementing the Wheatsheaf index are available on request from the authors.

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9 *windex*: analysing convergent evolution using the Wheatsheaf index in R

This chapter is published in *Evolutionary Bioinformatics* (Arbuckle and Minter. 2015. *Evol. Bioinform.* 11:11 - 14), and a copy of the final article is contained at the end of this thesis.

9.1 Author Contributions

Kevin Arbuckle and Amanda Minter

Institute of Integrative Biology, Biosciences Building, University of Liverpool, Crown Street, Liverpool, Merseyside, L69 7ZB, United Kingdom

Amanda Minter assisted with writing the R code (jointly with myself) and provided comments on the manuscript. I developed the initial concept, designed the study, collected the data for examples, conducted the analyses, and wrote the manuscript.

9.2 Abstract

`windex` is a package developed for the R statistical environment to provide novel tools for the analysis of convergent evolution. The recently described Wheatsheaf index provides quantitative measures of the strength of convergence and opens up new possibilities for exploring this evolutionary phenomenon. The `windex` package allows implementation of this method with additional functions which can be used to create plots and perform statistical tests. R provides compatibility with other packages, and the R environment is familiar to many researchers. The `windex` package is freely available from CRAN: http://cran.r-project.org/web/packages/windex/. Consequently, `windex` can be installed directly from R and is distributed under the GNU General Public Licence 2.0.

9.3 Main Text

The use of phylogenetic comparative methods in evolutionary biology has seen a remarkable increase in recent years (Freckleton, 2009; Harvey and Rambaut, 2000; Münkemüller *et al.*, 2012; Morlon, 2014). Much of this growth has resulted from the proliferation of newly developed methods (e.g. Alfaro *et al.*, 2009; FitzJohn, 2012; Ingram and Mahler, 2013) and a shift towards implementation of these methods in R, which has enhanced the flexibility and between-method compatibility of their implementation.

Convergent evolution, or the independent evolution of similar phenotypes, is a commonly observed phenomenon across the tree of life (McGhee, 2011). Nevertheless, methods designed to study convergence have traditionally been limited to identifying its presence (e.g. Muschick *et al.*, 2012; Ingram and Mahler, 2013), i.e. whether convergence has or has not occurred in a given case. Recently, Arbuckle *et al.* (2014) developed a new method which aims to provide a quantitative measure of the strength of convergent evolution - the Wheatsheaf index. By quantifying convergence, this method allows an expanded range of questions we can ask about such as 'do life history traits show greater convergence than morphological traits' or 'do limbs or eyes show stronger convergence in burrowing animals'? This more detailed understanding of how convergent evolution operates as a evolutionary mechanism can only be achieved once a suitable measure is available that can be used to analyse a wide range of traits.

Briefly, the Wheatsheaf index generates phenotypic (Euclidean) distances from any number of traits across species and penalises these by phylogenetic distance before investigating similarity (in order to weight close phenotypic similarity higher for distantly related species). It also takes an *a priori* designation of 'focal' species which are defined as species belonging to a 'niche' for which the traits are hypothesised to converge. The method then calculates a ratio of the mean (penalised) distances between all species to the mean (penalised) distances between focal species. In effect, the Wheatsheaf index detects stronger convergence as the focal species' diverge more in phenotypic space from the non-focal species', and also as the focal species' show a tighter clustering to each other.

Upon describing the Wheatsheaf index, Arbuckle *et al.* (2014) made available a MatLab script with which to implement the method, although this was quite inflexible and many potential users are not familiar with MatLab. Therefore in this paper we introduce a user friendly R package (`windex`) with which researchers can use the Wheatsheaf index to analyse convergent evolution.

To illustrate the use of the R package `windex` we analyse morphological convergence for burrowing in monitor lizards (*Varanus*) using the Wheatsheaf index and data taken from Thompson *et al*. (2008). `windex` contains three functions: `plotTrait`, `windex`, and `test.windex` (Table 9.1). These functions require up to two inputs, which we will herein refer to as the 'traits' and the 'tree' for convenience. The tree is a phylogenetic tree of the class 'phylo', as is standard for most phylogenetic packages in R. Traits is a data frame consisting of a few necessary columns. The first column must be named 'species' and contain species names which match the tip labels in the tree. One column must designate the focal taxa (see Arbuckle *et al.* 2014 for details of the method itself and further understanding of these terms) as 1 and non-focal taxa as 0. The focal taxa are those species for which you are interested in testing for convergence (e.g. burrowing species in our

Varanus example). Other columns contain the trait values, typically (semi-)continuous traits but if there are a large number of binary traits in the dataset then they can also be used, as they would similarly allow the calculation of meaningful phenotypic distances. By (semi-)continuous we mean ordinal or count data in addition to truly continuous measurements, as all of these types would generate a meaningful phenotypic distance. The data frame is then read into the R environment. The data frame for our example herein consists of three traits of interest: head depth (`headD`), upper-fore limb length (`UforelimbL`) and upper-hind limb length (`UhindlimbL`). The first six rows are shown below to illustrate the format of the dataframe (titled `dat`), called as follows:-

```
> head(dat)
        species focal headD UforelimbL UhindlimbL
1    acanthurus     1  10.9       14.2       20.5
2    brevicauda     1   6.4        5.9        7.4
3 caudolineatus     0   6.8        7.8       11.3
4       eremius     1   9.6       11.3       16.8
5     giganteus     1  25.7       43.6       55.9
6       gilleni     0   7.3        8.7       11.7
```

Table 9.1. Brief summary of functions in the `windex` package.

Function	Input	Output
`plotTrait`	Traits	A plot of phenotypic space for visualisation of raw (not phylogenetically corrected) data.
`windex`	Traits and tree	Wheatsheaf index along with 95% confidence intervals obtained by jackkniving the data.
`test.windex`	Traits and tree	P-value for a test of particularly strong convergence, including a graphical display of the result.

The `plotTrait` function only requires the traits (not the tree), and is intended as a tool for data exploration. It produces a plot that represents a phenotypic space with 1-3 dimensions (traits) with focal taxa highlighted to visualise where they appear relative to non-focals, although this plot does not take into account phylogenetic relationships. Nevertheless, it may often be a useful preliminary step for understanding how the data are structured.

For our monitor lizard example, we plot 1, 2 and 3 dimensional plots with the traits head depth (`headD`), upper-fore limb length (`UforelimbL`) and upper-hind limb length (`UforelimbL`) (Fig. 9.1), using the following code:-

```
> par(mfrow=c(1,3))

> plotTrait(dat,traits="headD")

> plotTrait(dat,traits=c("headD","UforelimbL"))

>
plotTrait(dat,traits=c("headD","UforelimbL","UhindlimbL"
))
```

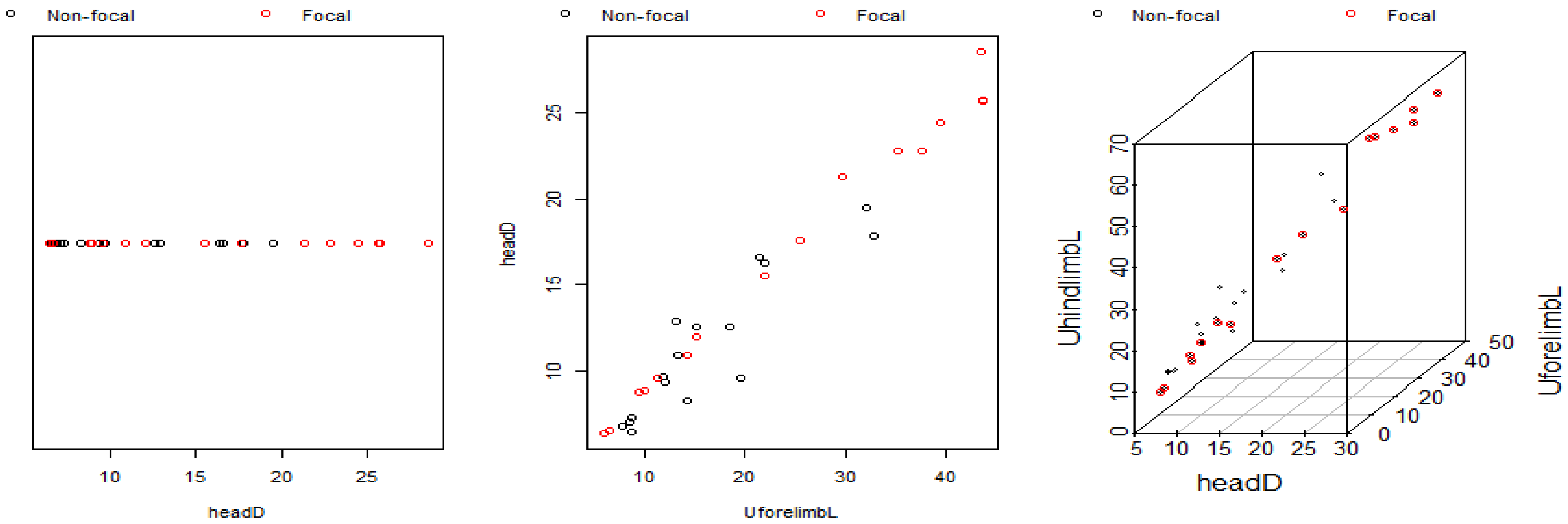

Figure 9.1. Illustrative example of `plotTrait` function for 1-dimensional, 2-dimensional and 3-dimensional plots using the traits head depth (headD), upper-fore limb length (UforelimbL) and upper-hind limb length (UforelimbL).

The core function of the package is `windex`, which takes both the tree and traits as input and calculates the Wheatsheaf index. This function also performs jackknife resampling of the traits as per Arbuckle *et al.* (2014) and uses these samples to return 95% confidence intervals alongside the calculated index. The method requires that measurements for each trait are standardised by the standard error for the trait across species. Although this can be done as pre-treatment of the datafile, the `windex` function includes an option that allows this step to be included as part of implementation, removing the need for any such pre-treatment of the data and so increasing the method's ease of use.

Here we use the function `windex` to calculate the Wheatsheaf index for a combination of three traits which are likely to be important in burrowing in our *Varanus* example: head depth (`headD`), upper-fore limb length(`UforelimbL`) and upper-hind limb length (`UforelimbL`):-

```
>
windex(dat,tree,traits=c("headD","UforelimbL","Uhindlimb
L"),SE=T)

$`Wheatsheaf Index`

[1] 1.322459

$`Lower 95% CI`

[1] 1.243233


$`Upper 95% CI`

[1] 1.389691
```

The final function in the package is `test.windex`, which implements the statistical test for exceptionally strong convergence given the topological constraints of the tree (see Arbuckle *et al*., 2014 for more details). The function takes the same arguments as the `windex` function (as this is called internally by `test.windex`), plus two additional arguments. The first (`reps`) specifies the number of bootstrap replicates from which the P-value is derived. The number of replicates is, of course, case dependent, but we have chosen 2000 in the example below as a compromise between computation time and accuracy. The user may wish to increase or decrease the number of replicates, though too few replicates may lead to unreliable results. The second additional argument (`plot`) is an option to plot a visualisation of the result in addition to returning the P-value for the test, and the default for this argument is set as `plot=TRUE`. The plot consists of a histogram of the distribution of the Wheatsheaf index from bootstrap replicates, with the calculated value and its 95% confidence interval marked on the plot. Additional arguments are passed to the basic `hist` function in R to allow the histogram to be customised. Since this function can take several minutes or longer on large datasets, we have incorporated a simple status bar to allow the user to monitor the progress of the function. To return to our monitor lizard example, we now illustrate the `test.windex` function on the same set of traits as were used above for the `windex` function:-

```
>
test.windex(dat,tree,traits=c("headD","UforelimbL","Uhin
dlimbL"),SE= TRUE,reps=2000,col="light grey")

$`P-value=`

[1] 0.097
```

The P-value obtained here is 0.097, marginally non-significant and therefore indicating that although convergence is present in *Varanus* (Thompson *et al.*, 2008), it is not exceptionally strong in the selected traits for burrowing. The plot generated by the code above can be seen in Fig. 9.2.

We hope that `windex` will greatly increase the ease of using the Wheatsheaf index to analyse convergent evolution. The method is not intended to overshadow currently existing analyses for studying convergence, but rather to complement them. Indeed, methods designed to test for the presence of convergence are strongly advised before using the Wheatsheaf index since it makes little sense to quantify the strength of something that does not exist in a given dataset. As such, we have developed `windex` as another component of the analytical toolbox available to investigators of convergent evolution, and one that provides an easy to use and useful extension to the suite of methods available in R (e.g. Paradis *et al.*, 2004; Revell, 2012; Ingram and Mahler, 2013).

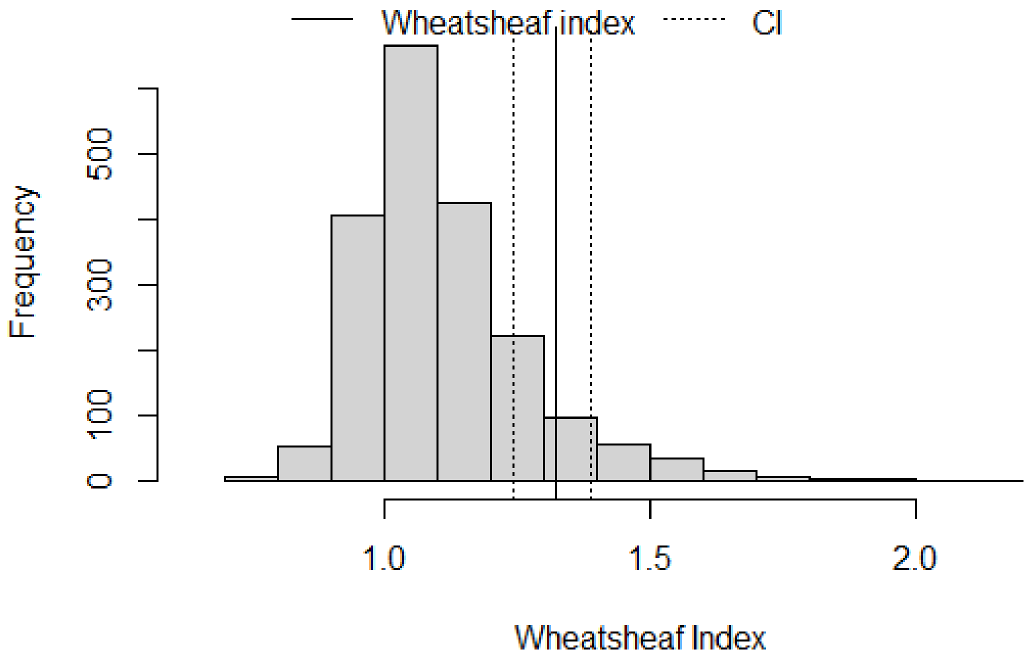

Figure 9.2. Histogram of the distribution of bootstrapped Wheatsheaf index values from our example of morphological convergence for burrowing in monitor lizards. The calculated Wheatsheaf index observed in the dataset is shown along with its jackknived 95% confidence interval.

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10 Teaching old data new tricks: reanalysis of neotropical butterfly defences reveals patterns of convergence related to type of mimicry

This chapter is currently in preparation to be submitted to the *Journal of Evolutionary Biology*, taking into account helpful discussions during my viva.

10.1 Author Contributions

Kevin Arbuckle[1], Robert B. Srygley[2] and Michael P. Speed[1]

[1] Institute of Integrative Biology, Biosciences Building, University of Liverpool, Crown Street, Liverpool, Merseyside, L69 7ZB, United Kingdom.

[2] USDA-Agricultural Research Service, Northern Plains Agricultural Research Lab, 1500 N. Central Ave, Sidney, MT 59270, USA.

Mike Speed provided comments on the manuscript and discussion of ideas. Bob Srygley collected the original dataset and provided comments on the manuscript and discussion of ideas. I developed the initial concept, designed the study and conducted the analyses, and wrote the manuscript.

10.2 Abstract

Protective mimicry, or phenotypic similarity between species which fulfils a defensive function, is among the best-studied antipredator mechanisms used by animals. Because in many cases the similarity observed within mimicry complexes is independently evolved, it represents an excellent (and common) example of convergent evolution. Nevertheless, although we know that mimicry can lead to similarities in multiple traits, our understanding of which traits show the strongest patterns of convergence is limited, and therefore so is our ability to predict the overall phenotypic outcomes of mimicry. Consequently, here we quantify the strength of convergence for several types of traits representing the defensive characteristics and appearance of a diverse group of neotropical butterflies. In particular, the butterflies in our dataset include several different mimicry groups and we compare the patterns of convergent evolution among our sets of traits with respect to each of these mimicry groups. We find evidence that the type of mimicry, Batesian or Müllerian, influences the observed pattern of convergence. Specifically, Müllerian mimics typically converge most strongly in traits that relate to defence (such as palatability, speed, and evasiveness), followed by size, and relatively weakly in biomechanical variables (which influence how the butterfly appears in flight). In contrast, Batesian mimics typically show the strongest convergence in the traits that relate most closely to appearance (i.e. size and biomechanics). Our study is the first attempt to quantitatively examine mimicry-type-specific patterns of phenotypic evolution, and as such provides novel insights into how mimicry influences convergence across a range of traits.

10.3 Introduction

Protective mimicry is among the best and longest-studied antipredator defences and involves the evolution of phenotypic similarity between prey species in order to signal unprofitability to predators (Ruxton *et al*., 2004). If the signal is honest such that multiple prey species which all possess repellent defences evolve a similar appearance to share the costs of educating predators about the meaning of the signal, it is termed 'Müllerian mimicry' (Endler, 1986; Ruxton *et al*., 2004). On the other hand, if the signal is dishonest such that edible prey evolve a similarity to an unprofitable species to deceive predators into ceasing (or failing to initiate) an attack, it is termed 'Batesian mimicry' (Endler, 1986; Ruxton *et al*., 2004). In practice, this distinction is often blurred to some degree (Speed, 1999; Balogh *et al*., 2008; Rowland *et al*., 2010), but nevertheless can here be considered a general pattern that is appropriate for further study.

An abundant literature on mimicry exists, much of which is focussed on mechanistic aspects (both functional and genetic mechanisms) and how mimicry originates and is maintained. For instance, mimicry has been demonstrated to reduce the frequency of attacks by predators in many prey taxa including spiders (Uma *et al*, 2013), insects (Kauppinen and Mappes, 2003; Barber and Conner, 2007), fish (Caley and Schluter, 2003), reptiles (Brodie and Janzen, 1995), amphibians (Kuchta *et al*., 2008), and birds (Rowe *et al*., 1986). Indeed, of the major groups of vertebrates, only mammals have not yet been convincingly demonstrated to procure reduced predation through the use of mimicry, although Müllerian mimicry in musteloids such as skunks is likely to operate. In addition to these individual benefits, often assessed with field or experimental studies, modelling approaches have been extensively used to investigate the evolutionary origins and maintenance of mimicry (e.g. Hadeler *et al*., 1982; Speed, 1999; Sherratt, 2008; Balogh *et al*., 2010) and the molecular underpinnings of some mimicry complexes have been interrogated with genomic methods (e.g. Joron *et al*., 2001; Jones *et al*., 2012; Kunte *et al*., 2014).

However, much less is known about the macroevolutionary patterns that characterise mimicry complexes. This is partly because direct tests of many potential hypotheses have had to await appropriate methodological developments, and partly because of a focus on other aspects of mimicry such as those mentioned above. Much of the existing comparative literature on mimicry has involved ancestral state reconstruction to assess whether mimetic similarity is derived independently or the result of a radiation which maintains ancestral colour patterns (e.g. Miller, 1996; Dumbacher and Fleischer, 2001; Symula *et al*., 2001; Sanders *et al*., 2006; Bocak and Yagi, 2010). Nevertheless, there are exceptions which consider broader questions such as whether mimics and models exhibit co-phylogenetic patterns (Ceccarelli and Crozier, 2007) or whether individual parts of mimicry patterns evolve as one functional unit or several (Simmons and Weller, 2002). Kunte (2009) also provided a reasonably comprehensive investigation of the phenotypic evolutionary path to Batesian mimicry in a complex of butterflies, but phylogenetic comparative studies still remain relatively rare in the context of work on mimicry.

There is, in particular, one evolutionary process for which mimicry often provides excellent examples (at least when mimicry patterns have evolved independently): convergent evolution. In his monograph on convergence, McGhee (2011) briefly makes this point but does not devote much discussion to mimicry on the basis that "entire books have been written on the evolution of mimicry". While this is true, few works have used mimicry as a good case study to ask questions about convergence *per se* or examined how convergence manifests itself in mimicry complexes, but are rather limited to the description of mimicry as an example of convergence. Just as studies of mimicry have rarely been addressed in the framework of convergent evolution, most reviews of convergence have devoted little or no time to mimicry as an example, despite its prevalence in nature (e.g. Arendt and Reznick, 2008; McGhee, 2011; Rosenblum *et al*., 2014). Therefore we contend that mimicry presents an underused opportunity to examine how convergence operates in nature and, consequently, how predictable the evolution of mimicry can be.

Because humans (and also some key predators such as birds) have good colour vision and are primarily visually orientated animals, mimicry complexes are often denoted by colour patterns. Examples of mimicry defined by other senses exist, such as acoustic mimicry of rattlesnakes by burrowing owls (Rowe *et al*., 1986), but are nevertheless focussed on one aspect of the phenotype. However, mimicry can involve multiple aspects of the phenotype which have evolved similarity in concert with, for example, colour patterns (Srygley, 1994; Uma *et al*., 2013; Penney *et al*., 2014). Despite some studies showing that different aspects of phenotype such as behaviours sometimes (but not always) converge alongside mimetic colour patterns (e.g. Penney *et al*., 2014), little is known about which or when traits should converge as a result of mimicry. Indeed, the answer may be dependent on the type of mimicry employed because Batesian and Müllerian mimicry have fundamentally different aims (deception vs mutualistic honest signalling respectively).

Herein, we use a combination of recently developed methods and a classic case study of mimicry evolution (neotropical butterflies) to investigate whether the type of mimicry influences the patterns of convergent evolution. Specifically, we predict that Müllerian mimicry complexes should most strongly converge on defensive traits such as palatability, evasiveness and flight speed, whereas Batesian mimicry complexes should be most strongly convergent on traits related to appearance in flight such as size and biomechanical attributes. This is because Batesian mimics have evolved similar phenotypes to their models for deception and rely solely on appearance for protection, but Müllerian mimics have evolved a more honest signalling strategy to inform predators of other defensive traits. Mimicry complexes which include both Batesian and Müllerian mimics should show characteristics pertaining to the predictions of each type due to each type of mimic exerting its own effects. We investigate these predictions using the recently developed Wheatsheaf index (Arbuckle *et al*., 2014) and in doing so also provide a case study for the use of that method in mimicry research.

10.4 Methods

10.4.1 Data collection

Data were obtained from 110 species of neotropical butterflies for a range of defensive traits, appearance, locomotion ability, and biomechanics variables (described below). Specifically, the data were taken from the data set collected and used in three previous studies (Chai and Srygley, 1990; Srygley and Chai, 1990; Srygley and Dudley, 1993), plus unpublished data for some additional species using the same methods. Full methodological details of data collection are available in those two papers and so will only be briefly described here to allow an understanding of what the variables represent in the context of this study. The data, R script, and the phylogeny (see below) used in this study are available from http://dx.doi.org/10.6084/m9.figshare.1428556.

For each species, palatability was measured as the proportion of butterflies that were eaten during presentations to a neotropical, insectivorous bird (rufous-tailed jacamars, *Galbula ruficauda*). Evasiveness (termed 'manoeuvrability' in Srygley and Dudley, 1993) was measured using presentations, similar to palatability, as the proportion of attacks that failed (a reasonable proxy for evasiveness based on behavioural observations, as used in the previous study). Flight speed was measured as wing lengths per second using recorded video clips of horizontal flight in insectaries, and both mean and maximum flight speed was recorded. The body length, mass, and wing length were measured in freshly euthanised specimens and then, following removal of wings and legs, the abdominal length and mass, and the thoracic length, diameter, and mass were measured. The wing area was measured from traces of wing pairs on graph paper, which served as a ready means of obtaining measurements from wings, and the wings themselves were weighed. From these specimens we also measured the following biomechanical variables: aspect ratio, wing loading, wing area centroid, radial moment of inertia, centre of wing mass, centre of body mass measured from the head, and centre of body mass measured from the wing base. All of these measurements were obtained from R.B. Srygley, and the dataset provided for use in the present work.

Because we were interested in convergence in functional groups of traits that correspond to biologically meaningful aspects of the overall phenotype rather than individual traits *per se*, we combined our variables into five trait sets for analysis (all our convergence analyses can handle multiple traits). The first two of these (palatability and evasiveness) consisted of single variables as these were relatively distinct traits. Another, which we term 'speed' combined both mean and maximum speed. Our 'body size' trait set consists of body length, body mass, thorax diameter, relative (to body length) abdomen and thorax length, and relative (to body mass) abdomen and thorax mass. Finally, our 'biomechanics' trait set included wing length, wing area, aspect ratio, wing loading, wing area centroid, centre of wing mass, radial moment of inertia, centre of body mass measured from the head, and centre of body mass measured from the wing base. We note that, because our analyses of convergence work on the basis of relative positions in an n-dimensional (for n traits) phenotypic space, correlations between individual variables within a trait set is not problematic for the methods, and so collinearity was neither tested nor accounted for in any way.

Butterfly species were assigned to one of 11 mimicry groups (or none of these), denoted based on colour pattern on the upper side of the wings in the same manner as Srygley (1994). These mimicry groups are named in this paper as follows: *Battus*, *Dione*, *Parides*, tiger1, tiger2, tiger3, tiger4, *Oleria*, *Laodamia*, black-white, and black-yellow-red. Also, because the four 'tiger' mimicry groups are broadly similar (distinguished primarily by differences in hindwing patterns), we also analysed a combined tiger group defined as those species displaying any of the four subgroups. Finally, we defined mimicry types by considered the mimicry complex to be Müllerian if all members were unpalatable (proportion eaten ≤0.5), as Batesian if at least one member was palatable and one was unpalatable, and as both types if there were multiple unpalatable species and at least one palatable species. We note that a palatability cut-off of 0.5 to delimit mimicry types is arbitrary, but there were no values near this threshold because highly palatable species in mimicry groups were either 1 or very close to 1, and unpalatable species in these mimicry groups were much lower, typically <0.3.

10.4.2 Phylogenetic tree

To allow us to analyse our data in a comparative framework, we created a phylogenetic tree for all 110 species in our dataset in two stages. Firstly, we assembled the topology in Mesquite v3.0 (Maddison and Maddison, 2014) from a set of published phylogenies which together contained information on the relationships between all taxa in our dataset (Caterino *et al.*, 2001; Penz and DeVries, 2002; Silva-Brandão *et al.*, 2005; Braby *et al.*, 2006; Beltrán *et al.*, 2007; Penz, 2007; Warren *et al.*, 2008; Wahlberg *et al.*, 2009; Brower *et al.*, 2010; Garzón-Orduña, 2012; Ortiz-Acevedo and Willmott, 2013; Wahlberg *et al.*, 2013; Brower *et al.*, 2014; Wahlberg *et al.*, 2014; Lewis *et al.*, 2015). Secondly, we dated this tree using 25 calibration points obtained from previously published sources (Wahlberg *et al.*, 2009; Wahlberg *et al.*, 2013; Lewis *et al.*, 2015) and the BLADJ function in

Phylocom v4.1 (Webb *et al*., 2008). The final tree was used for all subsequent analyses and is shown in Fig. 10.1.

10.4.3 Analyses

To justify the analysis of mimicry in the context of convergent evolution, we must first ensure that the mimicry groups represent cases of convergence rather than similarity derived from a common ancestor. To do this, we used stochastic character mapping (Huelsenbeck *et al*., 2003) to reconstruct the ancestral states for each mimicry group in turn (as a binary trait denoting membership in the group or not) over the tree using the phytools package (Revell, 2012) in R v3.1.2 (R Core Team, 2014). Stochastic maps were based on an 'all rates different' model, which allows rates of gain and loss of a trait to differ, and the results from 1000 simulations were summarised using the densityMap function in phytools, from which we assessed whether each mimicry group was convergent or not. If mimicry groups were not convergent they were excluded from downstream analyses of convergence.

We also tested whether our five trait sets showed some evidence of convergence to justify their analysis in the context of convergent evolution. Because the trait sets are comprised of multiple traits, simple ancestral state reconstruction is insufficient as such methods are typically designed for single traits. However, more recent methods have been developed specifically to test for convergence in multiple quantitative (cf. categorical) traits simultaneously. For this purpose we used the SURFACE method implemented in the package of the same name (Ingram and Mahler, 2013). SURFACE fits Ornstein-Uhlenbeck models of phenotypic evolution to a tree, first starting with a single model over the whole tree then adding new models with different parameter sets (called 'selective regimes' here) to different branches if the added model provides a significantly better fit across the tree. Then SURFACE compares these (independently evolved) additional regimes and tests whether any are the same as another regime on the tree. If so, this is taken as evidence for convergence because the phenotype shows independently evolved similarity on the tree. If trait sets did not show evidence of convergence they were excluded from downstream analyses.

To compare patterns of convergent evolution across trait sets and mimicry groups, we need a quantitative measure of the strength of such convergence. Herein we use a recently developed method that was specifically designed for such quantification, the Wheatsheaf index (Arbuckle *et al*., 2014). Briefly, the Wheatsheaf index considers convergence in traits within a 'focal group' (the mimicry group in this context) to be stronger as the focal group become more similar to each other and more disparate from non-focal species. Furthermore, phenotypic similarity is penalised by phylogenetic distance such that convergence is considered more striking when a pair of species are more distantly related. In this study, higher values of the Wheatsheaf index represent 'stronger' convergence (as defined in Arbuckle *et al*., 2014) for a given trait set within each mimicry group. The method also provides a P-value for exceptionally strong convergence given phylogenetic constraints (note, not for whether convergence exists or whether it is stronger for one trait than another - direct comparison of index values provides information on the latter). All calculations involving the Wheatsheaf index were conducted in the R package windex (Arbuckle and Minter, 2015), and plots of index values were drawn using the plotCI function in the plotrix package (Lemon, 2006).

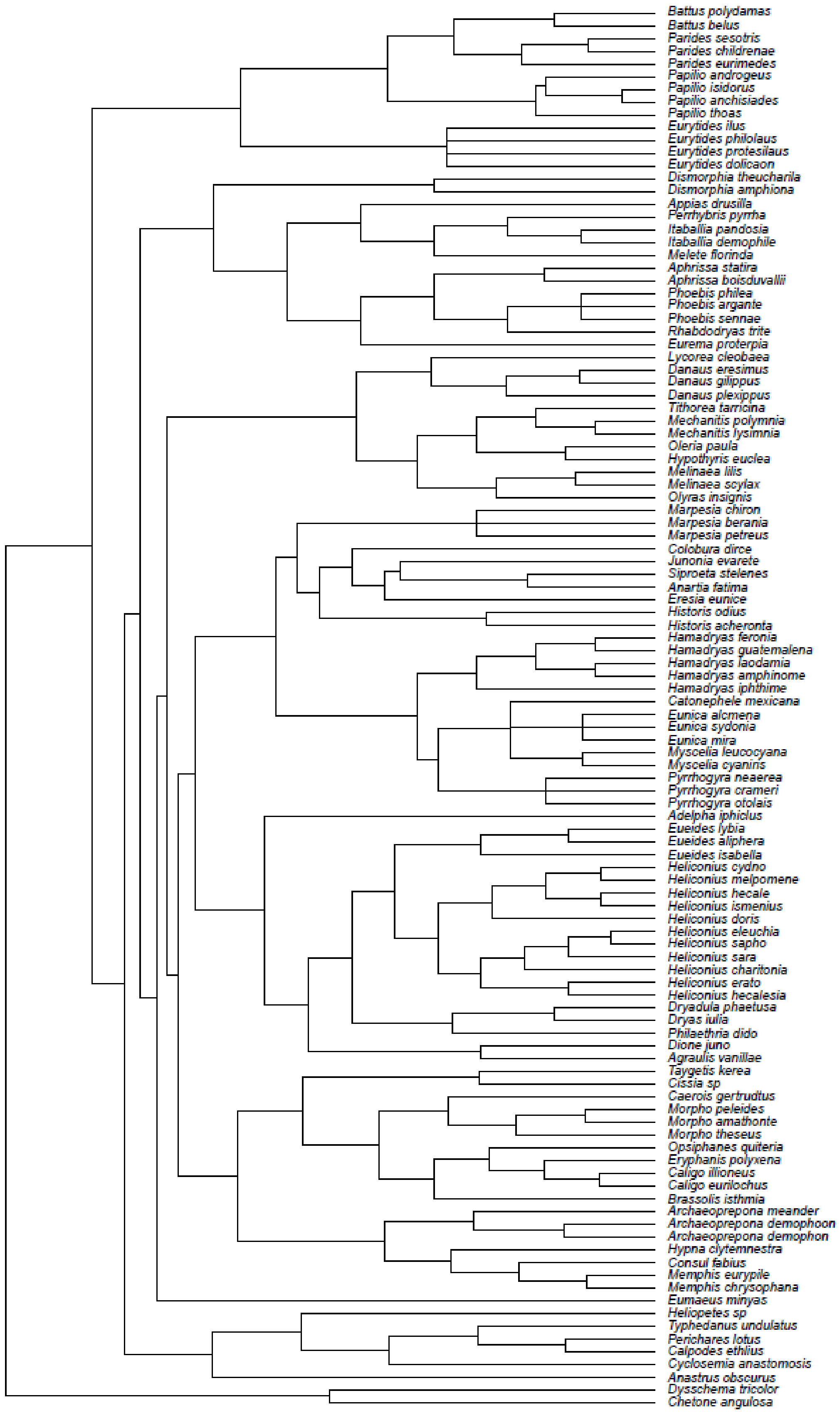

Figure 10.1. Phylogenetic tree for all 110 species used in this study.

10.5 Results

In our checks for convergence of mimicry groups, stochastic mapping revealed evidence for convergence in 8 of the 11 groups (plus the combined tiger group). In two cases (the *Dione* and *Laodamia* groups), the mimicry group consisted of a single origin in the ancestor of two sister species in our dataset and so was excluded from further analysis. In the other group which did not show evidence for convergence, the *Parides* group, the mimetic colour pattern was estimated as the root state which was lost several times in descendant lineages. While this is most likely to be a consequence of limited taxon sampling compared to the overall diversity of butterflies, rather than an accurate reconstruction, we exclude the *Parides* group to be conservative and uphold our *a priori* criteria for inclusion.

The SURFACE analyses to ensure that our trait sets exhibited convergent evolution found evidence for this in all cases. Specifically, we found 18 shifts to four convergent regimes for palatability, seven shifts to three convergent regimes for evasiveness, 12 shifts to three convergent regimes for speed, 10 shifts to four convergent regimes for body size, and six shifts to three convergent regimes for biomechanics. As such, all trait sets were included in further analyses of convergence.

Individual plots for each mimicry group displaying the calculated Wheatsheaf index for each trait set are presented in Fig. 10.2 (annotated above each plot with the type of mimicry in the group). In each case of Müllerian mimicry groups, palatability, evasiveness or speed are the most strongly convergent traits, whereas in the Batesian mimicry group (*Oleria*) size is the most strongly convergent trait set. Furthermore, the biomechanics set (relating to appearance in flight) is weakly convergent compared to others in all Müllerian groups, but is stronger than some other trait sets in mimicry complexes that include at least some Batesian mimics.

Mimicry complexes characterised by both Müllerian and Batesian mimics show intermediate patterns with some defensive traits (e.g. speed) and some traits.'appearance' traits (e.g. biomechanics) being amongst the most strongly convergent If the single Batesian mimic in the tiger1 group (*Consul fabia*) is excluded, creating a Müllerian only group, the results for tiger1 are almost identical (Fig. 10.3). The only quantitative difference is that using only Müllerian mimics results in slightly stronger convergence for evasiveness and palatability, with both of these traits showing evidence for exceptionally strong convergence (P-values of 0.038 and 0.051 respectively). When the same is done for the combined analysis of the tiger groups, we find a similar result in that the index values do not change a great deal except for palatability showing stronger convergence when the Batesian mimic is excluded (Fig. 10.3).

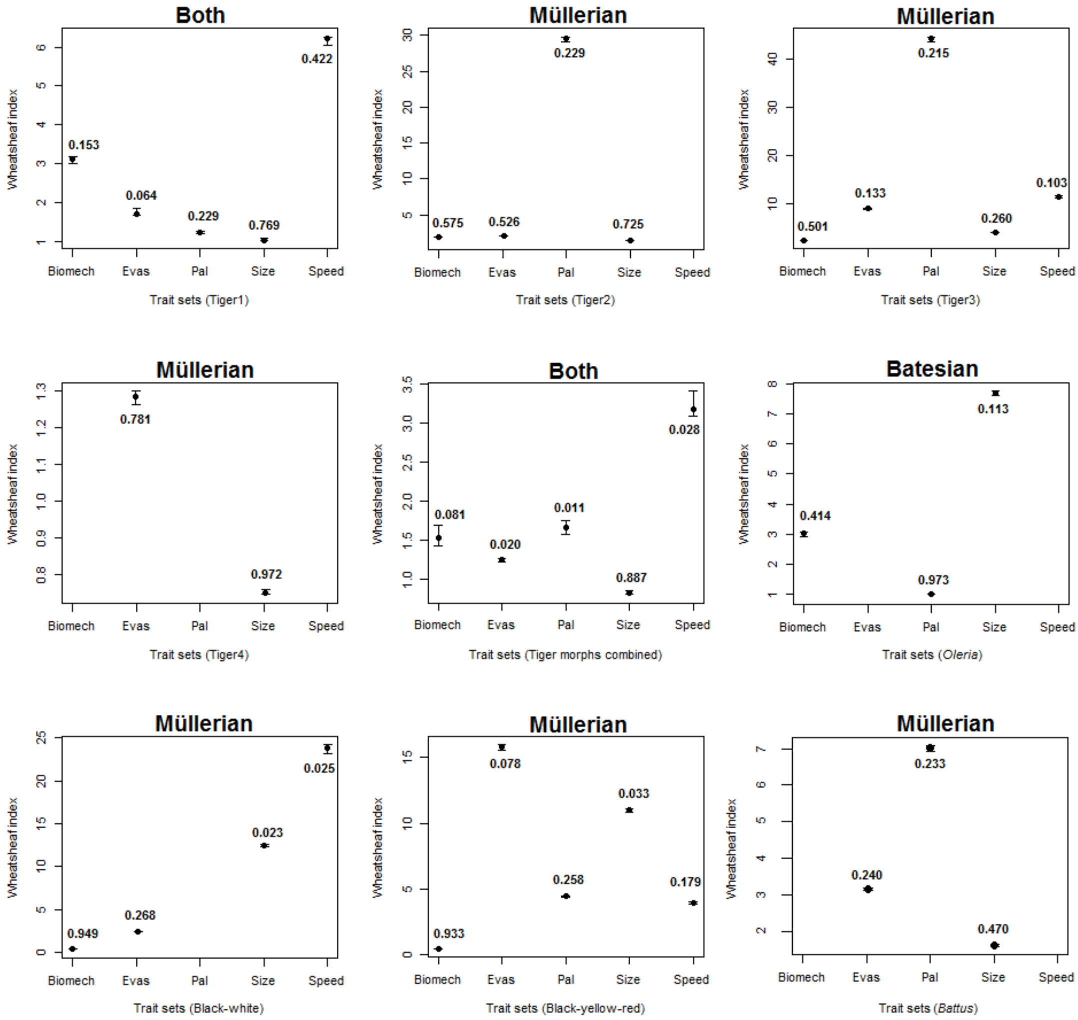

Figure 10.2. Plots of the Wheatsheaf index (with 95% confidence intervals) for each mimicry group. Each plot contains the index values for each trait sets, and is annotated above with the type of mimicry (Batesian, Müllerian, or both) found within each group. Numbers on the plots beside each point are the ‘P-values’ for tests of exceptionally strong convergence given the phylogeny, but note that these do not affect comparisons between trait sets within plots.

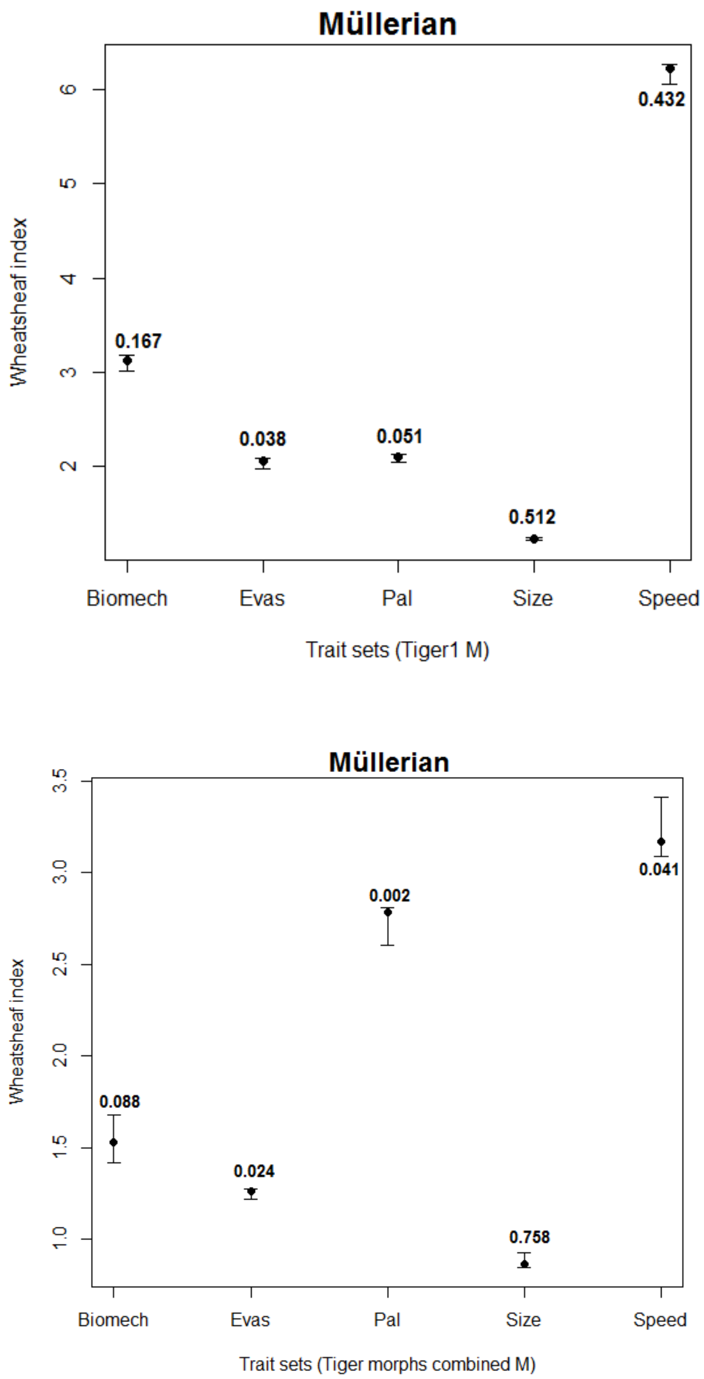

Figure 10.3. Plot of the Wheatsheaf index (with 95% confidence intervals) for the tiger1 and combined tiger mimicry groups excluding the single Batesian mimic (*Consul fabius*). Format and annotations on plot are the same as Fig. 10.2. Note that the exclusion of *C. fabius* has very little quantitative and no qualitative impact on the results.

Wheatsheaf index calculations are missing for some trait sets in many of the mimicry groups, and this is for one of two reasons. Where speed or biomechanics do not appear in a given plot, data were missing for members of the mimicry group in question. Where results are missing for palatability in Müllerian mimicry groups, this is due to all members being extremely unpalatable (with values of 0), and these 0 values cause mathematical difficulties in the calculation. Nevertheless, this situation likely represents strong convergence for low palatability in these groups, even if it cannot be strictly quantified by our measure. Therefore, in these two cases (tiger4 and black-white groups), palatability is likely the most strongly convergent trait despite its absence from the plots in Fig. 10.2. The same scenario as with palatability also explains the absence of a result for evasiveness in the *Oleria* Batesian mimicry group, in which both model and mimic had very low evasiveness with a value of 0 and the measure again became mathematically intractable.

We note that cases of exceptionally strong convergence (identified by low P-values on the plots) do not follow the general patterns described above. However, these are influenced by phylogenetic constraints and the distributions of the traits, and the number of species in the mimicry group (not just the strength of convergence), and so provide different information than the Wheatsheaf index itself (in which the patterns are observed). Note also that they do not influence our comparisons between the index values of different trait sets within a mimicry group. For example, a trait may be less strongly convergent than another but be higher than its own expected value (based on its phylogenetic distribution), and therefore be exceptional but not necessarily more strongly convergent than another trait. Nevertheless, the P-values indicate that convergence may often be stronger than we expect based on phylogenetic (topological) constraints, implying that traits are relatively free to evolve in this system.

10.6 Discussion

By reanalysing data on the form and function of 110 neotropical butterflies using new methods, we provide novel insights into the patterns of convergence found in mimicry complexes. Specifically, our results suggest that Müllerian mimics are generally characterised by stronger convergence in defensive traits than others, whereas our Batesian mimicry group was characterised by stronger convergence in traits relating to appearance than others. These findings are consistent with predictions based on the mechanisms by which different types of protective mimicry operate - Batesian by deceptive appearance, Müllerian by aposematic advertisement of other defences. However, we caution that our results for Batesian mimics were based on only one group and to a limited extent on comparisons between groups with Batesian mimics present vs remoed from analyses. We also provide some evidence for the Srygley's (1994) idea that Batesian mimics should diverge from Müllerian mimics (i.e. their models) in traits associated with escape from predators following attack. As a result of this analysis of convergent evolution in mimicry, we illustrate the usefulness of methods to quantify convergence for understanding evolutionary patterns.

Multimodal signals, such as combinations of morphology and behaviour, are commonly used in defensive contexts (Ruxton *et al.* 2004). The evolution of such signals has been discussed largely in terms of aposematism, in which multimodality may provide different signals to different predators (e.g. Ratcliffe and Nydam, 2008). Similarity in different traits within mimicry complexes may evolve for similar reasons (effective mimicry against multiple predators; e.g. Uma *et al*., 2013), or to increase the general resemblance of Müllerian mimics or Batesian mimics to their models in order to signal to predators which use several different cues in prey choice. However, results from previous work have been mixed. For example, Srygley (1994) found evidence for locomotor mimicry in colour pattern mimics in a subset of the neotropical butterflies included in this paper. In contrast, Rashed *et al*. (2009) found no evidence of acoustic mimicry in hoverflies (Batesian morphological mimics of Hymenoptera) and Penney *et al*. (2014) found that some hoverflies were also behavioural mimics but most (51 of 57 species tested) were not. Our results suggest that some of this variation may be a consequence of different mimicry types, although we do not claim it to be a complete explanation of variation in multimodal signalling in mimics (which is likely to consists of many reasons).

Our knowledge and understanding of broad-scale macroevolutionary patterns has perhaps been obscured by the focus on research at lower scales such as individual benefits, ecology, and microevolutionary principles such as origin and maintenance of mimicry. With some exceptions (e.g. maintenance of Batesian mimics dependent on model frequencies; Joron and Mallet, 1998) there is little need to discuss alternative types of mimicry with regard to many questions. This is largely because at this scale predators should perceive both types similarly and therefore the distinction is less important; a predator will treat all members of a given mimicry complex as unprofitable, by definition, although not necessarily equally so. However, at larger scales where we are concerned with the evolutionary relationships between overall phenotype (multiple traits), distinguishing patterns by mimicry type may be more important. In this case, we are not explicitly concerned with how the predator perceives prey *per se*, but what which aspects of the phenotype are most important, which is different in Batesian and Müllerian mimics. In the former, traits involved in creating a deceptive appearance are key to the mimics, rather than traits involved in underlying defences. In the latter, the more or less honest nature of the signals mean that creating a repellent or otherwise well-defended phenotype is more important than having a general similarity (providing the signal itself is shared).

Although Batesian mimicry was relatively rare in our study system, with only one mimicry group being characterised exclusively by Batesian mimicry, we can look at the effect of Batesian mimics in our tiger1 group (consisting of both mimicry types) to aid our interpretation (Figs. 10.2 and 10.3). Although the results before and after excluding the Batesian mimic from analyses are broadly similar, and qualitatively identical, we note two quantitative differences. These were a signature of stronger convergence in palatability and evasiveness when only Müllerian mimics were considered. This result indicates that the Batesian mimic is less convergent in both of these defensive traits than the Müllerian mimics, which is unsurprising in the case of palatability (as this was used to define mimicry type) but more interesting with respect to evasiveness.

Although we may expect Batesian mimics to both look and behave like their models, Srygley (1994) argued that models and mimics may diverge in behavioural (e.g. escape-related) defences. The basis of this view is that, whereas Müllerian mimics have little need for evasive flight because they rely on unpalatability if attacked, and in fact may use such slow flight as an aposematic signal in itself (Srygley, 1994; Sherratt *et al*., 2004), Batesian mimics are likely to be consumed if attacked, and therefore should retain the ability to evade capture. Our results lend support to this idea, because convergence in evasiveness was stronger within the tiger1 group when the single Batesian mimic was excluded.

In our *Oleria* Batesian mimicry complex, we note that one defensive trait (evasiveness) showed signs of very strong convergence in that both the model and mimic had values of 0. This reflects that all attacks by the avian predator in the original presentation experiments on both models and mimics were successful, although the number of attacks differed. This observation of seemingly strong convergence could arise if neither species prioritises evasiveness as an antipredator strategy. For instance, if the mimic relies mostly on deceptive similarity to the model, which relies mostly on chemical defences to avoid being eaten, a lack of investment into evasiveness as an alternative strategy may lead to low failure rates by predator that do decide to attack. If predators rarely attack due to the aposematic (or mimetic) signals displayed by butterflies, then relatively ineffective alternative defences such as evasiveness may not be strongly selected against, particularly if evasiveness is involved in a trade-off with other aspects of the biology (Srygley and Dudley, 1993).

Although we found that different types of traits (e.g. 'appearance' versus 'defensive') converged in different types of mimicry groups, no specific trait set was consistently the most strongly convergent in all groups, even when only Müllerian mimicry groups are considered. This suggests that general trends in convergent evolution (such as applied to mimicry complexes) may be predictable, but that species- or group-specific patterns can generate less predictable differences in details. More examples of Batesian mimics are necessary to explain this finding for those groups, but Müllerian mimics are perhaps more yielding to explanation with the data we present.

In Müllerian mimicry, the important point is that predators learn that the warning signal means that the prey possess a defence that renders it unprofitable to catch (Ruxton *et al*., 2004). This leaves open the possibility that different defences are signalled by the same warning coloration in Müllerian mimicry rings or that defences are shared among members of the complex but different species have different patterns of investment in each defence. In terms of our results, where we compare which traits are most strongly convergent across different mimicry groups, it is likely to be a version of the second explanation in action here: different mimicry groups may possess similar defences, but they are investing more or less in particular defence strategies depending on the particular mimicry complex. This, combined with the common need in all cases of Müllerian mimicry to advertise some underlying defensive trait, could readily explain our finding of broad-scale generalities but smaller-scale variation in patterns of convergence. If this can be generalised to other cases of convergent evolution, it might suggest a reconciliation of opposing views on the predictability of evolution (e.g. Gould, 2000; Conway Morris, 2003) by considering it to be both predictable and highly stochastic depending on the level of measurement.

Protective mimicry is a common antipredator defence and commonly falls under one of two types: Batesian or Müllerian. Despite an extensive history of research, patterns of phenotypic evolution (in addition to colour pattern) resulting from mimicry remain an understudied aspect of this defence, and even fewer studies exist which specifically compare the effect of different types of mimicry. In particular, mimicry complexes often provide excellent examples of convergent evolution. Therefore, in this study we show that the recently developed Wheatsheaf index can be applied to investigate patterns of convergence in multiple phenotypic traits in several mimicry complexes of neotropical butterflies and discuss the results in the context of Batesian vs Müllerian mimicry. We find evidence for a general pattern for relatively strong convergence in 'defensive' traits between Müllerian mimics and relatively strong convergence in 'appearance' traits between Batesian mimics and their models. However, we again stress that results for Batesian mimicry are based on very very comparisons and only one solely Batesian mimicry group. We also find that the particular traits within these broad generalisations which converge most strongly are species- or group-specific, and so suggest an underlying unpredictability of details within a coarser scale predictability of phenotypic evolution. Such work therefore provides insights into patterns of convergent evolution in nature, the macroevolution of a common antipredator defence, and suggests that different mimicry types can carry different evolutionary consequences.

10.7 Acknowledgements

K.A. was funded by a NERC Doctoral Training Grant.

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11 Conclusions and future work

11.1 Conclusions

This thesis aimed to "improve our understanding of the macroevolutionary patterns and consequences of antipredator defences in animals" (section 4.2). To this end I have presented six results chapters which collectively investigate how antipredator defence influences diversity, either by acting to generate diversity, or by imposing constraints which can limit diversity via convergent evolution. I believe I have achieved this aim and, in doing so, highlighted the importance of predation as an importance force shaping evolution. Although conclusions specific to individual chapters are discussed in the relevant section of the text, I will briefly summarise the overall conclusions of this thesis here by integrating results from different chapters.

Chapters 5-7 considered the consequences of antipredator defence on the ecology, evolution, and conservation of animals. Taken together, my results provide evidence that these three elements are linked together such that evolutionary effects are driven by impacts on the ecology, which has implications for the conservation of species using particular defence strategies. Specifically, my work supports the idea that repellent chemical defence can allow a species to take advantage of more ecological opportunities as a result of relaxed behavioural constraints (which are imposed by predators). This is reflected in a broader niche and activity period in chemically-defended species and advertisement of these defences with conspicuous colour patterns can enhance the effect.

The use of chemical defence and conspicuous coloration as antipredator strategies can lead to increased speciation rates as predicted by 'escape-and-radiate' theory and *a priori* expectations of greater propensity for speciation in lineages which occupy a broader niche. However, in the case of lineages possessing chemical defences, a heightened extinction rate means that they actually diversify slower than lineages lacking chemical defences. Unfortunately, because chemical defence seems to be readily gained but difficult to lose (presumably due to the strong benefits to individuals), lineages become 'locked in' to a strategy which increases the probability of their extinction. Moreover, it seems that contemporary extinction risks are somewhat reflective of background (macroevolutionary) extinction rates in the case of antipredator defence, because chemically-defended species are also more likely to be considered threatened by the IUCN.

In Chapters 8-10, I developed new methods to enhance the study of convergent evolution and use these to investigate patterns of convergence found in protective mimicry complexes. This work described a new conceptual framework that enabled me to define, and subsequently measure, a 'strength' of convergent evolution. Once a flexible implementation of the method was created in the R environment for statistical computing, I was able to explore how convergence shapes patterns of phenotypic evolution in mimicry. I was able to derive a commonly held expectation, that convergence in appearance is stronger in Batesian mimics while convergence in defence is stronger in Müllerian mimics, providing an empirical demonstration of how the Wheatsheaf index can be used. However, despite broad-scale patterns such as this, the specific group of traits which showed the strongest convergence differed by mimicry group. Therefore, it seems that phenotypic evolution may be predictable at broad levels, but far less predictable in the details. I also find support for previous predictions that Batesian mimics should diverge (i.e. show weaker convergence) from their models in evasiveness, suggesting that certain traits vital to escaping predator if the deception fails are not tightly constrained in their evolution by mimicry.

I therefore contend that predation has been an important driving factor in evolution, in that antipredator defence mechanisms can modulate both the generation and constraints of biodiversity. In this thesis, I refer to biodiversity in the broad sense to include diversity of phenotypes (constrained by mimicry, Chapter 10), diversity of ecology (enhanced by certain defences, Chapter 5), and the more commonly used diversity of species (different effects for different defences, Chapter 6). These effects may remain of importance in the present day, since repellent chemical defences are related to higher extinction risk in contemporary species. Nevertheless, further work is required in order to evaluate whether the relationship between chemical defence and conservation status still holds after other traits (such as body size and latitude) are taken into account. If it does however, it may lead to the first potential application of this work.

Knowledge of the extent of the threat of extinction is vital for conservation programmes because resources are limited and so must be allocated carefully. The decision of how to allocate such resources is often informed by the risk faced by the organisms in question, with priority given to the most threatened species. However, knowledge on the state of wild populations is lacking for many species, and this is perhaps most severe for the most threatened species because rarity makes population surveys difficult. Consequently, a great deal of effort has been expended in predicting extinction risk from various traits. Good predictive models are likely to require information on many different traits and so understanding which traits are linked to conservation status provides information on what should be included in a general and predictive model.

The other potential application of work in this thesis is the use of the Wheatsheaf index and associated windex package in R by researchers working on convergent evolution. The method provides a way to directly compare the strength of convergence in different (sets of) traits and therefore should enable a quantitative approach to broad scale question of how convergence operates as a process shaping phenotypic evolution. Consequently, in addition to contributing to the specific field of antipredator mechanisms, I believe that the work contained in this thesis may be applicable to the wider evolutionary biology community.

11.2 Caveats and Limitations

This thesis has used a range of phylogenetic comparative methods to address a set of questions related to the evolution of antipredator defences. However, as with all methods, this approach comes with several assumptions, caveats, and limitations that could potentially influence the results. The most important of these are therefore discussed in this section.

Perhaps the most important limitation is that the methods used can provide only limited evidence for causality and no direct evidence for the mechanistic underpinnings of the results. In contrast, the analyses presented throughout this thesis are limited to demonstrating evolutionary patterns rather than mechanisms and processes. Nevertheless, I hope that the patterns reported herein can stimulate further research into the underlying mechanisms that produce these patterns.

The various phylogenetic methods used herein make their own indidivual assumptions and therefore the results obtained have caveats specific to each method. A common assumption made by all the methods in this thesis is that the phylogeny used in the analysis is correct. In reality, we can never know what the true phylogeny is, and the chapters herein all rely on a single phylogeny - not taking into account phylogenetic uncertainty. Furthermore, many comparative methods including those used in Chapter 5 may be prone to giving false positives when the traits of interest are clumped in the phylogeny and exhibit few independent origins. It is possible that this is the case in some of my analyses (for example, see Fig. 5.1) and therefore this caveat applies almost throughout the thesis. Additionally, the BiSSE models used in Chapter 6 are considered to perform poorly when some of its assumptions are violated. An important one is the assumption that diversification rates are constant throughout the phylogeny, in that they do not show substantial patterns of variation through time or between clades. This is unlikely to be the case in any large phylogeny, and although some of the other analyses in Chapter 6 attempt to limit the risk of false inference due to such factors it is a caveat that could nevertheless influence my results. If this is problematic for Chapter 6, then it also applies to Chapter 7 as the idea for that chapter originated from the results of Chapter 6.

A broader limitation and caveat to some of the chapters in this thesis, particularly Chapters 5 and 7, is that the statistical models contained therein are univariate - containing only a single explanatory variable. This leaves open the possibility that the results are susceptible to covariation of the explanatory variables with other traits not included in the methods. For instance in Chapter 7, where I asked whether chemical defence predicts conservation status, it would be instructive to include additional variables in the model such as body size and latitute. This extended analysis will be added before the manuscript is considered ready to submit for publication, as it is an important caveat given the numerous factors that have been shown to influence extinction risk.

11.3 Future work

Throughout this thesis, I have suggested a number of places where the work presented here could be extended in future studies. In this section, I will briefly highlight the areas which I believe are likely to be the most beneficial ways to take this research forward.

In Chapter 6, I suggest three hypotheses that offer potential explanations for the increase in extinction rates observed in chemically defended amphibians. In Chapter 7, I discuss these again in light of the concordance of the results between these two chapters despite the different temporal scales of the analyses. However these competing (though not mutually exclusive) hypotheses remain to be tested, and doing so would begin to provide a mechanistic insight into the findings presented in these chapters. Such a project would require substantial effort to compile a dataset of numerous variables related to geographic distribution, habitat preferences, life history traits, and perhaps estimates of energetic costs for as many of the 857 species of amphibian for which I have data on chemical defence. Nevertheless, understanding this rather surprising result would be fruitful and would likely outweigh the costs associated with conducting the study.

In Chapter 7, I suggest that because chemical defence is associated with a higher extinction risk (or at least a more severe IUCN Red List status) it is possible that it could be included in models designed to predict extinction risk for establishing conservation priorities. However, this requires substantial additional work to confirm the link between defence and conversation status. Furthermore, given that other traits have also been linked to extinction risk it would be enlightening to conduct a study directly comparing the predictive ability of different traits and combinations of traits. Evaluating the relative importance of different traits in predicting threat status would enable an objective assessment of which variables should be included in an optimal predictive model. The consequences of making wrong decisions in conservation scenarios are severe, potentially leading to the extinction of species. Therefore accuracy and applicability are key requirements of any estimation method for extinction risk and so devising an optimal model for this is vital.

In Chapter 8 I describe the Wheatsheaf index as a method to measure the strength of convergent evolution. However, there are a number of extensions to this method which would be useful if implemented, so there is scope to continue working on it. For instance, most or all phylogenetic comparative methods are likely to benefit from the inclusion of fossil taxa, but the current formulation of the Wheatsheaf index was designed for extant taxa only. This could be implemented by using a co-phenetic phylogenetic distance matrix rather than a variance-covariance matrix derived from the tree as the representation of phylogenetic distance used by the method. The Wheatsheaf index does not explicitly assume any model of evolution, but does implicitly assume a model whereby similarity between a pair of species is a function of the time since their last common ancestor. Since it might be useful in some circumstances to measure convergence while incorporating a particular model (or a range of different models) of trait evolution, such as an Ornstein-Uhlenbeck process or Early-Burst models, adding this functionality to the method and the R package 'windex' would be a benefit. One other extension that could prove very useful is to use the Wheatsheaf index as a 'prospecting tool' to evaluate which traits (or set of traits) are most (or least) convergent for a given focal group. In essence, this would involve an iterative approach whereby traits are added singly and in all possible combinations, the index is calculated for each set, and the set of traits with the highest (or lowest) value of the index is deemed to be the most (or least) convergent trait set for that focal group.

In addition to the above-mentioned extensions to the Wheatsheaf index, there are other studies which could be based on the method. For example, although the original paper describing the index (Chapter 8) examined its performance under a range of conditions, it did not evaluate how sensitive it is to taxon sampling. A simulation study comparing the results generated by running the index on variously sized subsets of taxa sampled from one large clade would therefore add to our understanding of how the results should be interpreted under incomplete taxon sampling.

Finally, using the same conceptual framework which prompted the development of the Wheatsheaf index, other related methods could be developed. For example, a similar metric of divergence (cf. convergence) would be a straightforward complement to the Wheatsheaf index but permit comparable analyses of the opposite side of this spectrum of phenotypic evolution. The use of a method for categorical variables (such as the common case of binary traits) composed within the same framework would also open up quantitative analyses of convergence to a wider range of traits that possible with the Wheatsheaf index, although this method would be more challenging to devise.