Colour polymorphism in the leaf beetle genus *Oreina*

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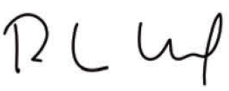

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Colour polymorphism in the leaf beetle genus *Oreina*

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Abstract

This dissertation investigates the maintenance of colour polymorphism in the leaf beetle genus *Oreina* CHEVROLAT (Coleoptera; Chrysomelidae). The remarkable colour variation in this seemingly aposematic and chemically defended genus forms a paradox with current scientific views concerning predator learning and mimicry. Using a variety of approaches we explore the selective forces that influence the dynamic colour polymorphic equilibrium. On the basis of a literature review we argue that there isn't just one selective force of overriding importance but a multitude of factors in a heterogeneous landscape producing a geographic mosaic of coevolution in space and time. Using a phylogenetic approach we analysed the genetic structure and habitat use of *Oreina speciosissima* populations from the Swiss Alps. Specimens grouped according to their habitat, which was in turn mostly defined by plant associations. Via field experiments we test Müller's theory of warning colour and mimicry in the wild and prove frequency-dependent selection using tethered beetles exposed to natural predators. By making use of TEM microscopy we prove that colour in *Oreina gloriosa* is structural and produced by microstructures in the epicuticle. Differences in colour are the result of minute differences in thickness of the electron lucent layers within the chirped multilayer reflector in the beetle's elytra. I conclude by arguing that there is a multiplicity of factors driving the persisting prevalence of colour polymorphism in *Oreina* and provide suggestions for further investigation.

KEYWORDS: Colour polymorphism, Chrysomelidae, *Oreina*, positive frequency-dependent selection, ecological speciation, DNA sequencing, AFLP structural colour, chirped multilayer reflector.

one.

1. Introduction

The dissertation in front of you focuses on the leaf beetle genus *Oreina* CHEVROLAT (Coleoptera; Chrysomelidae). The main objective at the start of the project was to examine possible causes for the remarkable colour variation observed within this genus. Not only can we observe intra-specific colour variation between the two extreme ends of a distributional range, but also within the tiniest of host plant patches, found across European mountain ranges.

Working with *Oreina* beetles has been something of a blessing and a curse, and as Svensson *et al*. rightfully state:

"*Although research on* Drosophila *and similar model organisms allow high experimental precision, most studies are performed under artificial laboratory conditions, which limit the inferences that can be made about natural populations. On the other hand, research on non-model organisms is hampered by the difficulty of obtaining information about the heritable basis of ecologically important traits that influence the speciation process. Thus, workers interested in the ecology of speciation are caught in a methodological dilemma: either study well-characterized model organisms in artificial laboratory conditions and obtain high genetic precision, or study organisms in their natural environments but sacrifice high genetic precision.*" [1].

This dilemma is perfectly applicable to *Oreina* research. The genus is good for field experiments and served as a textbook example for the early work on chemical defence, but not for projects interested in examining the maintenance of colour variation under field conditions.

Despite more than thirty years of research we have not succeeded in establishing the (genetic) basis of variable colours. Furthermore little is known about their biology or ecology. To come back to the point made earlier, these gaps in our knowledge leave plenty of room to develop and test one's own ideas but make interpretation of results difficult.

Distribution of the genus

The genus *Oreina* consists of approximately 28 species [2,3] ,found throughout the montane to alpine strata of the European mountain ranges, with the centre of their distribution in the Alps.

Three exceptions are *Oreina caerulea,* which occurs in lowland areas, and *Oreina sulcata* and *Oreina redikortzewi* found in Siberia [4].

Host plants

Most members of the genus *Oreina* inhabit syntaxa that belong to the hydrophilic montane to sub-alpine, tall-forb communities like the Petasition officinalis and Adenostylion association of the Mulgedio-Aconitetea class [5]. Within the Mulgedio-Aconitetea plant communities *Adenostyles alliariae* and *Peucedanum osthrutium* may reach very high degrees of cover. Alpine beetle species, including *Oreina speciosissima,* can also be found in exposed stone runs patchily vegetated by plants of the Petasition paradoxi, or Androsacion alpinae plant communities where *Doronicum clusii* and *Doronicum grandiflorum* are important host plants [6]. *Oreina* beetles are generally considered oligophagous, feeding only on some host plants, although *Oreina gloriosa* is regarded as monophagous on *Peucedanum ostruthium* [2].

Beetles

Beetles feeding on high-forbs can be found on top of their host plants throughout the day, whereas species living in sparse plant habitats are mostly nocturnal and can be found concealed under loose rocks close to their host plants during the day. Overall, most populations are presumed to be relatively small and reproductively isolated [7,8] due to the patchiness of the host plants and limited adult flight behaviour [9]. It should be mentioned, however, that the hostile mountain peaks that may form geographic barriers for the beetles, simultaneously deny researchers access to the sites which is essential to properly test this hypothesis.

Colours

Colours of *Oreina* beetles range from metallic green with or without a blue stripe to dark blue, almost black, bronze and green with blue and red stripes. Often as many as four or five species and many more colour morphs occur sympatrically within a host plant patch or site. Beetles of the subgenus *Protorina* WEISE have a dull colouration [2,3].

Chemical defence

Beetles within the genus *Oreina* have pronotal and elytral exocrine glands [10] where they store blends of various plant derived secondary metabolites and/or *de novo* synthesized compounds.

These secretions are believed to serve a purpose in defence against predators. The different chemical defence strategies are correlated with host plant use. Species feeding on *Apiaceae* or *Cardueae* produce cardenolides *de novo*, whereas species feeding on *Senecioneae* exploit pyrrolizidine alkaloid N-oxides (PAs) in their host plants. These alkaloids are incorporated and transferred to their defensive secretions [11,12,13]. Sequestered PAs from asteraceous host plants are stored both in the body and the elytral glands of adults. *Oreina* larvae store the defensive compounds in the whole body [11,14,15].

Sexual reproduction and phenology

The generation time of *Oreina* probably varies with altitude. Beetles from high altitudes (e.g. Le Petit Saint Bernard (F); 2200m) are semivoltine and might need two years to complete the life cycle from egg to adult [16]. A season usually lasts from early May to mid-September for beetles living at altitudes around 1500m. Higher sites obviously have shorter seasons. Individuals from lower altitudes are univoltine, using one growing season from egg to adult [personal communication Matthias Borer] or might even be bivoltine under exceptional circumstances. High altitude individuals overwinter as pupae during their first year and as a full grown beetle in their second year. Adult beetles are thought to live for up to three years [16].

Oreina cacaliae females are able to store sperm during overwintering and do not need to re-mate in early spring but have been observed to do so [17]. Within the genus *Oreina* several reproductive modes exist. *Oreina elongata* and *luctuosa* are oviparous, *cacaliae*, *gloriosa* and *alpestris* are viviparous while *luctuosa* is also facultatively viviparous. Vivipary in *cacaliae* evolved independently from *gloriosa* and *alpestris* (synonym *variabilis*) [12,17].

Dissertation outline

Exploring the maintenance of colour polymorphism is like a multi-faceted gem; there are so many different sides to it that it is hard to get a complete picture that deals with the topic from all angles. This dissertation should be regarded as an introduction to the topic with regard to leaf beetles and hopefully a starting point for future research. This document consists of chapters that deal with a single member of the genus as is the case in chapter 3 that focuses on *O. speciosissima* and chapters 4 and 5 that deal with *O. gloriosa* and chapters that deal with the genus as a whole, such as chapter 2. The combination of phylogenetics, field experiments,

literature research and lab work provides an insight in some of the important aspects of colour polymorphism in leaf beetles.

Chapter 2

In this chapter we review the current scientific literature with respect to the maintenance of colour polymorphism in chemically defended insects. We explore the most important selection pressures at stake and discuss their relative importance. We advocate the applicability of the geographic mosaic theory of coevolution.

Chapter 3

Here we investigate genetic structure *O. speciosissima* using three classical mtDNA markers and AFLP genotyping. We discuss possible mechanisms that are responsible for the observed genetic divergence within this species in an ecological speciation framework. A recent shift in host plant use seems to play an important role.

Chapter 4

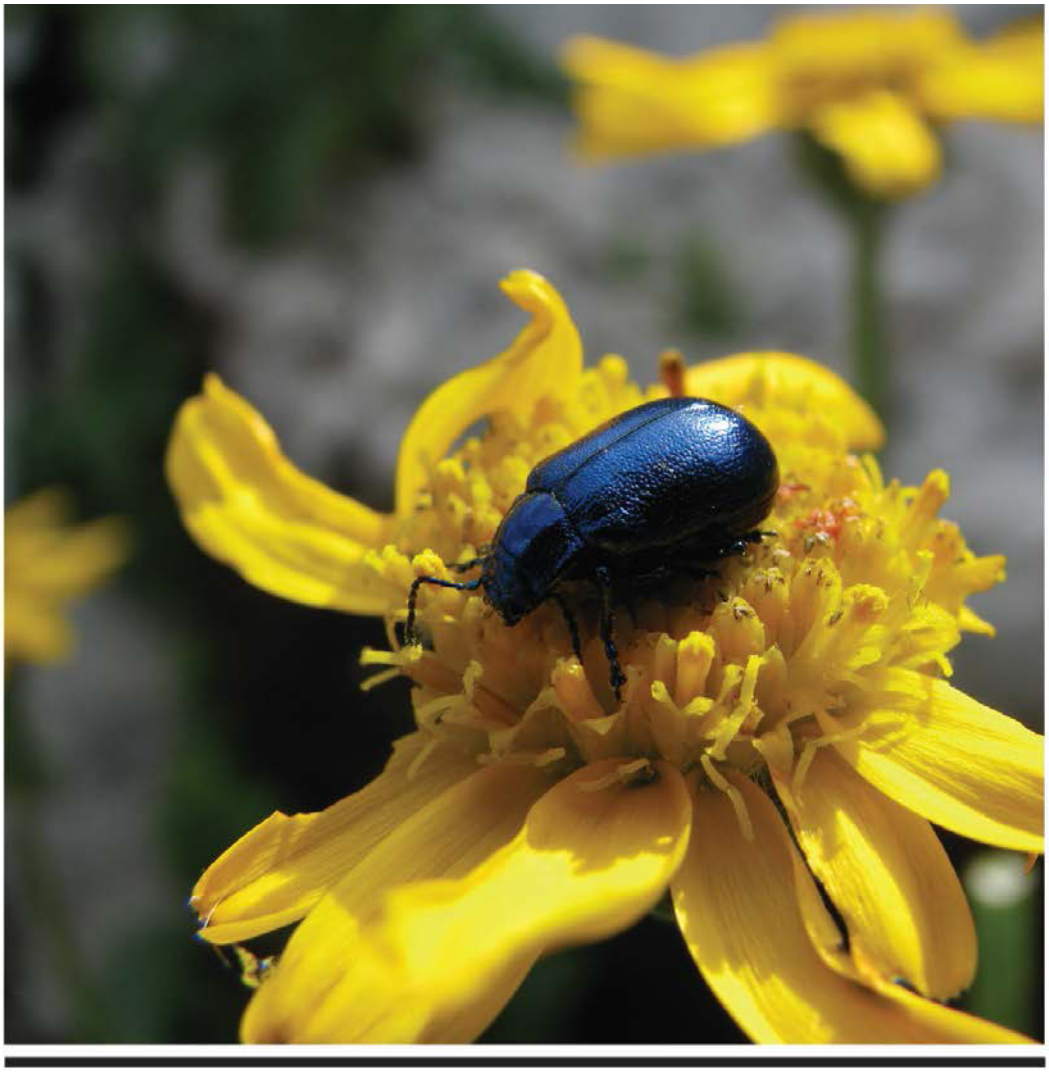
Using field experiments we demonstrate positive frequency-dependent selection in *Oreina gloriosa*. In order to test whether purifying selection by predators takes place in the wild we compared the survival of blue and green individuals in blue- or green-dominated background populations.

Chapter 5

Using Transmission Electron Microscopy, fibre optics and a freely available software package we explore the proximal causes of colour polymorphism in *O. gloriosa*. Differences in the thickness of electron lucent layers within the epicuticle of the beetle's elytron are shown to be responsible for the observed colour variation.

Chapter 6

In the general discussion I summarize the most important findings of this dissertation and elaborate on interesting topics for future research.



two.

2. Colour polymorphism in defended insects: the role of spatio-temporal variation in selection[1]

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Abstract

The study of colour polymorphism is a recurrent theme within the field of evolutionary biology, and the question of its maintenance in nature is a central point in many studies. For obvious practical reasons, researchers often focus on the role of a single selective force on the maintenance of colour polymorphism. But under natural circumstances, it is likely that many factors may be responsible. By means of this review we explored the various selective forces that have been demonstrated to play a role in the maintenance of colour polymorphism and the way they interact leading to (variation in) the balance in the occurrence of the different colour forms. Since chemical defence and the often associated aposematism seem to be at odds with colour polymorphism, we concentrated on chemically defended species where possible. In order to additionally investigate the role of environmental factors with respect to colour polymorphism we restricted ourselves to animals that are likely to express the most prominent effects: small ectothermic animals with a short generation time, specifically insects. We argue that there is no single explanation for the mosaic of colours and patterns that are manifested but a plethora of selection pressures that vary in strength and direction, in space and time.

Key words: frequency-dependent selection, population dynamics, melanism, single-locus polymorphism, adaptation, visual predators, sexual conflict, geographic mosaic theory of coevolution.

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[1] Submitted to Biological Reviews

Introduction

Since the dawn of the field of evolutionary biology, colour polymorphism has received almost continuous academic attention, as pattern differences are noticeable and easily scored. This area of research brings together scientists with interests in speciation, sexual selection, aposematism, and mimicry [18]. Moreover, the usually relatively simple genetic basis of many polymorphisms (one or a few loci) make colour polymorphic species suitable candidates to study evolutionary processes under natural conditions [1]. Laboratory and field studies are often restricted to the role of a single selective force on the maintenance of colour polymorphism, but may, unintentionally, lead to an oversimplification of the situation in nature. In the few cases where scientists study the biological systems more in depth, a multi-faceted and far from straightforward reality appeared time and again. In their classical work, Jones, Leith and Rawlings [19] acknowledge the danger of oversimplification stating the following: *"not all biological observations must necessarily be explicable in a simple and unitary way and not all hypotheses to be tested need to be mutually exclusive"*. Their exhaustive study of polymorphism in the genus *Cepaea* discloses many genetic and environmental factors that influence morph frequency down to the population level. Muggleton (1978) voices the same worry stating: "*There has, however, been a tendency to overlook the possible complexity of selective factors and to search instead for some single factor which will explain the entire picture*" [20]. The influential geographic mosaic theory of coevolution of J.D. Thompson [21,22,23] follows the same line of thought and posits that the interplay of different sets of variables and the constantly changing mixture of selection forces in space and time results in highly dynamic evolutionary landscapes. In the present paper we will use the theoretical framework proposed by these leading scientists and present an overview of the multiple agents of selection that have been shown to play a role in the maintenance of colour polymorphism in chemically defended insects. This paper will give an overview of the factors that maintain colour polymorphism of insects in general, but will make use of examples of chemically defended insects whenever possible and occasionally examples from other phyla to strengthen the argument. We hope that by bringing together the numerous empirical studies (see table 1.) with regard to this topic we will be able to provide: **a) better awareness of the complexity of the evolutionary processes that lead to the maintenance of colour polymorphism, b) better understanding of the interconnectedness and the net effect of all those individual agents of selection and c) provide guidelines for a possible approach to disentangle the complex interactions between selection pressures that influence colour polymorphism.**

Colour polymorphism is defined as the existence of two or more distinct, genetically determined colour morphs within a single interbreeding population at a frequency too high to be solely the result of recurrent chance events [24]. Colour differences in the chrysomelid genus *Oreina* seem to be positioned along a continuum (i.e. they lack the discrete colour forms that define colour polymorphisms). Despite frequent usage of the designation 'morph' in *Oreina* literature, it is at least debateable whether the beetles differ enough, both in a genetic and visual sense, to classify colour variation in *Oreina* as a typical case of colour polymorphism. Regardless of this discussion the genus *Oreina*, embodies a textbook example of the theoretical paradox colour polymorphic insects present. The genus *Oreina* shows remarkable variation in elytral colours and is generally regarded as chemically defended and aposematic [25]. This combination of traits leads to the counter intuitive observation that these insects use a multitude of visual signals to advertise the single message of 'unprofitability', which seems rather paradoxical from an evolutionary point of view [26,27]. Indeed, convergence in warning signals among aposematic species is a commonly observed phenomenon known as Müllerian mimicry (in contrast to Batesian mimics, where profitable prey exploits the warning signals of defended/unprofitable model organisms). Müllerian mimics benefit from increased warning efficiency by sharing the same visual signal to predators. Individuals that deviate –in the eyes of the hungry beholder- are thus not recognized as unpalatable and stand a smaller chance to produce offspring. Polymorphism and diversification in defended, aposematic species is therefore theoretically unlikely [27,28]. Additionally, Borer *et al*. demonstrated that there is positive frequency dependent selection on colour in *O. gloriosa* [26]. On its own, this form of directional selection would eventually lead to convergence of colours and the subsequent fixation of one colour morph. Within the genus *Oreina* however, colour polymorphism seems to have been maintained over a long period of time, which implies the existence of selective forces that counterbalance this positive frequency dependent selection.

Factors favouring the apparition and maintenance of colour polymorphisms

The 'null hypothesis' of adaptive evolution is neutrality. Which role do genetic drift and gene flow play in the maintenance of colour polymorphism compared to other processes, such as selection? Backed-up by examples from [29,30] Gray and McKinnon concluded that genetic drift, usually thought to lead to fixation of one colour morph, can contribute to the maintenance of colour polymorphism in concert with other evolutionary forces such as gene flow, frequency-

dependent selection and temporal variation in selection [24]. In the following, we will focus on the main selective agents that have been linked with colour polymorphisms.

(1) Thermal effects

There are various examples where colour polymorphisms are linked to thermal effects. Most of these examples involve different degrees of melanism. Melanism is commonly defined as the occurrence of variants that are mostly or completely dark in pigmentation either as intraspecific polymorphisms or as fixed differences between closely related species. Melanism can refer to variation involving discrete melanic and non-melanic phenotypes (melanic and typical morphs) or to cases where pigmentation varies along a continuum. For more details about melanism and its associated selective pressures we refer to a very elaborate and detailed review on *Colias* butterflies [31] where increased melanism can dramatically raise flight activity (flight initiation temperature, flight distance) and indirectly influence life history traits connected with fitness in Alpine regions [32]. Hence, this study provides strong evidence for the "thermal melanism" hypothesis whereby melanism is beneficial in cold environments. Under conditions with ambient temperatures that are sub-optimal for activity of ectotherms, a dark colouration -which enables the organism to absorb solar radiation more efficiently than a lighter coloured conspecific- will be favoured by selection through an elevated (reproductive) activity. The large variation in microclimatic conditions in Alpine regions and subsequent different local optima with respect to the degree of melanisation which could leave room for the coexistence of different colour morphs according to Roland [32]. The presence of different colour morphs implies the existence of factors acting against high degrees of melanism; this may simply involve a cost associated with the production of melanin, but may also involve other selective pressures. Within *Adalia bipunctata*, Brakefield and de Jong [33] observed a decrease in the frequency of melanic morphs (from 10-60% in 1980 to an overall 20% in 2004), most probably caused by a genetic response to climate change along a transect in the Netherlands. Laboratory tests revealed significant differences in thermal budgets for melanic and non-melanic morphs and subsequent pronounced differences in activity (especially at low ambient temperatures) between the two morphs under identical conditions [33,34]. The influence of convection (heat transfer through air flow in this case) should not be underestimated since it can greatly reduce differences in body temperature between morphs [34]. Another study (between 1971 and 2004) including *A. bipunctata* recorded no differences in the proportions of non-melanic (90.1%) and melanic morphs (9.9%) in the Czech Republic over the years [35]. Morph frequencies in *A. decempunctata*, addressed in the same study, did not differ significantly over the years (1976-

2004) either [35]. Thus, although the studies above [32,33,34] suggest that increased melanism provides considerable benefits to ectotherms under colder climatic conditions, these results are not ubiquitous across different areas and/or species, suggesting that the story is more complex than a simple climatic selection on melanism with trade-offs on other traits. For example, Brakefield [36] points out that if thermal melanism would be the overriding selective pressure influencing the melanism in the two-spot ladybird beetle, the elytra would be expected to be completely black in areas where melanism would be favoured. The existence of contrasting red colour spots on the elytra of melanic individuals suggests that another factor, such as warning colouration through contrasting colour patterns, or mimicry, also plays a role (see fig. 1). This selective factor may act to a different extent and in a different direction than the thermal one in particular areas, leading to "compromises" and hence variation in correlations between melanism and climate across different geographical areas, and between species. Indeed, a few years earlier Muggleton (1978) reported thatwhile19 bird species reject ladybirds as food in laboratory experiments,an astounding 121 bird species did eat ladybirds, mostly under natural conditions [20]. Inspection of Common House Martin (*Delichon urbicum*) droppings revealed that the remains of melanic beetles greatly outnumbered those of the typical aposematic morphs [20]. These results are concordant with recent observations reporting that the dark *carbonaria* morphs suffer higher predation by insectivorous birds in comparison with the *typica* morphs of British Peppered Moths (*Biston betularia*) [37]. The size of the selection coefficient representing the evolution of melanism obviously depends on the relative cost-benefit relationship compared to other adaptations such as camouflage [38]. Forsman reports temperature excess differences between morphs that were independent of colour. Bigger grasshoppers attained higher body temperatures relative to small individuals under laboratory conditions which is in concordance with results from Stewart and Dixon [39]. Results from studies on the leaf beetle *C. lapponica* largely overlap with results from *A. bipunctata*. At lower temperatures melanic individuals show more feeding and walking activity than pale morphs [40]. The fact that these tests were performed with melanic individuals from Finland, locally adapted to low but stable winter temperatures, and non-melanic individuals from the Czech Republic, locally adapted to mild but variable winter temperatures, might have influenced the outcomes of the study. Under laboratory conditions dark morphs reached a higher body temperature quicker than the non-melanic morphs [40]. Forsman and co-authors report female preference for higher body temperature in males, and significant differences in variation among colour morphs in preferred body temperatures in females, but no differences in preferred body temperatures between male colour morphs of *Tetrix subulata* [41,42,43]. Under laboratory

conditions, dark morphs did not only reach higher temperatures when exposed to increased illumination, but also showed a preference for elevated temperatures [42]. This variation between morphs is probably a result of the underlying genetically determined physiological variation with regard to temperature optima, and it is consistent with the idea that colouration, behaviour and physiology co-evolve, which is sometimes called 'the co-adaptation hypothesis' [44].

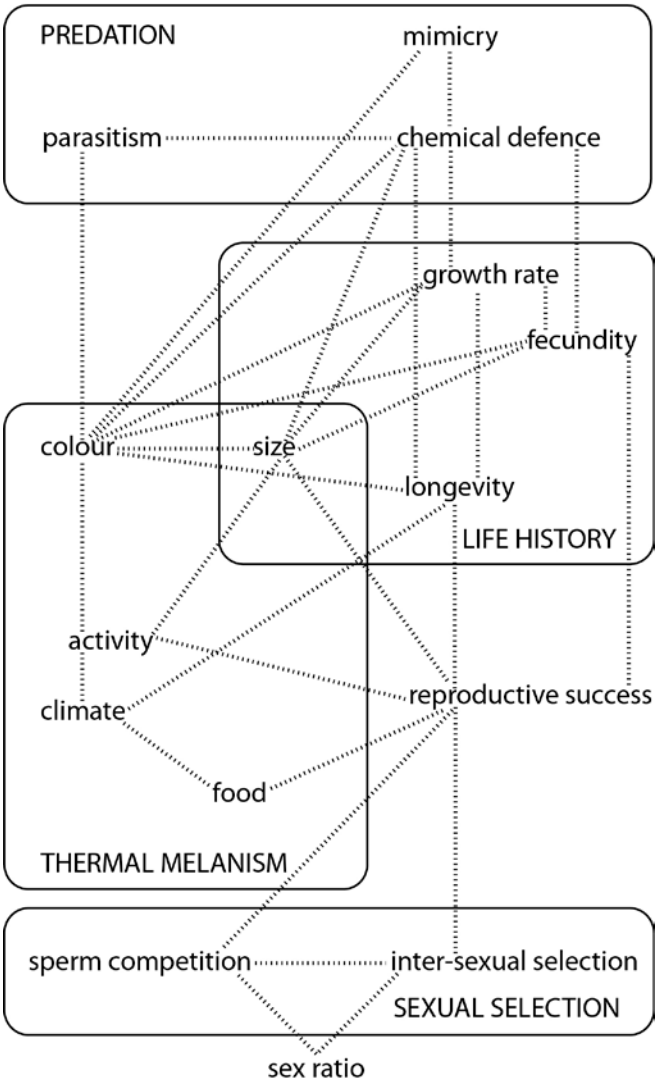

Figure 1. Hypothetical factors and their interactions influencing colour polymorphism in the two-spotted ladybird beetle, *Adalia bipunctata*. The factors are grouped into four different domains: sexual selection, life-history, thermal melanism, and predation, which are all interrelated with respect to their differential effect on the fitness of different colour forms.

The two important messages that can be distilled from this work are: first, Forsman and Appelqvist argue that the effect of dorsal colouration on survival, together with the correlations between colour pattern and biologically important traits such as body size, behaviour, thermal capacity, physiology and reproductive performance suggests it is to be expected that colour pattern significantly influences individual fitness [45]. Moreover, polymorphism is likely to be maintained by spatially variable selection in combination with gene flow [45]. Colour pattern in combination with morph-specific behaviour has a large influence on the 'susceptibility' to visual predators. And second, that the differences in thermoregulatory capacity do not necessarily translate into morph-specific differences in performance. Pale morphs may have lower physiological optima and may thus perform equally well as darker morphs with their subsequent elevated body temperatures under the same conditions [41]. These findings appear to contradict the widely accepted view that increased melanisation in ectotherms leads to increased fitness under temperature-limiting conditions (see for instance [46]). The physiological optimum argument seems to have gained ground in recent years [38,41].

(2) Mimicry, crypsis and aposematism

In a study focussing on *A. bipunctata,* Brakefield argues scale of distribution is important when considering Müllerian mimicry and colour pattern variation in coccinellids, [36]. Within particular mimicry rings (i.e. protected species resembling one another, can form groups or rings that possess the same colouration and shape) aposematic species might be monomorphic throughout their range or polymorphic between different rings or within certain subrings [36]. These ranges might span broad geographical regions [36]. Brakefield [36] agrees with Thompson [47] that aposematic and apostatic selection balance each other in some cases, thereby maintaining polymorphisms for warning colour. Müllerian mimicry reinforces aposematic selection but the chemical defence of the moderately unpalatable coccinellids is not completely effective as proven by the variable responses by different avian predators and the seasonal changes in predation levels (see [36,48]). Finally Brakefield concludes that aposematic selection by certain predators will favour the most abundant or apparent colour patterns whilst apostatic selection by other predators might favour rarer morphs [36]. These statements might, to a certain extent, also hold true for *Oreina.* Most *Oreina* species are close to monomorphic in some areas and extremely polymorphic in other places. Similarly to what [36] and [20] describe for coccinellids, and [37] reports for moths, chemical defence in *Oreina* is far from infallible given the numerous examples of predation witnessed in nature [TVN, personal observation]. Crypsis minimizes the probability of detection against the visual background [49,50]. Colour

polymorphism might enhance crypsis since the existence of numerous colour morphs within one population might hamper the establishment of a search image within a visual predator as a result of a decreased number of opportunities to receive positive reinforcement [51]. Warning signals aim at manipulating predator behaviour by advertising unprofitability through (bright) colours, foul smells or defensive behaviour. The association between such a signal and a potential prey item's unprofitability is called aposematism [52]. Aposematic signals work best when easily detectable and memorable [53].

(3) Sexual selection

Sexual selection is defined as competition within one sex for members of the opposite sex and the differential choice by members of one sex for members of the opposite sex [54]. Important aspects of the sexual selection theory, such as female choice and male-male competition, sexual conflict and mate choice play an important role in the maintenance of colour polymorphism. In a non-negligible part of the colour polymorphic taxa, only one of the sexes is polymorphic. The insect orders Odonata and Lepidoptera incorporate some well-studied examples of this sex-dependent polymorphism. Below we will highlight some cases.

(a) Female-limited polymorphism

Female limited polymorphism has been observed in several species of damselflies belonging to four genera of the Coenagrionidae family and one species within the Lestidae (see Table 1 With females being either 'andromorphs' or 'androchrome' females (female that resemble conspecific males) or gynomorphs (female with a typical female phenotype). Andromorphs are thought to benefit from their resemblance to males by decreased harassment by mate-searching males that do inflict a negative frequency-dependent selection on the gynomorphs [55,56] [57,58]. Although it may be expected that males and andromorphs might suffer higher predation due to their conspicuous colouration van Gossum and his co-workers proved otherwise using 'human predators' [59]. Following a general rule in colour polymorphic insects that different morphs are often associated with a distinct set of alternative reproductive or life history strategies, andro- and gynomorphs (the cryptic *infuscans* and *infuscans-obsoleta* morphs) of *Ischnura elegans* not only differ in colour pattern but also show differences in behavioural pattern. They differ in mate avoidance tactics with gynomorphs showing more evasive flight activity whereandromorphs use more open habitats [60]. As is true for a lot of polymorphic systems, the *Ischnura* polymorphic system is highly dynamic showing rapid changes in relative fitness of the different morphs across

generations [57]. Sexual conflict plays a conservative role in the maintenance of different morphs in the *Ischnura* system, preventing one morph going to fixation and subsequent monomorphism [57]. Additionally, Svennsson, Abbott and Härdling point out that recent theoretical work by Gavrilets and Waxman [61], suggests that sexual conflict can promote the establishment of genetically distinct groups of females, in other words, the formation and maintenance of different colour morphs and colour polymorphism, in contrast to the widely accepted concept of continuous coevolutionary arms race and subsequent speciation [57].

At the end of the last century Miller and Finke [62] proposed the Learned Mate Recognition (LMR) hypothesis after showing that male *Enallagma ebrium* and *E. civile* damselflies could distinguish between conspecific andromorph and gynomorph females after several encounters. Under field conditions, male damselflies of the abovementioned species seem to select mating partners in a frequency-dependent manner. In other words, they prefer the most abundant morph. The two aforementioned features of male behaviour seem to contribute to the maintenance of female colour polymorphism [62]. The authors state that their results demonstrate that *Enallagma* andromorphs should not be considered as male mimics with respect to colouration since other, morph independent, female colour traits seem to function as much stronger cues to males [63]. However, andromorphs could still be behavioural mimics [62]. Interestingly, fitness of female morphs in the damselfly *Ischnura senegalensis* is inversely correlated with their frequency in the population [64]. This Negative Frequency Dependent Selection (NFDS) contrasts with the example above. Takahashi *et al.* conclude that male mating preference is a result of prior experiences and prove that individual behavioural plasticity can maintain genetic diversity and colour polymorphism within a population [64]. This type of selection causes cyclical fluctuations of female morph frequencies, with small populations showing large oscillations, and large populations showing smaller ones. The maintenance of female colour polymorphisms in odonates seems to be the result of population density effects, morph frequency and fitness correlates [65].

Female limited colour polymorphism in *Papilio dardanus* is maintained through a rather complex interplay of selection pressures [66]. Mate choice seems pivotal and according to Cook *et al*. male preference finds its origin in the fact that two out of three female morphs (*P. dardanus var. trimeni* (andromorph) and *P. dardanus var. lamborni*) are difficult to recognize as females upon approach, might be difficult to detect in general, or –due to their lower frequencies- might not be recognized as conspecifics. From a distance males are most attracted to the Batesian mimic *P.*

dardanus var. hippocoonides. These observations are congruent with the male avoidance hypothesis [55]. Cook and co-workers argue that the co-existence of *hippocoonides, trimeni* and possibly *lamborni* can be explained through the fact that *hippocoonides* females have a frequency-dependent selective advantage being Batesian mimics. However, their reduced fitness due to high levels of male harassment is just the other side of the same coin. In sharp contrast with *hippocoonides, trimeni* females (andromorphs) have a selective advantage because of lower male harassment but a disadvantage through the increased predation as a result of not being a Batesian mimic [66]. Females of the *trimeni* morph might also have a frequency-dependent selective advantage if it is a result of male mimicry or rarity rather than just a result of reduced conspicuousness. An increase in andromorph frequency will result in a strong selection formales to approach male-like butterflies. Females of the rare *lamborni* morph might be maintained by a similar principle as they gain a frequency-dependent selective advantage by mimicking faded males. Since faded males are less common than yellow males this hypothesis is congruent with the low frequency of *lamborni* females. [66]. Apart from the melanism in *Colias* discussed earlier, this genus harbours also another polymorphism. Females of the species *Colias alexandra* and *C. scudderi* can either occur as yellow (homozygote recessive) or white (dominant) colour morph [67]. Just as *I. senegalensis* discussed above, frequencies of colour morphs show a cyclical pattern. Here, harassment by males not only happens within the species boundaries or exclusively between males and females, but the white coloured morphs of both sexes, especially *C. alexandra*, lose valuable time through unproductive courtship behaviour, or 'interference competition' by pierid butterflies [67]. This extra-specific interference competition is a likely driver of *Colias* male mate preference for yellow morphs. Small scale variation in morph fitness in relation to habitat requirements leads to a migration-selection balance.

(b) Male-limited polymorphism

Colour polymorphism limited to males onlyseems less common. One case of male-limited polymorphism concerns the damselfly *Mnais costalis* [68]. Within *M. costalis* two male morphs exist; clear-winged non-territorial 'sneaks' and orange-winged territorial 'fighters' [69]. Variation in wing colour is correlated with nutrient availability and age, and the bright orange wings of the territorial males are thought to serve as a true handicap signal [70]. These alternative reproductive tactics affect male longevity, with the territorial 'fighters' having a shorter lifespan. Since wing colour of individual males changes over time, this example might not classify as a strict case of colour polymorphism as defined in the Introduction [69]. More generally, Clarke and co-authors argue that sex-limited polymorphism tends to be randomly distributed among

males and females within arthropods, reptiles and amphibians in groups of animals that do not use colour (pattern) as a social signal and primarily limited to female butterflies and lizards that do use colour in intraspecific signalling [71].

Modes of selection

In the following sections we will review different types of selection by means of examples of (intraspecific) colour polymorphism, predominantly acquired from field research. It is just as difficult to assign one key factor that is responsible for the maintenance of colour polymorphism in one species, as it is to find examples that exclusively fall within the confines of just one paragraph.

(1) Divergent selection and gene flow

Divergent selection is selection on two distinct populations inhabiting different environments resulting in the genetic divergence of the two populations with a change in morph frequencies as a result [72]. Divergent selection is expected to occur in landscapes with large, homogeneous patches and resulting homogeneous selection regimes. Disruptive selection is the selection for extreme phenotypes over intermediate forms within a population [73]. This type of selection is thought to occur in landscapes with a lot of fine-scale variation where individuals tend to experience multiple microhabitats, for instance in mountainous areas or along environmental gradients. Since the concepts 'large and small scale' are relative and largely dependent on the mobility of the animals, or the (a)biotic factors studied, the difference between divergent and disruptive selection might prove in reality to be merely an academic discussion. Seminal work by Endler [74,75,76] and work on the famous *Heliconius* system showed that different visual environments, especially when stable and predictable, can trigger divergent selection. Differential light conditions produce more than one peak in the virtual 'fitness landscape' that spans one population and consequently give room to the persistence of more than one colour morph [77]. The multiple Müllerian mimicry rings in *Heliconius* are maintained due to their spatial separation [78,79]. Under gene flow these interconnected mimicry rings create a mosaic of alternative selective environments that allow for inter-population morph persistence [80]. Evidence in *Heliconius cydno galanthas* and *H. pachinus* showed that reproductive isolation based on male mating preference for wing colour can occur in mimicry rings with a more extensive spatial distribution [81]. It seems that on a small scale environmental heterogeneity can support colour polymorphism by divergent selection and gene flow, while at a larger scale due to lower gene flow it can lead to speciation [24,78]. Fujiyama [82] reports a very

pronounced transition in morph frequencies of *Chrysolina aurichalcea* along an altitudinal gradient. More than the difference in altitude, differences in habitat, or more precisely differences in light conditions, might play a role here. In a later paper Fujiyama and co-authors [83] hint at "*variable selection in time and space"* as a possible force helping to maintain colour polymorphism. Extensive work on the colour polymorphic walking stick insect *Timema cristinae* [84,85,86,87,88,89,90,91,92,93] showed that divergent selection against less cryptic migrants by means of frequency-dependent predation reinforces pre-zygotic reproductive isolation. Mating pairs that consist of locally cryptic females and (conspicuous) immigrant males suffer higher predation rates by animals that use mainly visual cues during hunting than do local tandems [94]. Intermediate migration frequencies assure a certain encounter rate between different morphs which strengthens assortative mating and thereby fuel reinforcement. Unlike *Heliconius* butterflies, mate choice in walking sticks seems to be largely influenced by olfaction rather than by colour [94].

(2) Disruptive selection

Gray and McKinnon [24] state that the non-overlapping nature of the phenotypic distribution of colour polymorphic species *sensu stricto* can be a result of developmental or genetic constraints but that it is far more likely to stem from disruptive selection supported by negative frequency-dependent selection. In concrete terms, this means that intermediate males may experience lower fitness as a result of female preference for the rare morph at one end of the colour spectrum. Until now, evidence for the role of disruptive selection in the maintenance of colour polymorphism has been mostly indirect, possibly because this type of selection can act on suites of traits rather than on individual traits and, as a result, might be difficult to quantify [18]. Such correlations within suites of traits may be caused by linkage disequilibrium due to tight physical linkage or pleiotropy [18]. Furthermore they argue that the assemblage of correlated traits can strengthen polymorphism if subjected to the same type of selection, as can be the case when they contribute to alternative foraging or reproductive strategies [18]. In insects, correlational selection has been observed in *T. subulata* [43], *Drosophila polymorpha [95,96]* and *H. cydno* and *H. melpomene* [97]. Evidence for (disruptive) correlational selection promoting colour trait correlations gives way to the possibility that correlational frequency-dependent selection might also be relatively prevalent because disruptive selection and frequency-dependent selection establish themselves in a similar way [18]. Analysis of the genetic mechanisms behind disruptive correlational selection in *Heliconius* revealed that major genes that code for colour polymorphism are closely linked, resulting in reduced production of individuals with a mixed

trait set diverging from the co-models [97]. Something similar has been observed in the two-spot ladybird beetle where so called 'supergenes' have been hypothesized to influence the overall colour pattern. These supergenes are then thought to consist of tightly linked genes of which at least one influences the pronotal colour and another one the elytral colour [98]. Kronforst and co-authors reported linkage between genes that code for male mate-preference and forewing colour genes in *H. cydno* and *H. pachinus* [81]. Natural selection that forces a shift in forewing colour patterns in these two species also causes evolution of corresponding male mate-preference. When frequency-dependent correlational selection produces more and more co-adapted gene complexes in multiple colour morphs, assortative mating should become increasingly prevalent. Thus, the production of individuals with a poorly adapted set of traits should be further reduced [24]. This type of disruptive sexual selection on suites of traits that are closely linked to colour might thus eventually lead to speciation.

(3) Density dependent selection

Within the leaf beetle *Chrysomela lapponica,* the dark morphs suffer higher mortality than light morphs at overwintering sites, possibly as a result of Delayed Inducible Resistance (DIR) that causes a decrease in host-plant quality [99]. DIR is thought to be induced by heavy herbivore damage [100] and has an adverse effect on *C. lapponica* life history traits such as fecundity, development time, and larval survival[101,102]. DIR is responsible for the negative feedback loop that causes temporal cycles in morph frequencies and is closely linked with post-outbreak populations in case of *C. lapponica*. It is hypothesized that frequency-dependent non-random mating (rare morph advantage) is the driver behind the recovery of dark morph frequencies as soon as DIR selection pressure decreases in response to the drop in population size (and consequential decrease in foliar damage) [99].

(4) Frequency-dependent selection

For a long time frequency-dependent predation has been thought to play a major role in the maintenance of colour polymorphism [103,104,105] but see [26]. Theoretically, negative frequency-dependent selection, also known as apostatic selection, results in elevated survival rates of rare morphs. Evidence in support of this hypothesis comes from laboratory studies by Bond using virtual prey items [106,107,108,109]. In order to optimize foraging, predators form a search image for the most common prey items making them less likely to detect and remove rare morphs from a population. This type of selection prevents one colour morph to go to

fixation and promotes co-existence of several morphs, or in short, supports maintenance of colour polymorphism. However, it could also potentially promote conditions that lead to speciation [110]. Negative frequency-dependent selection can also crop up as a by-product of sexual selection when mate preference in females consistently (and heritably) shifts towards the rare morph (rare morph advantage). In insects experimental evidence for this type of selection mainly stems from the work on *A. bipuncta* [111,112,113]. Majerus and co-workers observed strong frequency dependent, non-assorting preference for *A. quadrimaculata* (a melanic form of *A. bipunctata*) in mating experiments where the frequency of *A. quadrimaculata* <0.5 [111,112] although these conclusions also met considerable criticism [114,115]. Roulin presented ample evidence for frequency dependent, non-assorting preference in birds [116]. Consistent heritable differences in female mating preference can facilitate the evolution of reproductive isolation within a sympatric speciation model, but constantly shifting preference of females for rare males has an opposite effect by preventing divergence through gene flow between morphs. A third mechanism of divergent selection leading to frequency dependent selection can come to pass when microhabitat variation leads to temporal variation in visual backgrounds that help morphs to blend into a certain 'visual niche' while rendering its colour maladaptive in another niche. If sexual selection is microhabitat-dependent and mate choice occurs entirely within these visual niches, rare morphs may be favoured and colour polymorphism maintained [117].

Polymorphisms at the genomic level

Colour polymorphisms often depend on a just few genes. Thompson presented theoretical evidence that concurrent apostatic selection can stabilise colour polymorphisms for one, two or three aposematic morphs determined by two alleles at a single locus [47]. The scarlet tiger moth (*Callimorpha dominula*) is polymorphic for wingspots [118]. The common typical form, *C. dominula var. dominula* is homozygotic, the *medionigra* morph is heterozygotic and finally the rare *bimacula* morph is homozygotic for the *medionigra* gene [118]. Sheppard & Cook showed that the *medionigra* morph is less fertile than the wild-type *dominula* morph and that this is caused by an increased incidence of infertile *medionigra* males as compared with *dominula* males [119]. The fitness of two carriers of the mutant gene, the *medionigra* morph and the *bimacula* morph, is further compromised by a fifty percent decreased survival during the immature stages [119]. Disassortative mating, with male preference for different phenotype females occurs [119]. Via classical capture-mark-recapture assays Fischer & Ford (1947) observed annual fluctuations in morph frequencies exceeding levels that could be reached due to stochastic effects and conclude fluctuating selection is responsible [118]. Half a century later

O'Hara draws the same conclusion by stating that both drift and selection are significant factors affecting changes in the allele frequency of the *medionigra* gene [29]. The same author argues that the question regarding the relative contribution of drift and selection remains open but his hypothesis that fitness of the *medionigra* genotype is affected by temperature, allele frequency or a change in the environment should be rejected [29].

Within *Ischnura graelsii* the inheritance of female colour polymorphism is controlled by an autosomal locus (*p*) involving three alleles (p^a: androchrome; p^i: *infuscans* and p^o: *aurantiac*a) which co-occur with a hierarchy of dominance ($p^a > p^i > p^o$) each one coding for a different morph. Androchromes have three possible genotypes (p^ap^a, p^ap^i and p^ap^o). Gynomorphs of the *infuscans*-type have two possible genotypes (p^ip^i and p^ip^o) and gynomorphs of the *aurantiaca*-type only one genotype (p^op^o) [65,120]. Both in *Ischnura elegans* and *I. graelsii* the androchome allele (p^a) is dominant and the inheritance of colour morphs is identical, but in *I. damula*, *I. demorsa* and *Ceriagrion tenellum* this allele is recessive [65,121]. The genetics behind the colour polymorphic *Biston betularia*, a textbook example of the effect that anthropogenic environmental change can have on a population, have only recently been uncovered. Majerus already established that the difference between the *typica* and the *carbonaria* morph are controlled by a single-locus dominant allele p^C some years ago [122]. The non melanic, black and white coloured *typica* morph is specified by the recessive p^c allele, intermediate melanic *insularia* alleles that are inherited at the same locus are dominant to *typica* and recessive to *carbonaria* [37]. The genetic architecture and the developmental mechanism behind this polymorphism were uncovered only recently by van 't Hof and co-authors (2011). The pattern of genetic variation in genomic region, that is orthologous to a segment of chromosome 17 of *Bombix mori*, suggests a selective sweep around a single mutational origin of melanism [123]. Cook and co-authors recently confirmed long standing theory which points towards predation by birds as a major cause for the rapid changes in frequency of melanic morphs of *Biston betularia* [124] [37].

Joron and co-authors provide evidence for the existence of a homologous gene or a complex of genes that regulates pattern diversity in *Heliconius numata*, *H. melpomene* and *H. erato* [125]. Despite the shared genetic architecture, previously posed as a possible constraint to morphological differentiation [126], natural selection has shaped a developmental switching mechanism that can respond to a wide variety of mimetic pressures and is able to produce locally adapted and highly divergent colour patterns [125].

In contrast to the previous examples, a whole series of alleles located at a single pigmentation locus (*p*) located at an autosome form the genetic basis of colour polymorphism within the meadow spittlebug (*Philaenus spumarius*). An elaborate dominance and co-dominance system comprising seven alleles coding for eleven morphs (within brackets); p^T (*trilineatus*), p^M (*marginellus*), p^F (*flavicollis-F*), p^L (*lateralis*), p^C (*flavicollis-C*, *gibbus* and *leucocehaplus*), p^O (*quadrimaculatus*, *albomaculatus* and *leucophthalmus*) and p^t (*typicus*) is recognized [127] (but see also [128] for opposing ideas). *P. spumarius* is widely known for its remarkable constancy of morph frequencies across time (and space) [129]. On the basis of direct and indirect evidence Halkka and co-authors report p^T to be the top dominant allele and p^t the bottom recessive allele in female spittlebugs whereas in males p^T is also the top dominant allele with p^t directly below it in the hierarchy [127]. More conclusive evidence shows the dominance of p^T over p^L, p^T over p^O and p^T over p^t as well as p^M over p^O [127]. Furthermore Halkka and co-authors found co-dominance relationships between p^L and p^F, p^L and p^C and p^M and p^F [127]. The *marginellus* morph was shown to be expressed via three different allele combinations; p^Mp^t, p^Lp^F and p^Lp^C Halkka, 1973 #1801}. Non-allelic modifiers influence the expression of the genes p^C and p^O and are pivotal for the existence of the *flavicollis-C*, *gibbus*, *quadrimaculatus* and *albomaculatus* colour morphs [127]. The colour genes are believed to have a tight physical linkage with suites of trades that control physiological responses to the environment [127]. Stewart & Lees report that in contrast to Scandinavian populations, melanic morphs in British *Philaenus* populations are dominant to *typicus* in both sexes. This results in a more or less equal frequency of melanics within males and females [130]. This difference might be a result of dominance breakdown through the absence of modifier genes at certain locations [131]. Stewart & Lees conclude that melanism in *Philaenus* seems to be an exceptional case where the direction of dominance in one sex was reversed in different independently-studied populations [130]. Contrary to the conclusions of a number of scholars throughout this review, Halkka and co-workers argue that regional differences in colour morph frequencies are not likely to be explained by variable selection pressure by -yet unidentified- visual predators [127,129,132]. Halkka, Vilbaste & Raatikainen report a positive correlation between altitude and frequency of the p^O allele [133]. Allele frequencies in Finland [134], the UK [130,135] and the USA [136] seem to stay relatively stable as a result of site specific selection that outweighs the effects of gene flow and random drift. However, Drosopoulos concludes that random processes rather than selection are responsible for the occurrence of colour polymorphism in *Philaenus* [128]. Populations on the smaller islands in the Scillies and the Gulf of Finland show signs of recurrent extinction

recolonization cycles and an important role of founder effects and genetic drift [135]. To what extent random processes versus selection are responsible for the occurrence of different colour morphs in *Philaenus* is a question that typically lends itself to a population genomics analysis such as proposed in [137] and [138].

Discussion

(1) Frequency-dependent selection in the form of male harassment seems be a very important factor in the maintenance of female-limited polymorphism when considering *Ischnura, Enallagma, Pappilio* and *Colias* systems. Sexual conflict can, therefore, be considered as an important driver of selection leading to colour polymorphism.

(2) However, the evolutionary mechanism behind colour polymorphism cannot be explained by a single selective force. Instead, maintenance of colour polymorphism is rather the result of the interaction between numerous selective forces that are variable in direction, space and time [60]. Muggleton noted: "*In conclusion it is proposed that the melanic frequency of a population is the result of the interaction of a number of factors whose importance varies from locality to locality*" [20]. Clusella Trullas and co-authors point out that no studies have attempted to simultaneously differentiate those selective forces in terms of their relative costs and benefits in ectotherms [38]. With respect to thermal melanism and the multiple modes of selection for distinct colour patterns, this seems to hold true for maintenance of colour polymorphism in general. The relative contribution of the selective forces that lead to the maintenance of colour polymorphism remains to be elucidated and will most probably turn out to be highly species- and even site specific [38]. Joron comes to the conclusion that limited spatial movement of predators, in concert with niche specialisation of prey animals, might allow for the coexistence of different mimicry rings on a larger scale [28]. Completely in congruence with the geographic mosaic theory of coevolution of Thompson [21,22,23] Joron and Punzalan, Rodd & Hughes conclude that landscapes which consist of a mosaic of microhabitats influence the form of selection on prey colouration. Hence they are more likely to incorporate and enhance local diversity in mimicry and ultimately determine whether polymorphism is maintained [28,105]. And once more: The same principle that holds for the evolution of structural colours in beetles applies to the maintenance of colour polymorphism in chemically defended insects: there is no single evolutionary pathway or pressure that maintains colour polymorphism, and there is no single function for colour polymorphism [139]. Following Seago and co-authors, similar patterns and colours are likely to serve different functions in different taxa and can be either adaptive in

relation to sexual signalling, aposematism, mimicry or thermoregulation or simply be anatomical artefacts [139].

(3) At a genomic level, colour polymorphism seems to be largely controlled by single locus autosomal genes. Genes having alarge effect, so-called 'major genes' are often modified by closely linked genes that regulate ecophysiological traits (co-adapted gene complexes) that aid local adaptation. As expected, this conclusion is completely in line with conclusions by Mallet and Joron who state that colour patterns of Müllerian and Batesian mimics are often regulated by relatively few loci with major effects that can be classified as 'supergenes', comprising multiple tightly linked epistatic elements [27].

(4) The balance between the various selective factors influencing colour polymorphisms determines the frequency with which the colour forms locally occur. The fact that it is unlikely that a single factor is responsible for a particular colour form frequency explains the deviation from the expected monomorphism in chemically defended insects (see introduction).

(5) And last, not discussed in an elaborate fashion in this review since predator psychology and –vision are whole subjects of their own, is a suggestion by Machado & Valiati (2006). In order to understand the maintenance of colour polymorphism in Müllerian mimics we must understand the elements that predators use to identify their prey [140]. The same authors state that colour polymorphism may well be less common in aposematic species than it appears to the human eye when seen through the eyes of a predator. We must know what predators see and how they 'interpret' the information they receive but these elements of predator psychology and sense perception are inextricably linked to predator physiology and environmental conditions and therefore difficult to decipher under natural conditions.

Conclusions and further outlook

Reconsidering the wide array of colour polymorphic insects that came to the spotlight and the selection mechanisms that combine into a dynamic equilibrium maintaining colour polymorphism, the geographic mosaic theory of coevolution of J.D. Thompson [21,22,23] seems very applicable in this context. In order to unravel the complex interactions between different, variable selection pressures that aid the maintenance of colour polymorphism in insects, large scale field studies that asses both geographical and temporal variation of colour and the associated genetic variation seems pivotal. Combined with field experiments designed to filter

out the most important selection forces, these studies should be able to designate and quantify the relative importance of the forces involved. A set up that studies several polymorphic (sibling) species in parallel might be powerful enough to disentangle general principles from site or species specific effects. The population genomics approach, advocated by Vermeer, Dicke and De Jong [138] with respect to studies focussing on the evolution of plant-insect interactions seems perfectly suited to be applied in colour polymorphism research. To illustrate this line of thought we refer to the earlier mentioned example of *A. bipunctata*. Striking observations include clinal variation in melanic forms in an opposite direction in different geographic areas [141,142,143,144], the absence of clinal variation in this trade in the closely related *A. decempunctata* [36], and a temporal change in the percentage of melanic forms in the *A. bipunctata* in some areas but not in others [35]. The study of such contrasts is highly likely to yield cues about selective factors that influence the polymorphism.

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Table 1. Summary of colour-polymorphic insect species and the selective factors that have been empirically shown to influence the different colour forms. This table is based on similar overviews by Gray & McKinnon [24] and McKinnon & Pierotti [18] but limited to insects and supplemented with extra examples found in the literature.

Species name (Family)	Chemical defence	Colour polymorphism	Sex	Selective factors	References
Damselflies					
Argia vivida (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[55,145]
Ceriagrion tenellum (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[121,146,147]
Enallagma boreale (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[148,149]
Enallagma civile, E. aspersum (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[150,151]
Enallagma cyathigerum (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[152,153,154]
Enallagma damula (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[155]
Enallagma erbium (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[62]
Enallagma praevarum (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[151]
Ischnura elegans (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[57,65,156,157]
Ischnura graellsii (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent	[65,148,158,159,160,161]

Species name (Family)	Chemical defence	Colour polymorphism	Sex	Selective factors	References
				selection (male harassment)	
Ischnura ramburi (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment), morph specific predation	[59,146,162,163,164,165,166]
Ischnura senegalensis (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[64,167,168]
Lestes disjunctus (Lestidae)	Unknown	Body colour pattern	F	Morph specific predation, sexual selection, morph specific behaviour	[169,170,171]
Mnais costalis (Calopterygidae)	Unknown	Wing colour	M	Unknown	[68,69]
Nehalennia irene (Coenagrionidae)	Unknown	Body colour pattern	F	Frequency dependent selection (male harassment)	[172]
Butterflies					
Biston betularia	Unknown	Wing colour	M/F	Morph specific predation	[112,122,123,124,173]
Callimorpha dominula (Arctiidae)	Pyrrolizidine alkaloids	Wing colour patterns	M/F	Disassortative mating, genotype x environment effects	[29,118,119,174,175]
Colias nastes, C. meadii (Pieridae)	Possibly host plant derived chemicals	Wing colour	M/F	Local adaptation, thermal effects	[32,176]
Heliconius cydno, H. pachinus (Nymphalidae)	Cyanogenic glycosides	Wing colour pattern	M/F	Sexual selection, divergent positive frequency-dependent selection (Müllerian mimicry)	[24,80,81,177,178,179]
Hypolimnas mysippus (Nymphalidae)	Unknown	Wing colour pattern	F	Density-dependent selection, mimetic selection, apostatic selection, epistatic selection	[180,181]

Species name (Family)	Chemical defence	Colour polymorphism	Sex	Selective factors	References
Papilio aegeus, P. phorcas (Papilionidae)	3-hydroxy-n-butyric acid	Wing colour pattern	F	Frequency dependent selection, male harassment	[71,182]
Papilio dardanus (Papilionidae)	Pyrrolizidine alkaloids	Wing colour pattern	F	Frequency dependent selection, male harassment	[66,71,183,184]
Beetles					
Adalia bipunctata (Coccinellidae)	Adalinine	Elytral and pronotal colour pattern	M,F	Frequency dependent (apostatic) selection, assortative mating	[33,185,186,187]
Adalia decempunctata (Coccinellidae)	Adalinine	Elytral and pronotal colour pattern	M,F	Aposematic and apostatic selection, Local adaptation	[35,36,187]
Agriotes ustulatus (Elateridae)	Unknown	Dorsal colour	M/F	Unknown	[188]
Ceroglossus suturalis, C. chilensis, C. buqueti, C. darwini (Carabidae)	Various unidentified prey derived chemicals	Body colouration	M,F	Unknown	[189,190]
Chauliognathus flavipes, C. octomaculatus, C. expanses, C. fallax, C. lineatus (Cantharidae)	Pyrrolizidine alkaloids	Elytral and pronotal colour pattern	M,F	Unknown	[140,191,192,193,194]
Chrysolina aurichalcea (Chrysomelidae)	Unknown	Dorsal colour	M/F	Unknown	[82,83,195]
Chrysomela lapponica (Chrysomelidae)	Salicylaldehyde	Elytral colour	M/F	Local adaptation, thermal effects	[40,99,196,197,198]
Chrysophtharta agricola (Chrysomelidae)	Unknown	Body colouration	M/F	Colour seems neutral, random mating	[199]
Cylas formicarius (Brentidae)	Unknown	Elytral colour	M/F	Unknown	[200]
Goniotecna artica (Chrysomelidae)	Various unidentified plant derived	Elytral colour	M/F	Unknown	[196]

Species name (Family)	Chemical defence	Colour polymorphism	Sex	Selective factors	References
	chemicals				
Harmonia axyridis (Coccinellidae)	Harmonine	Elytral colour	M/F	(Assortative mating), thermal effects, local adaptation	[201,202,203,204]
Lissorhoptrus oryzophilus (Curculionidae)	Unknown	Elytral colour	M/F	Unknown	[205,206]
Oreina cacaliae (Chrysomelidae)	Pyrrolizidine alkaloids	Elytral colour	M,F	Unknown	[2,3,10,12,207,208,209,210,211,212,213]
Oreina gloriosa, O. bifrons, O. speciosa, O. virgulata, O. alpestris (Chrysomelidae)	Cardenolides	Elytral colour	M,F	Frequency dependent selection	[2,3,12,25,26,207,213,214,215,216,217]
Oreina intricata, O. frigida, O. elongata, O. speciosissima (Chrysomelidae)	Pyrrolizidine alkaloids and cardenolides	Elytral colour	M,F	Unknown	[2,6,12,207]
Oreina sulcata (Chrysomelidae)	Various unidentified plant derived chemicals	Body colouration	M,F	Unknown	[196]
Grasshoppers					
Ephippiger ephippiger (Tettigoniidae)	Unknown	Body colouration	M,F	Unknown	[218]
Pterochroza ocellata (Tettigoniidae)	Unknown	Body colouration	M,F	Unknown	[51]
Roxelana crassicornis (Tettigoniidae)	Unknown	Body colouration	M,F	Unknown	[51]
Ruspolia differens (Tettigoniidae	Unknown	Body colouration	M,F	Unknown	[219]
Tetrix subulata (Tetrigidae)	Unknown	Body colouration	M,F	Disruptive correlational selection, gene flow	[41,42,45,220,221,222]
Typophyllum trigonum, T. bolivari (Tettigoniidae)	Unknown	Body colouration	M,F	Unknown	[51]

Species name (Family)	Chemical defence	Colour polymorphism	Sex	Selective factors	References
Other orders					
Acyrthosiphon pisum (Aphididae)	Unknown	Body colouration	M/F	(Negative frequency dependent selection)	[223]
Drosophila mediopunctata (Drosophilidae)	Unknown	Abdominal pigmentation	M,F	Developmental temperature, mating preference	[224]
Drosophila polymorpha (Drosophilidae)	Unknown	Abdominal pigmentation	M,F	Local adaptation	[95]
Drosophila simulans (Drosophilidae)	Unknown	Thoracic pigmentation	M,F	Unknown	[225]
Philaenus spumarius (Aphrophoridae)	Unknown	Body colouration	M,F	Apostatic selection, random genetic drift, gene flow and bottleneck effects	[127,128,129,130,133,134]
Timema cristinae (Timematidae)	Unknown	Body colouration, stripe pattern	M,F	Divergent selection, mate preference	[18,85,88,94,226,227]

three.

3. Does a shift in host plants trigger speciation in the Alpine leaf beetle *Oreina speciosissima* (Coleoptera, Chrysomelidae)?[2]

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Abstract

Within the Coleoptera, the largest order in the animal kingdom, the exclusively herbivorous Chrysomelidae are recognized as one of the most species rich beetle families. The evolutionary processes that have fuelled radiation into the more than thirty-five thousand currently recognized leaf beetle species remain partly unresolved. The prominent role of leaf beetles in the insect world, their omnipresence across all terrestrial biomes and their economic importance as common agricultural pest organisms make this family particularly interesting for studying the mechanisms that drive diversification. Here we specifically focus on two ecotypes of the alpine leaf beetle *Oreina speciosissima* (Scop.), which have been shown to exhibit morphological differences in male genitalia roughly corresponding to the subspecies *Oreina speciosissima sensu stricto* and *Oreina speciosissima troglodytes.* In general the two ecotypes segregate along an elevation gradient and by host plants: *Oreina speciosissima sensu stricto* colonizes high forb vegetation at low altitude and *Oreina speciosissima troglodytes* is found in stone run vegetation at higher elevations. Both host plants and leaf beetles have a patchy geographical distribution. Through use of gene sequencing and genome fingerprinting (AFLP) we

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analysed the genetic structure and habitat use of *Oreina speciosissima* populations from the Swiss Alps to examine whether the two ecotypes have a genetic basis. By investigating a wide range of altitudes and focusing on the structuring effect of habitat types, we aim to provide answers regarding the factors that drive adaptive radiation in this phytophagous leaf beetle. While little phylogenetic resolution was observed based on the sequencing of four DNA regions, the topology and clustering resulting from AFLP genotyping grouped specimens according to their habitat, mostly defined by plant associations. A few specimens with intermediate morphologies clustered with one of the two ecotypes or formed separate clusters consistent with habitat differences. These results were discussed in an ecological speciation framework. The question of whether this case of ecological differentiation occurred in sympatry or allopatry remains open. Still, the observed pattern points towards ongoing divergence between the two ecotypes which is likely driven by a recent shift in host plant use.

Introduction

The debate about the relative importance of ecological speciation in species diversification spans several decades [228,229,230,231,232,233,234,235,236,237,238,239,240,241,242,243,244,245,246,247]. However, concrete cases based on empirical evidence remain relatively scarce [228,248,249,250,251,252]. In essence, ecological speciation is related to the "ecological species concept", which was defined as follows [253]: "a species is a lineage (or a closely related set of lineages), which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range". The driving force behind ecological speciation is thus divergent natural selection between environments or, in other words, reproductive isolation of populations by means of adaptation to different environments or niches [72,245,246,248,254]. Ecological selection is a consequence of individual-based interactions with the environment. From this interaction follows that divergent selection between ecological niches is a major driving force differentiating lineages until reproductive isolation occurs [244]. Ecologically divergent pairs of populations will show higher levels of reproductive incompatibility and lower levels of gene flow than ecologically more similar population pairs [255]. A resulting corollary is that ecological speciation is more likely to arise in regions with patchworks of contrasting habitats and/or distinct environmental gradients.

The number of taxa within the insect order Coleoptera exceeds that of any known plant or animal group [256]. More than half of the beetles are phytophagous, including the species rich

superfamilies Curculionoidea and Chrysomeloidea, of which a majority feeds on angiosperms [257]. The increase in phytophagous beetle diversity was facilitated by the rise of flowering plants [257]. The family Chrysomelidae currently consists of more than thirty-five thousand recognized species including economically important pest species such as the Colorado potato beetle (*Leptinotarsa decemlineata*), the Northern corn rootworm (*Diabrotica virgifera*), the Cereal leaf beetle (*Oulema melanopus*), and the Striped turnip flea beetle (*Phyllotreta nemorum*). The biological and economic importance of the superfamily Chrysomeloidea make it vital to understand the factors that drive diversification in this group.

Here, we present a case of ecological niche differentiation in the alpine leaf beetle *Oreina speciosissima* that may represent the early stages of ecological speciation. The genus *Oreina* currently includes twenty-eight species, of which only seven early-diverging taxa do not exclusively occur in high forbs (i.e. five develop in stone run vegetation and two can be found in both high forbs and stone runs) [2]. According to current knowledge [25], the most parsimonious explanation is that high forbs vegetation is the ancestral niche for the remaining twenty-one *Oreina* lineages, among which only our focal taxon *Oreina speciosissima* shows a partial reversal, since it is found both in high forbs and stone run vegetation.

Our focal taxon *Oreina speciosissima* is distributed across nearly the entire range of the genus *Oreina* (from the Pyrenees in the west to the Carpathian Mountains in the east) through a wide altitudinal gradient (ranging from 800 to 2700 m above sea level). At lower elevations it generally colonizes the very abundant high forbs vegetation whereas at higher elevations it is found in stone run habitats across a small portion of its distribution range [unpublished observations MB, TVN][2]. Kippenberg [2] and personal observations suggest that *Oreina speciosissima* feeds exclusively on *Asteraceae* (*Achillea, Adenostyles*, *Cirsium, Doronicum, Petasites*, *Senecio* and *Tussilago*) and colonizes four distinct habitats represented by well-established plant associations: two occurring in high forbs – Petasition officinalis and Adenostylion – and two in stone run –Androsacion alpinae on siliceous bedrock and Petasition paradoxi on calcareous bedrock – (see Fig. 2). These plant communities are often patchily distributed due to the myriad of spatially proximate microclimates that occur in the Alps, especially sites with calcareous bedrocks which regularly present a mosaic of microhabitats. For instance, sinkholes or dolines, formed through water erosion in so-called karstic areas represent ecological islands inhabited by high forbs vegetation surrounded by areas covered by stone run vegetation [unpublished observation TVN].

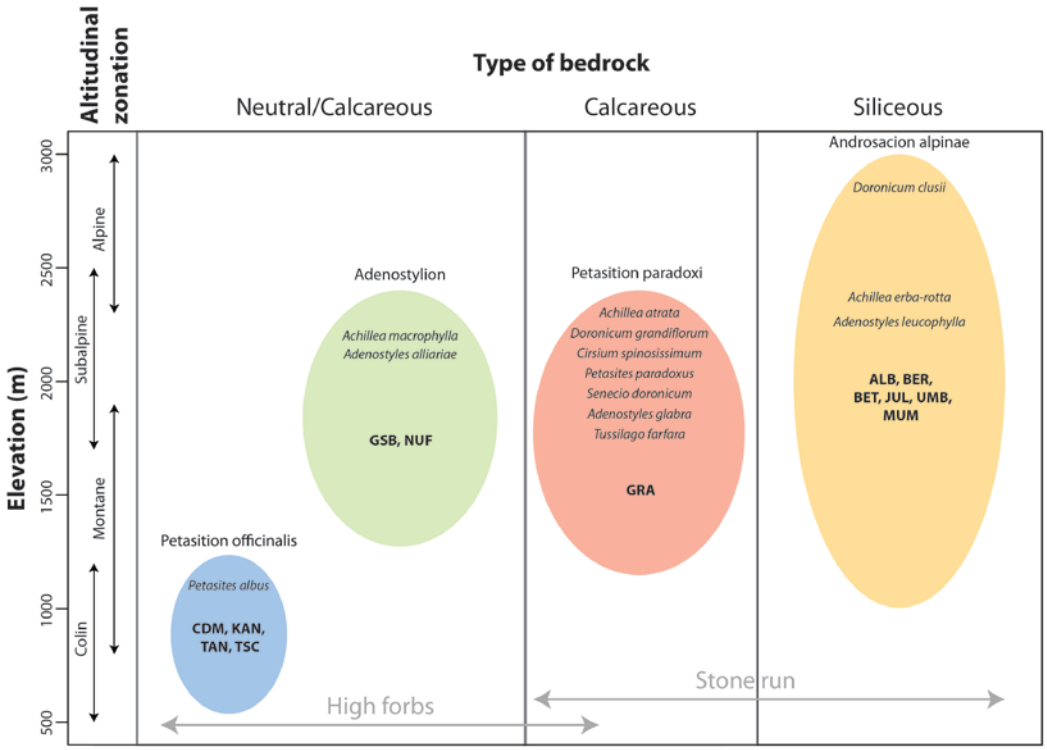

Figure 2 – Host plants of *Oreina speciosissima* and their altitudinal zonation and habitat

According to literature [14] and personal observations of the authors, 13 plant species were referred to as putative host plants for *Oreina speciosissima*. These species belong to four plant associations, i.e. Petasition officinalis, Adenostylion, Petasition paradoxi and Androsacion alpinae [42], which segregate along altitudinal zonation and bedrock type gradients. Whereas Petasition officinalis and Adenostyles correspond to high forbs habitat, Petasition paradoxi and Androsacion alpinae represent stone run habitats. In particular conditions, the Petasition paradoxi can merge with high forbs (see text). Each association includes bold written codes (see Table 2 for details) of sampled populations. Plant associations are written from left to right as a function of their mean elevation, and also according to a putative scenario of colonization of central Alpine silicious stone run vegetation by specific *Oreina speciosissima* lineages (high forbs representing the original habitat for most *Oreina* species). Main host plant species of *Oreina speciosissima* in each of the four associations are as follows: *Petasites albus* in Petasition officinalis, *Adenostyles alliariae* in Adenostylion, *Doronicum grandiflorum* in Petasition paradoxi, and *Doronicum clusii* in Androsacion alpinae.

Beetles inhabiting the highly divergent habitats have been categorized in two different subspecies, namely *Oreina speciosissima sensu stricto* (distributed across the whole species range) and *Oreina speciosissima troglodytes* (restricted to the Swiss and neighbouring Italian Alps), on the basis of differences in elytral colouration and the shape of male genitalia

(aedeagus) [2]. *Oreina speciosissima sensu stricto* beetles are bright metallic green or blue whereas the colouration in *Oreina speciosissima troglodytes* is generally darker and mat [2]. It is not known whether the morphological differences between ecotypes are in any way adaptive and/or have a genetic basis, although colour patterns in another species of *Oreina* are known to influence natural selection through predation pressure [26]. Like most other members of the genus, *Oreina speciosissima sensu stricto* can be found feeding or mating throughout the day on, or in the vicinity of its host plants. In contrast, *Oreina speciosissima troglodytes* is usually found adjacent to its host plants, concealed in crevices and under loose rocks. Previous studies by the authors [258,259] greatly challenged the existence of clear species boundaries within the genus: it is therefore realistic from a biological point of view to refer to these taxonomic entities as ecotypes rather than subspecies. Leaf beetles from the genus *Oreina* are generally thought to make only limited use of their dispersal capabilities [8,212] even though Kalberer *et al.* [260] reported an average flight dispersal of approximately one hundred meters for *Oreina cacaliae* beetles during autumn migration. Rowell-Rahier [34] showed that low vagility in concert with a patchy host plant distribution resulted in a low level of genetic structuring in *Oreina speciosissima. Oreina speciosissima sensu stricto* beetles inhabit high forbs patches that are generally larger in size, lie closer together and harbour more beetles per unit of surface area than stone run patches inhabited by *Oreina speciosissima troglodytes* [unpublished observations MB, TVN].

The present work investigates 13 populations representative of the two ecotypes, using sequencing of nuclear (*ITS2*) and mitochondrial (hereafter mtDNA) (*16S*, *COI* and *COII*) DNA regions as well as AFLP genome fingerprinting in a way to address the following questions:

1. Are ecotypes monophyletic?
2. Is adaptation to different habitats and host plants associated with genetic divergence?

Methods

Sampling

During the summers of 2004, 2005 and 2008, specimens of *Oreina speciosissima sensu stricto* and *Oreina speciosissima troglodytes* were collected from 13 populations (Table 2) and stored in pure ethanol at -20°C. All sampled beetles were found on four distinct plant associations, namely Petasition officinalis, Adenostylion, Petasition paradoxi and Androsacion alpinae. The Petasition officinalis (populations CDM, KAN, TAN and TSC) and the Adenostylion (populations GSB and NUF) occurred on neutral to slightly calcareous bedrocks, at low and medium altitudes,

respectively. The Petasition paradoxi (population GRA) and the Androsacion alpinae (populations ALB, BER, BET, JUL, UMB and MUM) grew on medium-high altitude calcareous and siliceous bedrocks, respectively (see Fig. 2). Three individuals from each population were selected for genetic analysis, using only males to ensure accurate identifications based on genitalia. Following the reasoning of Nosil *et al.* 2002, 2003 [91,92] a 'population' is defined as all of the insects collected within a homogenous patch of plants belonging to one of the four abovementioned plant communities. 'Parapatric' populations are those in contact with a second population using host plants of a different plant community. If we take the maximum migration distance of *Oreina cacaliae* as reported in [260] as a proxy for migration ability of *Oreina speciosissima*, and thus the possibility for gene flow, this study incorporates only one true parapatric pair (TAN – GRA). As a result of this our study is not suitable to test the influence of geographical distance with regard to genetic distance between beetles that use different plant communities as host plants. The dataset was completed with two individuals of *Oreina virgulata* (i.e. a closely related species) that were used as the outgroup [25].

DNA sequence data and phylogenetic analyses

The DNA extraction, amplification and sequencing protocols as well as primers for the nuclear (*ITS2*) region and the three mtDNA markers (*16S*, *COI*, *COII*) are provided in [258]. The alignments of mtDNA markers (using the Clustal-Wallis algorithm [261]) were combined in a total evidence approach [262] after having performed pair wise incongruence length difference ILD tests [263]. We followed the snowball procedure as implemented in the program MILD [264].

Phylogenetic analyses were performed using the maximum parsimony (MP) and Bayesian Markov chain Monte Carlo (MCMC) criteria. Each partition and the combined data set were analysed using parsimony ratchet [265] as implemented in PAUPRAT [266] and further run in PAUP* 4b10 [267]. Ten independent searches were performed with 200 iterations and 15% of the parsimony informative characters perturbed [265]. The shortest most parsimonious trees were combined to produce a strict consensus tree. Branch supports were calculated using the Bremer support (also known as 'decay index') [268] as implemented in TREEROT [269] and further run in PAUP* 4b10 [267]. The Bremer support measures the number of extra steps in tree length required before a node collapses [268,270]. Model selection for the mtDNA data partitions in the MCMC was carried out with MRMODELTEST2 v.2.3 [271] based on the 'Akaike information criterion' [272]. Two Metropolis-coupled Markov chains with incremental heating temperature of 0.1 were run in MRBAYES 3.1.2 [273] for 30 million generations and sampled every 1000th

generation. The simulation was repeated six times, starting from random trees. Convergence of the MCMC was checked using the Potential Scale Reduction Factor (PSRF) [274] implemented in MRBAYES 3.1.2 [273] and the effective sample size (ESS) criterion for each parameter as implemented in TRACER 1.4 [275]. To yield a single hypothesis of the phylogeny, the posterior distribution was summarized in a 50% majority rule consensus tree (the "halfcompat consensus tree" from MRBAYES) after burn-in (for each analysis 10000 trees were discarded). The combined dataset was analysed using partition specific model parameters [271].

AFLP

Genome fingerprinting was performed using the AFLP protocol described in [276]. The selective amplifications were performed using 5-FAM fluorescently labelled *Eco*RI primer (i.e. *Eco*RI + ACA) with one of the following: MseI primer + AXX (AGC, ACG and AAC). All amplifications were run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany). Samples were randomly displayed on a 96-well PCR plate, with ten individuals being replicated to assess the overall reproducibility of reactions. PCR products were analysed using the GeneScan technology with a capillary sequencer (ABI 3730XL, Applied Biosystems, Foster City, CA; the service was provided by Macrogen Inc. Seoul, South Korea).

Resulting electropherograms were analysed with PeakScanner (ABI, peak detection parameters: default parameters with the addition of a light peak smoothing) in order to detect and calculate the size of AFLP bands. The scoring was performed using an automated scoring R CRAN package, RAWGENO 2.0 [277,278]. The library was settled as follows: scoring range = 100 – 250 bp for EcoRI-ACA/MseI-AGC, EcoRI-ACA/MseI-ACG and 100-280 for *E*coRI-ACA/MseI-AAC, minimum intensity = 50 rfu, minimum bin width = 0, maximum bin width = 1 bp and closely sized bins (5%) were removed. Finally, the matrices of the three scored primer pairs were concatenated into a single binary matrix where individuals and bands were stored as lines and columns, respectively.

Phylogenetic and clustering analyses of the AFLP data set

Phylogenetic analyses of the AFLP data were performed using the MP and Bayesian MCMC criteria. The MP analysis (including Bremer support analysis) was performed as described above. Parameters for the Bayesian MCMC analysis performed in MRBAYES 3.1.2 were set as follows: "datatype = restriction" and "coding = noabsencesites". Four metropolis-coupled Markov chains with incremental heating temperature of 0.1 were run for 5 million generations and sampled

every 1000th generation. The simulation was repeated six times, starting from random trees. Convergence of the analysis was checked using the PSRF and ESS criteria (see above for more details). The posterior distribution was summarized in a halfcompat consensus tree (see above) after burn-in (for each analysis 1500 trees were discarded).

Two independent clustering algorithms were used to assign *Oreina speciosissima* specimens into a user-defined number of groups (hereafter *K*). First, we used non-hierarchical *K*-means clustering [279], a distance-based algorithm that proves reliable in an AFLP framework [280,281,282]. A total of 100 000 independent runs was carried out for each value of *K* clusters assumed (i.e. ranging from two to seven) and only runs yielding the highest inter-cluster variance were considered for further analysis. The optimal *K* value was determined based on the second derivative of the inter-cluster inertia, as in [282]. Computations were performed using R CRAN [283] (script available upon request to NAR). Second, we performed a model-based Bayesian inference clustering as implemented in STRUCTURE 2.2 [284,285]. The analysis assumed an admixture model and independent allele frequencies between clusters. Five independent runs were carried out for each value of *K* (i.e. ranging from one to seven), with parameters and model likelihood estimated over 1 000 000 MCMC generations (following a burn-in period of 200 000 generations). For each *K* value, only runs that obtained the highest likelihood value were taken into account for further analyses. The majority-rule criterion (>0.5 in the assignment probability) was applied to assign samples to a given cluster as in [282]. Both clustering approaches provided fully congruent insights and therefore only results from STRUCTURE are displayed here.

Results

Phylogenetic reconstruction of the DNA sequence data sets

The nuclear *ITS2* region showed no variation for *Oreina speciosissima* and was thus excluded from further analyses. In contrast, the three mtDNA regions were polymorphic with a total alignment length of 1632 bp; 529 bp for *16S*, 470 for *COI* and 633 bp for *COII.* Excluding the outgroup, 30 characters were potentially parsimony informative (hereafter PPIc) among 37 variable characters. The three mtDNA regions contributed as follows: *16S* (3 PPIc among 5 polymorphic sites), *COI* (13 PPIc among 16 polymorphic sites) and *COII* (14 PPIc among 16 polymorphic sites). The best substitution models were Hasegawa-Kishino-Yano (HKY) [286] for *16S* and Hasegawa-Kishino-Yano plus Gamma (HKY+G) [286,287] for *COI* and *COII*. The alignments of the mtDNA markers were combined in a total evidence approach, after pairwise

incongruence length difference ILD test [263] revealed no incongruence among the three mtDNA markers (*COI* and *COII*, *P* value = 1.00; *COI* and *16S*, *P* value = 1.00; *COII* and *16S*, *P* value = 1.00). The resulting dataset was investigated using maximum parsimony (hereafter MP) and Bayesian phylogenetic inference methods [265]. Both approaches produced highly congruent topologies with the same major nodes. The MP topology with Bremer supports [268] and corresponding Bayesian posterior probabilities from the Bayesian analysis (hereafter bpp) are shown in Fig. 3. The ingroup is well supported with a Bremer support of 42 and a bpp of 1.00. The ingroup splits into two groups, a well-supported clade (Bremer support = 10 and bpp = 1.00) containing all individuals from the GRA population and a polytomy (Bremer support = 5 and bpp = 1.00) containing all other individuals. Apart from a clade containing all individuals of GSB and one with two individuals from CDM and one from TSC, there is no resolution within the polytomy. Sample NUF_3 failed to amplify and is therefore not shown in Fig. 3. Only samples that rendered both satisfactory DNA sequences and AFLP fingerprints were used for phylogenetic analysis.

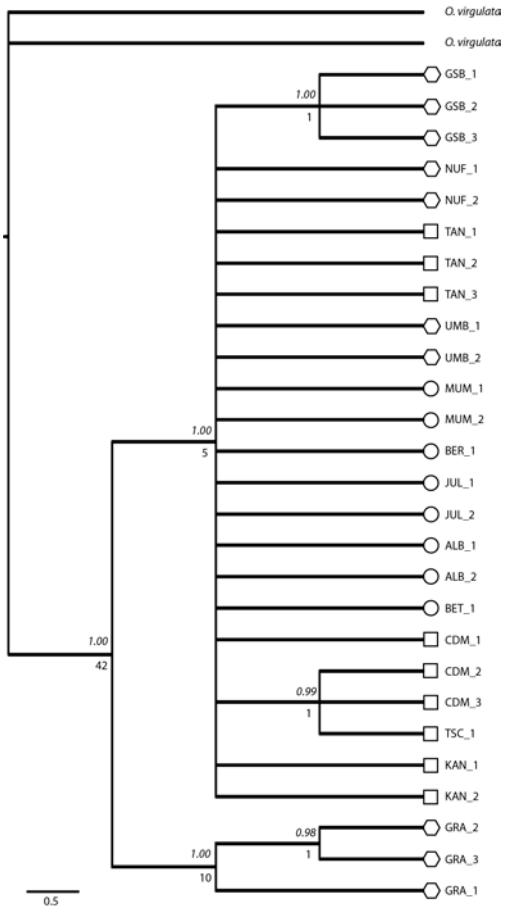

Figure 3 - Strict consensus tree of *Oreina speciosissima*, as revealed by mtDNA regions *16S*, *COI* and *COII* (maximum parsimony tree). Node supports are given by Bremer supports (decay index) ≥ 1 and Bayesian posterior probabilities (italic). Specimens are labelled according to morphotypes (i.e. square - *Oreina speciosissima sensu stricto,*circle - *Oreina speciosissima troglodytes* and polygon - intermediate forms).

AFLP

The AFLP analysis produced a total of 530 bands (171, 166 and 173 for EcoRI-ACA/MseI-AGC, EcoRI-ACA/MseI-ACG and *E*coRI-ACA/MseI-AAC, respectively) with an average of 254 bands per individual and an average reproducibility rate of 96.1%. Among 510 variable characters, 458 were potentially parsimony informative. Just as the mtDNA data, the AFLP dataset was investigated using MP and Bayesian phylogenetic inference methods. Again, both approaches were highly congruent as the MP and Bayesian trees shared the same major nodes. Consequently, only the Bayesian phylogeny (including the bpp and Bremer supports) is displayed in Fig. 3. Due to the lack of an outgroup, we present an unrooted topology (with supports extracted from the corresponding midpoint-rooted topology), which led to a separation of specimens into two well supported clans *sensu* Wilkinson *et al.* [288] (clan I and clan II), each with a bpp of 0.94 (Fig. 4). Clan I includes three sub-clans supported with bpp values of 1.00 (Ia), 0.79 (Ib) and 0.98 (Ic) respectively. Clan I contains nine specimens with a strict *Oreina speciosissima sensu stricto* morphology (sub-clan Ic) and six specimens with an intermediate morphology (sub-clans Ia and Ib). Within clan II, two sub-clans were well supported with a bpp of 0.96 (IIa) and 0.91 (IIb) respectively. Clan II contains eight individuals with strict *Oreina speciosissima troglodytes* morphology (all of sub-clan IIb, except UMB specimens) and five individuals with an intermediate morphology (all of sub-clan IIa and UMB specimens from sub-clan IIb). Notably, specimens with intermediate morphologies were sorted close to the midpoint root of the tree topology. The AFLP dataset was further investigated using a Bayesian (i.e. STRUCTURE see [284,285]) and a distance-based (i.e. *K*-means; see [280,282]) clustering algorithm. The approaches produced fully congruent relationships and only results of the former are provided here. The STRUCTURE analysis showed highly likely clusters when considering *K* values ranging between two and five (see box Fig. 4). The obtained results were largely congruent across *K* values (Fig. 4) and highly compatible with the phylogenetic relationships. The only incongruence that could be observed when considering all *K* values, or when comparing STRUCTURE results with the tree topology, involved specimens with an intermediate morphology. Hereafter, we will consider and discuss results based on *K*=5, given that they are the most informative. When viewed from a host plant perspective it becomes apparent that all leaf beetle specimens in clan II occur in the same stone run habitat with individuals from sub-clans IIa and IIb developing respectively in alkaline Petasition paradoxi and in acidic Androsacion alpinae habitats (Figs.2 and 4). In contrast all individuals from clan I occurred in high forbs, in

Adenostylion or Petasites officinalis habitat, either on alkaline, neutral or slightly acidic soils [5] (see Figs. 2 and 4).

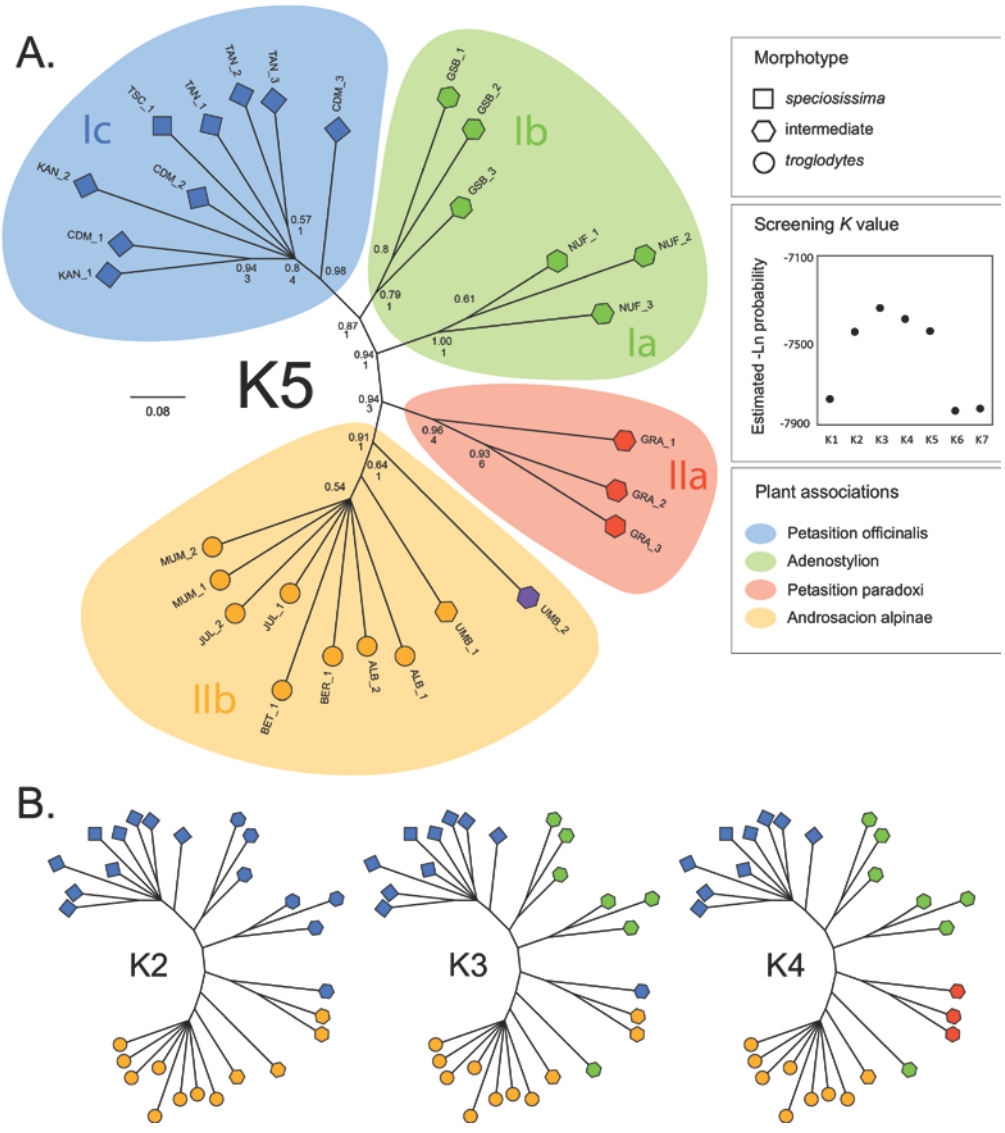

Figure 4 - Half-compat consensus tree and clustering of *Oreina speciosissima*, as revealed by AFLP data (Bayesian tree and STRUCTURE clustering) a) Specimens are labelled according to morphotypes (i.e. square - *Oreina speciosissima sensu stricto,* circle - *Oreina speciosissima troglodytes* and polygon - intermediate forms) and clusters defined using the Bayesian model-based STRUCTURE algorithm applied to AFLP data (i.e. colours of tips according to K = 5 groups; legend centre panel - log-likelihood values of the best STRUCTURE runs for K1 to K7 groups, see text for further details). In addition, the corresponding habitat types (translated into plant associations) and bedrock are displayed as colour coded backgrounds. The names of clans (Ia,b,c and IIa,b, based on the Bayesian AFLP tree topology) and the node supports (i.e.

above - Bayesian posterior probabilities and below - Bremer supports ≥ 1) are provided. b) Insights from alternative STRUCTURE results (i.e. K2 to K4). The tree and morphotype symbols are as in a).

Discussion

Are ecotypes monophyletic?

The phylogenetic tree based on mtDNA markers provides high support for the monophyly of *Oreina speciosissima sensu lato* (Fig. 3). However, very little polymorphism and genetic structure are revealed within the ingroup, although the mtDNA markers proved variable enough to reconstruct intra-specific phylogenetic relationships in other arthropod systems (e.g [289,290,291]). Indeed, the resulting topology supports neither geographical nor ecotypic grouping of the beetles, possibly suggesting a recent divergence of *Oreina speciosissima* lineages, with the exception of specimens from the GRA population, which cluster as the sister lineage to all other specimens. Beetles from this latter population thus form an orphan clade [292], which may correspond to an isolated refugial lineage. AFLP data on the other hand shows a clear-cut differentiation of specimens (Fig. 4). However, this pattern does not appear to have a geographical basis (Fig. 5) and instead correlates with the beetle ecotypic definition, or in other words, to the plant habitat (Fig. 2). In contrast, AFLP genetic structuring only partly correlates with morphotypes (sub-clans Ic and the larger part of IIb, see Fig. 4). Ecotypes *per se* are thus not monophyletic, although there is a strong tendency for specimens and populations to cluster within the boundaries set by plant associations and their intrinsic ecologies.

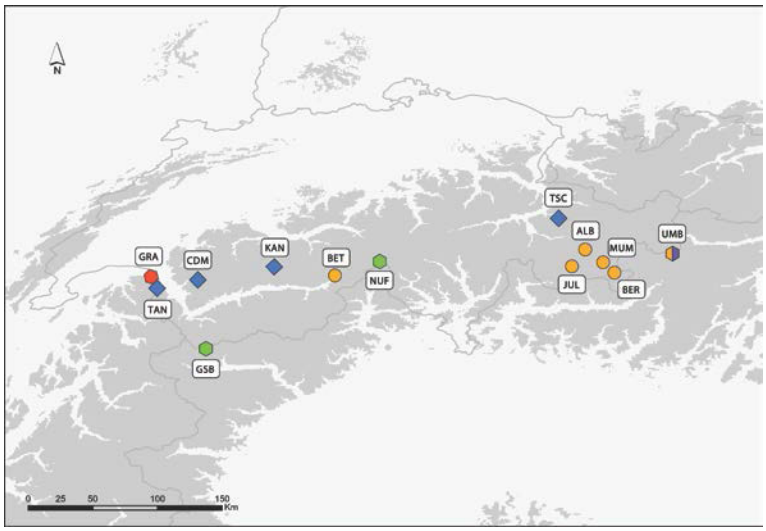

Figure 5 - Geographical distribution of the 13 sampled populations of *Oreina speciosissima* and results from AFLP Bayesian clustering. Populations are labelled according to morphotype (i.e. square - *Oreina speciosissima sensu stricto,* circle - *Oreina speciosissima troglodytes* and polygon - intermediate forms)

and clusters defined using the Bayesian model-based STRUCTURE algorithm (i.e. colours of tips according to K = 5 groups). Grey shaded areas represent elevated regions with altitudes above 1000m. For full names of the populations see Table 2.

Code	Population	Altitude	Coordinates	Morphotype	Habitat	Year
KAN	Kandersteg	1314m	46°28'21"N, 07°39'23"E	*speciosissima*	hf	2004
TSC	Tschiertschen	1325m	46°48'55"N, 09°36'31"E	*speciosissima*	hf	2004
CDM	Col des Mosses	1716m	46°23'26"N, 07°07'30"E	*speciosissima*	hf	2005
TAN	Lac Taney	1389m	46°20'38"N, 06°50'01"E	*speciosissima*	hf	2008
GRA	Le Grammont	1974m	46°21'15"N, 06°49'04"E	intermediate	sr	2008
NUF	Nufenenpass	2172m	46°28'41"N, 08°22'36"E	intermediate	hf	2008
GSB	Grand St. Bernard	2410m	45°52'04"N, 07°10'27"E	intermediate	hf	2008
BET	Bettmerhorn	2628m	46°24'44"N, 08°04'33"E	*troglodytes*	sr	2008
UMB	Umbrailpass	2647m	46°32'53"N, 10°25'43"E	intermediate	sr	2008
BER	Berninapass	2315m	46°24'37"N, 10°01'36"E	*troglodytes*	sr	2008
ALB	Albulapass	2324m	46°34'46"N, 09°50'15"E	*troglodytes*	sr	2008
MUM	Muottas Muragl	2735m	46°30'27"N, 09°56'29"E	*troglodytes*	sr	2008
JUL	Julierpass	2373m	46°28'02"N, 09°43'35"E	*troglodytes*	sr	2008

Table 2. Sampled populations of *Oreina speciosissima* Sample locations with altitude (meters above sea level), coordinates (WGS 84) and year of collection with their codes, morphotype and habitat (hf: high forbs; sr: stone run).

Is adaptation to different habitats and host plants associated with genetic divergence?

Our results showed that genetic differentiation among *Oreina speciosissima* lineages was clearly associated with plant communities (Fig. 4). Accordingly, clustering in *Oreina speciosissima* is well explained by differences in bedrock type and host plants (translated here into different plant associations) (Figs 2 and 4). While specimens feeding in the Petasition paradoxi association (in which the calcicolous *Doronicum grandiflorum* is the main host plant for *Oreina speciosissima* [unpublished observations MB, TVN]) cluster in sub-clan IIa, specimens developing in the Androsacion alpinae association (in which the silicicolous *Doronicum clusii* is largely dominant as a host plant for *Oreina speciosissima* [unpublished observations MB, TVN]) are restricted to sub-clan IIb. The effect of soil acidity is less striking in clan I, probably because the Adenostylion and Petasition officinalis associations, which are characteristic of all specimens within this clan, are defined by intermediate soil pHs. These two plant communities include species showing an intermediate tolerance to acidic-alkaline variation, such as *Achillea macrophylla, Adenostyles*

alliarae and *Petasites albus* [293]. Whereas the latter two represent the main host plant species of *Oreina speciosissima* in high forbs habitat [unpublished observations MB, TVN], other species (particularly in the Petasition paradoxi association) could play or have been playing the role of subalpine bridge species between the montane high forbs and alpine stone runs (see below). We are confident that these results are robust to potential shortcomings inherent to our limited sampling size (see [294] for a review). First, specimens were collected throughout the common geographical range of both ecotypes, a strategy that maximized both the phylogeographic and ecological representativity of our sampling. Second, robust and consistent results were obtained using both phylogenetic and clustering algorithms.

Towards a scenario of ecological speciation in Alpine *Oreina speciosissima*

Although our data does not allow for divergence time estimates between *Oreina speciosissima* ecotypes, it seems likely that they diverged relatively recently. Indeed, the current distribution of *Oreina* populations suggest that the ecotype divergence might have arisen after one of the last glacial maxima, given that populations were probably not able to survive cold periods at high altitudes due to the presence of ice caps (with the possible exception of the GRA population; see above). This hypothesis is consistent with the low levels of genetic variation observed in nuclear sequences and the low resolution in the mtDNA topology, as well as with a preliminary dating of the *Oreina* genus, in which the origin of *Oreina speciosissima* is estimated at circa 0.4 million years ago [258].

Our results suggest that from an ancestral niche associated with high forbs (see above) beetle populations were able to colonize new habitats along an altitudinal gradient (Fig. 2) and invaded the acidic siliceous stone run habitat (corresponding to the Androsacion alpinae association), which is typical for Alpine regions in Central Europe. We propose that this habitat change could have been associated with host shifting events. Accordingly, the plant communities on which *Oreina* ecotypes feed appear to be connected by phylogenetically related host species. In a framework of plant-insect coevolution [21,22], adaptation to a given plant species might allow beetles to spread to other similarly-defended congeneric species [295,296]. Accordingly, *Doronicum* species occur in the Petasition paradoxi and the Androsacion alpinae, *Petasites* species link the Petasition officinalis to the Petasition paradoxi and finally, *Adenostyles* species are shared among the Adenostylion, the Petasition paradoxi and the Androsacion alpinae. Assuming host-plant conservatism, the connections described above might represent “shifting” routes that could explain how *Oreina speciosissima* lineages transited among habitats via host

switching. Furthermore, these connections could account for the presence of putatively admixed specimens showing intermediate morphologies (e.g. UMB), thereby outlining a possible ongoing migration of beetles from one habitat to the other.

Conclusions

Our study reveals a genetic structure in *Oreina speciosissima* as a function of the plant community in which beetles develop. We discussed several possible ecological features that could cause the divergence between ecotypes, among which the habitat and host-plant switches seem key factors. These results could be consistent with an ecological speciation scenario. Still, non-adaptive processes such as genetic drift, founder events and population bottlenecks might also have produced the observed pattern. Hence, further investigation is needed, for instance, fine scale studies relying on genomic approaches and targeting populations from a patchy distribution of the two ecotypes following an approach such as described by [138] could provide a powerful framework for detecting adaptive signatures associated to ecological speciation. Additionally reciprocal transplantation experiments in concert with crossings using local and non-local beetles could possibly reveal performance differences between locally adapted and non-adapted beetles and strengthen our argument for the existence of host races and ongoing or incomplete speciation (cf. [86,297]).

Authors' contributions

MB and TVN collected the samples, carried out the morphological and genetic analyses, participated in phylogenetic analysis and drafted the manuscript. NAR, SB and NAL designed phylogenetic tools, participated in phylogenetic analysis and revised the manuscript.

Acknowledgements and funding

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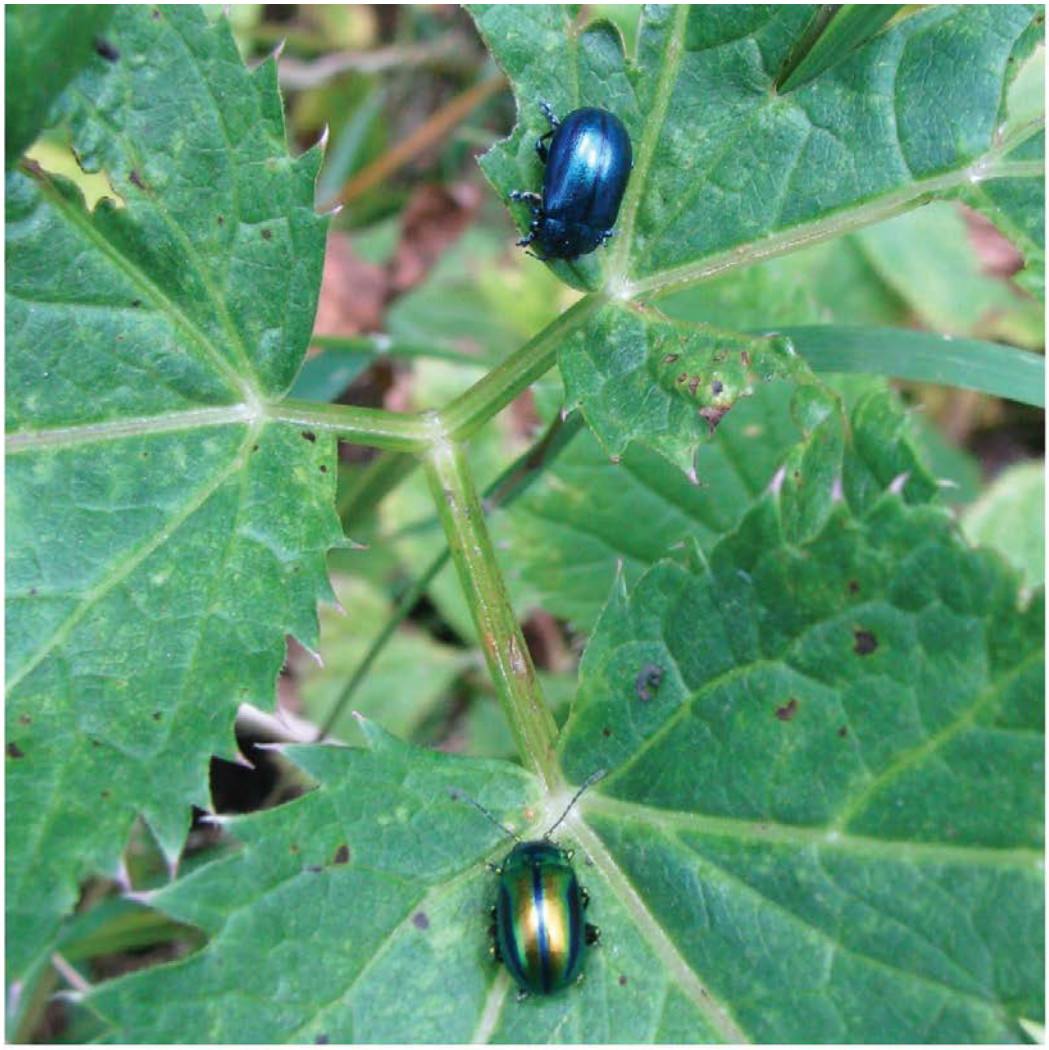

four.

4. Positive frequency-dependent selection on warning colour in Alpine leaf beetles[3]

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Abstract

Müller's theory of warning colour and mimicry, despite forming a textbook example of frequency-dependent selection, has rarely been demonstrated in the wild. This may be largely due to the practical and statistical difficulties of measuring natural selection on mobile prey species. Here we demonstrate that this selection acts in alpine beetle communities, by using tethered beetles exposed to natural predators. *Oreina gloriosa* leaf beetles (Coleoptera: Chrysomelidae) possess chemical defence in the form of cardenolides, accompanied by what appears to be warning colour in bright metallic blues and greens. Individuals that match the locally predominant colour morph have increased survival, with odds of week-long survival increased by a factor of 1.67 over those that do not match. This corresponds to selection of 13% against foreign morphs. Such selection, acting in concert with variation in community composition, could be responsible for geographic variation in warning colour. However, in the face of this purifying selection, the within-population polymorphism seen in many *Oreina* species remains paradoxical.

KEY WORDS: Aposematism, chemical defence, Chrysomelidae, Müllerian mimicry, natural selection, purifying selection.

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Introduction

Müller's theory of warning colour and mimicry is based on the following argument: in two unpalatable species that share a habitat, if they are dissimilar, predators must eat a certain number of each to learn that they are distasteful, whereas if they are identical, members of both species benefit by sharing the cost of predator education [53,298,299]. This generates selection for resemblance between unpalatable species within a community (Müllerian mimicry) and positive frequency-dependent selection (purifying selection) within a species, because in both cases common forms benefit from protection whereas rare variants suffer increased per capita predation. The principle is widely used as a textbook example of frequency-dependent selection and has considerable theoretical and comparative support (reviewed in [53,300]), with renewed interest in the literature including suggestions that mimetic shifts may influence both speciation and community structure [97,301,302,303]. Despite this, neotropical butterflies provide the only examples for which the survival advantage enjoyed by common forms has been demonstrated using natural prey and predators in the wild [80,304,305]. This is probably in large part due to the difficulty of measuring selection in mobile species, because the confounding effect of dispersal complicates the practical and statistical techniques needed to estimate survival from recapture probabilities.

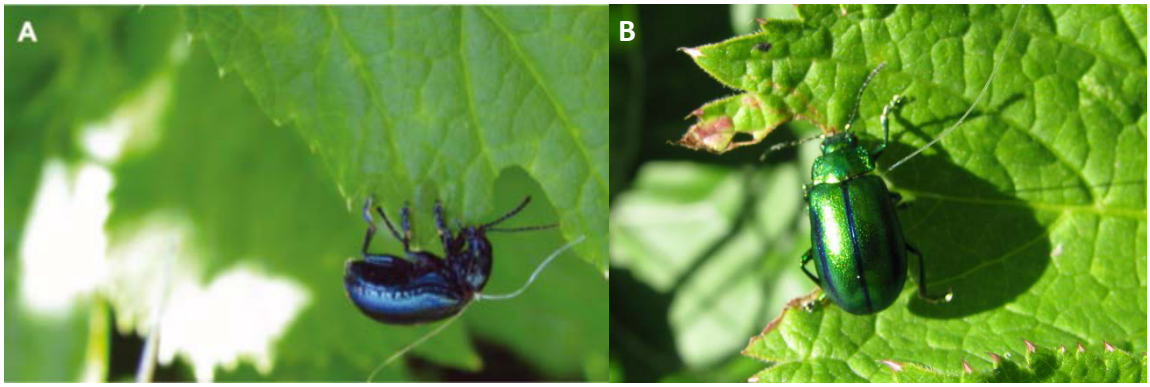

Figure 6. (A) Blue and (B) green *Oreina gloriosa* beetles tethered on fine plastic leashes. Photographs by TVN.

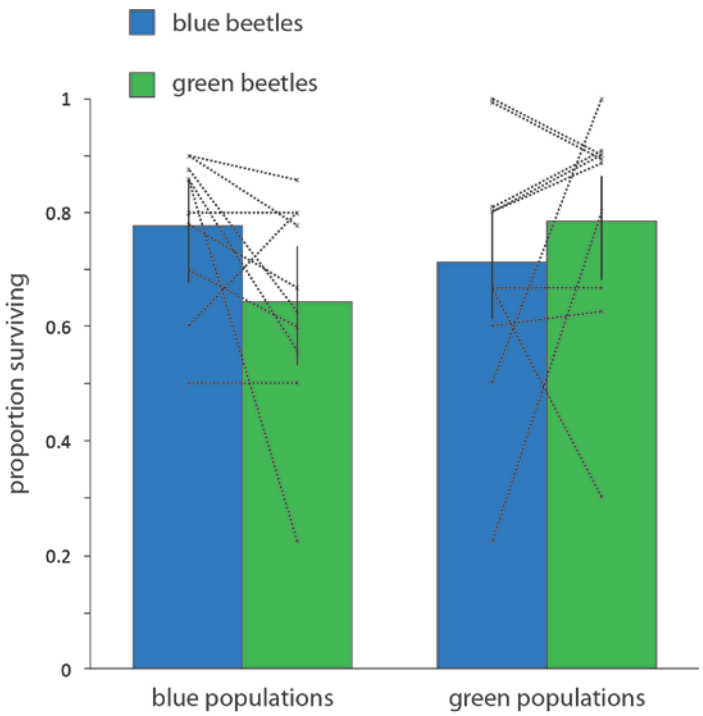

Figure 7. Week-long survival estimates for blue and green beetles at blue- and green-dominated sites (10 replicates for each bar). Error bars show exact binomial (Clopper–Pearson) 95% confidence intervals for survival probability. Crosses and dashed lines indicate the results for each individual site.

Here we avoid these problems by measuring the survival of tethered individuals (Fig. 6) in natural populations, to test for positive frequency-dependent selection on colour in the alpine leaf beetle, *Oreina gloriosa* (Fig. 7). Beetles of the genus *Oreina* (Coleoptera: Chrysomelidae) are found in isolated populations throughout the mountains of Europe [2,8,258]. They possess two chemical defence strategies correlated with their host plant use. Species feeding on Apiaceae or Cynareae (Asteraceae) synthesize cardenolides, whereas those feeding on Senecioneae (Asteraceae) are able to sequester plant derived pyrrolizidine alkaloids [12,306,307]. This chemical defence is accompanied by what appears to be warning colouration in bright metallic blues and greens, often in combination with blue or red stripes. Potential predators of adults include many that hunt visually, in particular birds such as the European Robin *Erithacus rubecula*, Winter Wren *Troglodytes troglodytes* and Dunnock *Prunella modularis* (TVN personal observation), as well as predators that are less reliant on vision, like shrews, ants, and spiders. These beetles are relatively sedentary and feed exposed on the upper leaves of their host plants, so tethering them on fine plastic leashes does not greatly interfere with their natural behaviour. It does, however, allow us to be certain in the identification of predation events, thereby avoiding the need to simultaneously model the unknown probabilities of resighting, dispersal, and predation from mark-recapture data [80,305]. Our focus is on *Oreina gloriosa,* a species that

is chemically defended by cardenolides and is monophagous on *Peucedanum ostruthium* (Apiaceae). It occurs throughout the Alps and varies in colour from blue to green, in combination with three longitudinal blue stripes on the elytra. Beetles also reflect in the UV region of the spectrum, with around 25% stronger reflectance in blue compared to green morphs. We test for frequency-dependent selection on colour by comparing the survival of blue and green individuals from mixed populations when exposed to predation in sites dominated by either blue or green beetles.

Material and methods

Experiments were carried out in communities close to the tree line (altitudes of 1592–2182 m above sea level) along the side valleys of the Rhone valley in south western Switzerland. In these sites the beetles are found in forest understory and open habitats in patches of a high-forb plant community that is often dominated by *Oreina* host plants, including *Peucedanum ostruthium*, *Adenostyles alliariae*, *Chaerophylum villarsii*, and *Heracleum sphondylium*. Beetle colour variation does not appear to be associated with habitat type. Tethered experimental beetles were placed in 20 natural *Oreina* communities during the alpine summers between July and early September in 2005–2008. In each replicate, 10 green and 10 blue *Oreina gloriosa* beetles (always all collected from the same mixed population, either at La Fouly or Col des Mosses) were attached individually to randomly chosen *Peucedanum* plants throughout a host-plant patch. The plants were marked with plastic tags and the beetles attached using 0.4 m-long leashes made of fine transparent plastic thread (0.1 mm diameter), tied at one end around the body in the constriction between the prothorax and elytra, and at the other end to an upper node of the host plant. At the same time, the predominant colour of beetles at the site was recorded, taking the entire natural *Oreina* community into account (all sites were strongly dominated by one or the other colour, and included from one to six common species dominated by *O. gloriosa*, often together with *O. cacaliae* and *O. speciosa*). Sites were then visited one week later to record the survival of each tethered beetle. Preliminary trials suggested that this period would result in moderate levels of predation. On 38 out of the 400 occasions the plants could not be found again and these individuals were excluded. The association between colour and survival was evaluated by using logistic regression to analyse the proportion of beetles surviving at each site. Generalized linear mixed modelling (GLMM) was used (Chapter 13 in [308], applying the lmer function in the lme4 package run within R version 2.9.2 [309,310]. Site name was treated as a random effect, thereby allowing us to control for differences in the overall level of predation among sites and to incorporate the correlation introduced by the paired design of the

experiment. It should be noted that this term includes all factors contributing to differences in the overall level of predation among sites, including effects of year of experiment, as well as actual site-specific influences such as variation in predator communities. Beetle colour was introduced as a fixed effect, coded as “local” for the beetles sharing the predominant colour of the site and “foreign” for those of the other colour. Two supplementary analyses were also carried out. First, beetle colour was recoded as “blue” or “green,” providing a test for an overall survival advantage of one morph over the other. Second, to exclude the possibility of consistent differences in the overall level of predation at blue- and green-dominated sites, the site term was instead introduced as a fixed effect with two levels (“blue-dominated” or “green-dominated”). For all these analyses, the data were well approximated by a binomial distribution (with dispersion factor of 1.19, compared to a value of 1 for an ideal binomial distribution) so there was no need to use quasi-binomial estimation. The effect of beetle colour was evaluated using a likelihood ratio test: when comparing models with the term included and excluded, double the difference in likelihood (2_*L*) asymptotically follows a Chi-square distribution, with the degrees of freedom given by the difference in the number of parameters (one in this case).

Results

The experiment was carried out at twenty sites, half blue-dominated and half green-dominated, with overall survival values varying between 43% and 95% (Fig. 1). Beetle colour had a significant effect on survival probability (2_L = 4.385, df = 1, P = 0.036). Matching the locally predominant colour increased the odds of week-long survival by a factor of 1.67 (with 95% confidence interval of 1.03 to 2.71). This advantage was similar for blue beetles in blue-dominated sites (odds ratio 1.93) and green beetles in green-dominated sites (odds ratio 1.44). There was no significant difference between blue- and green-dominated sites in their overall level of predation (2_L = 0.613, df = 1, P = 0.434), and no overall difference in survival between blue and green beetles (2_L = 0.493, df = 1, P = 0.483).

Discussion

Colouration had a strong effect on survival, with a significant benefit to matching the locally predominant colour. Learned avoidance by visually hunting predators is therefore an important factor in the survival of *Oreina* beetles. Because our experimental sites were dominated by *Oreina gloriosa*, this represents an example of positive frequency-dependent selection on colour, but there is also likely to be a contribution from Müllerian mimicry across the entire *Oreina* community. There was variation among sites in the overall levels of predation, but this is

not unexpected and would be influenced by many factors, including the weather during the replicate and the local predator density. There was also variation in the relative predation on local and foreign colours. This might be a result of natural differences in the relative frequency of blue and green morphs at each site leading to variation in the strength of frequency-dependent selection, as well as differences in the contribution of visual and nonvisual predators. The survival probabilities in our experiment can be used to estimate selection on colour, because life expectancy is proportional to these values if it is assumed that the majority of predation will occur in the initial period following introduction while predators are learning to avoid the foreign patterns [305]. The overall survival probabilities (*P*) of 67.7% for foreign morphs and 77.8% for local morphs translate into a selection coefficient(*s*) of 0.13 (using $s = 1 - P_{foreign}/P_{local}$). This strong selection is comparable with estimates from other Müllerian mimicry systems: 0.22 in *Heliconius erato* painted to produce novel morphs [304], 0.52 in *H. erato* transferred across a hybrid zone [305], and 0.64 in polymorphic *Heliconius cydno* [80]. In our experiment, the use of leashes allows us to be certain that death due to predation is the cause of the disappearance of individuals, and because all beetles within each replicate were taken from the same source population and varied only in colour, we can be sure that differential predation according to colour must underlie the selection. Our value is somewhat lower than earlier estimates, possibly because we can exclude the potential confounding effects of differential dispersal or resighting. However, variation in the estimates of *s* could also be a result of many ecological differences between the systems, such as differences in predator communities, prey memorability or predator learning ability, the strength of deterrence due to defences, or the background level of loss to nonvisual predators. This mode of positive frequency-dependent selection by predators would be expected to have opposite effects on the levels of inter- and intra-population polymorphism, acting to generate geographic variation and eliminate within-population polymorphism. It forms a mechanism by which geographic variation in community composition would alter the target of convergence, and could thereby generate geographic variation in colour within species [311]. *Oreina* communities are susceptible to vary geographically in the species present because of variation in the host plants available, and as a result of stochastic effects of their limited dispersal ability, the isolated nature of their habitats, and the fact that the entire region was subjected to repeated extinction and recolonization during Quaternary glacial cycles [8,312]. Local adaptation of populations towards the fitness peak formed by the predominant colour of the community could therefore be responsible for the observation that several species show great variation in colouration across their distribution [2,211]. In contrast, this form of frequency-dependent predation should eliminate within-population polymorphism

because it generates strong purifying selection. The remarkable variation in colour seen within populations of many *Oreina* species is therefore paradoxical [27,299]. Future work will be devoted to examining other factors, such as variation in unpalatability [313], neophobia [314], sexual selection, gene flow, or even the shape of frequency-dependent selection itself [27], that may be responsible for maintaining this diversity.

Acknowledgments

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5. Minute changes in the elytron generate colour variation in *Oreina gloriosa* leaf beetles (Coleoptera: Chrysomelidae)

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Abstract

Leaf beetles are famous for their cuticular iridescence. Members of the chrysomelid genus *Oreina* show a striking variation in colours. In this study we examine the elytra of several colour morphs of *Oreina gloriosa* in order to identify the source of colour variations using Transmission Electron Microscopy, Fibre optic spectrophotometry and a software package. We confirm that colour is produced by epicuticular microstructures in the elytron. Variation in the thickness of the electron lucent layers, that form bilayers with electron dense layers of constant width, is responsible for elytron colour. Model output proved not to be congruent with observed spectral peaks. This incongruence might be due to the use of wrong parameter values.

KEY WORDS: *Oreina gloriosa*, iridescence, structural colours, cuticle, entomology, TEM

Introduction

Colour is important in many different ways throughout the life of an animal. It can prevent detection by predators [59,171,315,316], signal unprofitability [317,318,319], fulfil a role in mate recognition [178,320], male-male competition [178,321,322,323,324], or help to maintain homeostasis [42,46,325,326,327,328,329]. Colours are generated by the interaction of incident light with either epicuticular microstructures or pigments (colours produced by means of bioluminescence or chemiluminescence will not be discussed here) of which the dimensions lie in the same order of magnitude as the wavelengths of light [330]. Structural colours in invertebrates can be roughly divided into three groups: scattering causing structures, diffraction gratings and thin-film reflectors [139,330]. The bulk of these colours are, once produced, permanent and unchangeable although some exceptions occur [331]. The leaf beetle genus *Oreina* harbours an impressive array of elytral colours. Members of a single species and even of

a single population can show remarkable variation in body colouration, commonly ranging from brilliant and uniformly coloured green, to polychromous striped blue specimens and the less abundant black and red phenotypes. The presence of such a polymorphism is noteworthy even among beetles, and calls for explanations accounting for both its biological or evolutionary significance and the related developmental processes that produce them. By using Transmission Electron Microscopy (TEM) we aim to uncover the morphological basis for the chromatic polymorphism found in many *Oreina* species. Fibre optic spectrophotometry will be used to provide a link between colour and TEM results. A software package will be used to verify correct interpretation of the TEM results.

Materials and methods

Transmission Electron Microscopy

Males of *Oreina gloriosa* (Coleoptera: Chrysomelidae) were collected near La Fouly, Switzerland. Six beetles were hand chosen to span a broad range of colours that occur naturally in the alpine regions of Europe. The beetles were put in phosphate buffer 0.2M (pH 7.0) and stored at 5°C until preparation. Small pieces of up to 10 mm^2 of the left elytron were removed and immersed in a fixative mixture consisting of 2.5% glutaraldehyde solution in phosphate buffer 0.1M (pH 7.0). After 1 hour pieces were washed with phosphate buffer (3 x 15 minutes) and post fixed in a 2% OsO_4 solution. After three washes in phosphate buffer, dehydration was carried out in a gradual ethanol series from 30% to 90% followed by steps in acetone (90% and 100%) and infiltration with 'Spurr' resin [332]. All treatments were carried out at room temperature. Pieces were subsequently embedded in Spurr resin for 24 hours at 60°C. Sections were cut perpendicular to the dorsal surface of the elytron with a diamond (Diatome) on a Reichert Ultracut S microtome (Leica). Ultra-thin sections (60-100 nm) were mounted on grids and counterstained for 5 minutes in uranyl acetate in 50% ethanol, followed by 5 minutes in lead citrate. Grids were examined in a Philips CM100 Transmission Electron Microscope at 60kV accelerating voltage. For measuring the thickness of the layers, micrographs are calibrated in the eucentric point of the CompuStage of the TEM.

Reflectance spectral analysis

Reflectance of the respective *O. gloriosa* elytra was measured under 0° incidence in the visible spectrum between 350 and 1100 nm using a fibre optic spectrophotometer (AVASPEC-3648-USB2; Avantes, The Netherlands). A diffuse PTFE reference tile was used as a white standard

(WS-2; Avantes, The Netherlands). The measured reflectance is a relative measure to the reflectance of the white standard.

Modelling reflectance spectra

The measurements we acquired during the TEM session form the basis of the following section. Here we calculate the reflectance spectra of the several multilayer reflectors found in the respective epicuticles of *O. gloriosa* using the transfer matrix approach described by Macleod (2001) [333] and which was applied by Kroiss and co-workers [334] in a similar study involving gold wasps. Typically multilayer reflectors in Coleoptera consist of alternating layers of chitin and lucid spacing material. The peak wavelength, $wavelength_{max}$ equals 2 ($n_a \cdot d_a + n_b \cdot d_b$), where n is the refractive index; d is the layer thickness; and a and b are the alternating translucent and dense layers. There is considerable variability in estimates for the refractive index of translucent and dense layers in insect epicuticle. A widely used estimate for the dense layers is n_{chitin}=1.56 [335], but Noyes and co-workers estimated n=1.68 due to inclusion of other substances in the chitinous matrix [336]. Epicuticle layers consisting of melano-protein might reach n=2.00 [337]. Bernard and Miller (1968) mention n=1.73 for electron dense layers and n=1.40 for the electron lucid layers [338] and see also Richards and references therein [339]. Land uses $n_{cytoplasm}$ =1.34 for the lucid layers [335]. It is unclear which values best estimate the refractive indices of the layers we observed in *O. gloriosa* best. As Parker and co-authors (1998) state: "*There is a general lack of information on refractive indices of materials involved in beetle reflectors, and such values are difficult to determine experimentally*" [330]. We therefore chose to use the values proposed by Hand as these have been used in many studies of this nature and modelled the reflectance spectra for the respective multilayer reflectors consisting of 5-7 lamellae with decreasing width. Each lamella comprises of a 22-48 nm layer of chitinous material (n=1.65) and a 81-167 nm layer of cytoplasm-like material (n=1.34) [335]. The multilayer reflector is topped by a single, semi-lucid band with a thickness of 96-105 nm representing the cuticulin. Since literature does not mention the refractive index of cuticulin we estimated the maximum Gray value of the cuticulin peak as a percentage of the maximum Gray value of the first reflective layer using the ImageJ software package [340]. On average the cuticulin peak proved to have a Gray value of 91.49% of the first reflective layer. We therefore estimated the refractive index of cuticulin to be (n=1.50). The reflectance spectra for wavelengths between 300-1000 nm with light incidences of 0°, 45° and 60° were calculated using the freeware package OpenFilters 1.0.2. and the TEM pictures (Fig. 11) as a blueprint [341].

Results

Transmission Electron Microscopy

Transmission Electron Microscopy revealed colour in *O. gloriosa* is structural. As shown in Figures 8. and 9 the epicuticle comprises of a stack of bilayers consisting of an electron dense (dark) and an electron lucent (light) layer.

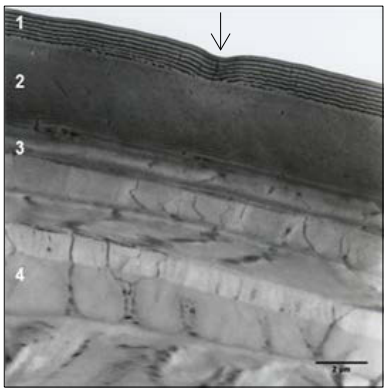

Figure 8. Perpendicular cross- section of dorsal surface of the elytron of male *O. gloriosa* (green morph) (8.580x magnification). 1) epicuticle, 2) outer exocuticle, 3) helicoidal cuticle, and 4) procuticle. The internal architecture shows strong similarity with that of *Cicindela scutellaris* studied in [337]. Scale bar = 1µm. The arrow indicates a dermal gland.

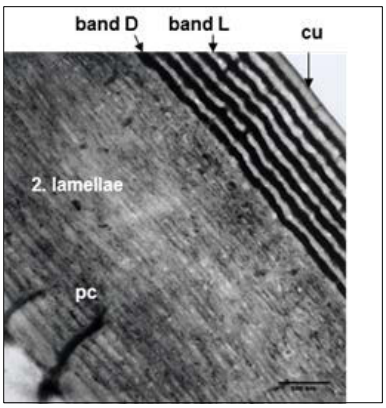

Figure 9. Perpendicular cross-section of dorsal surface of the elytron of male *O. gloriosa* (green morph) (36.960x magnification). The epicuticle consists of bilayers of alternating electron dense (band D) and electron lucent (band L) layers that determine colour. The cuticulin (cu) appears to consist of slightly more

dense material than the electron lucent layers of the epicuticle. Layer 2. or outer exocuticle consists of 32-37 packed non-sclerotized lamellae traversed by pore canals (pc). Scale bar = 1µm.

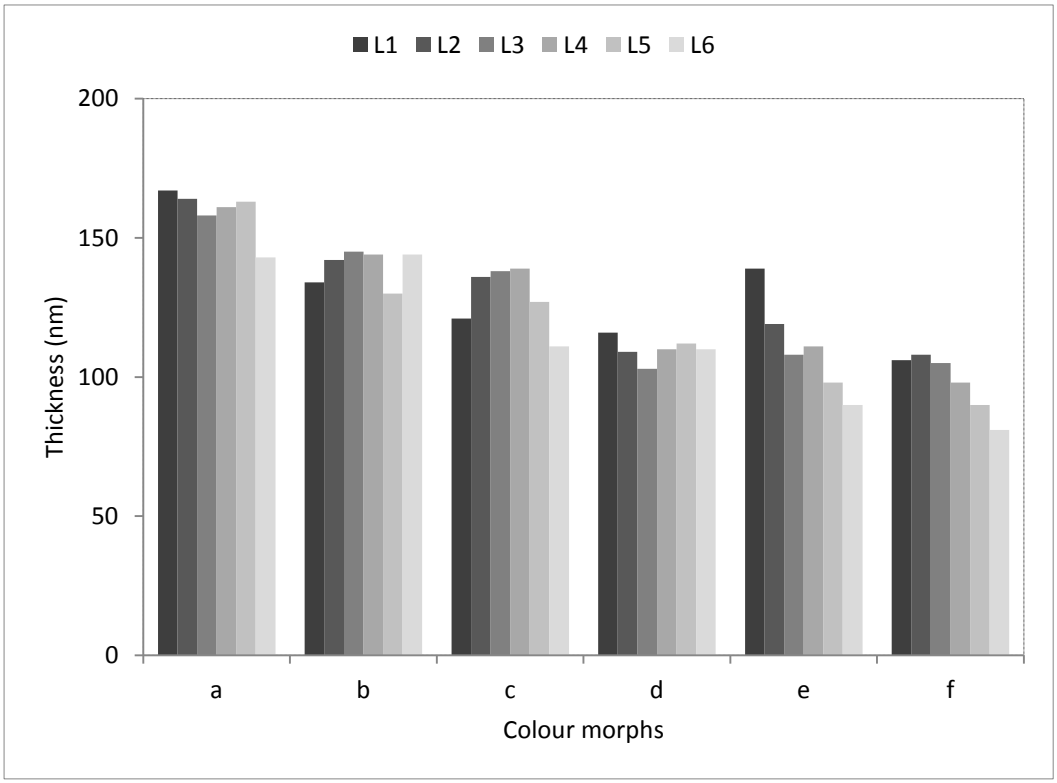

Figure 10. Layer thickness of the electron lucent (L) layers of the respective colour morphs.

Electron dense layers (D) have a more or less constant thickness throughout the elytron and across colour morphs. However, as is shown in Figure 10, the electron lucent layers (L) are more variable. Not only does the average layer thickness decrease as the beetles become bluer, it also decreases with the increase of distance from the elytron surface. Deeper layers are thinner. For example the lucent layer closest to the surface (L1) of the red morph (a) is 167 nm thick whereas the deepest layer (L6) is 106 nm thick.

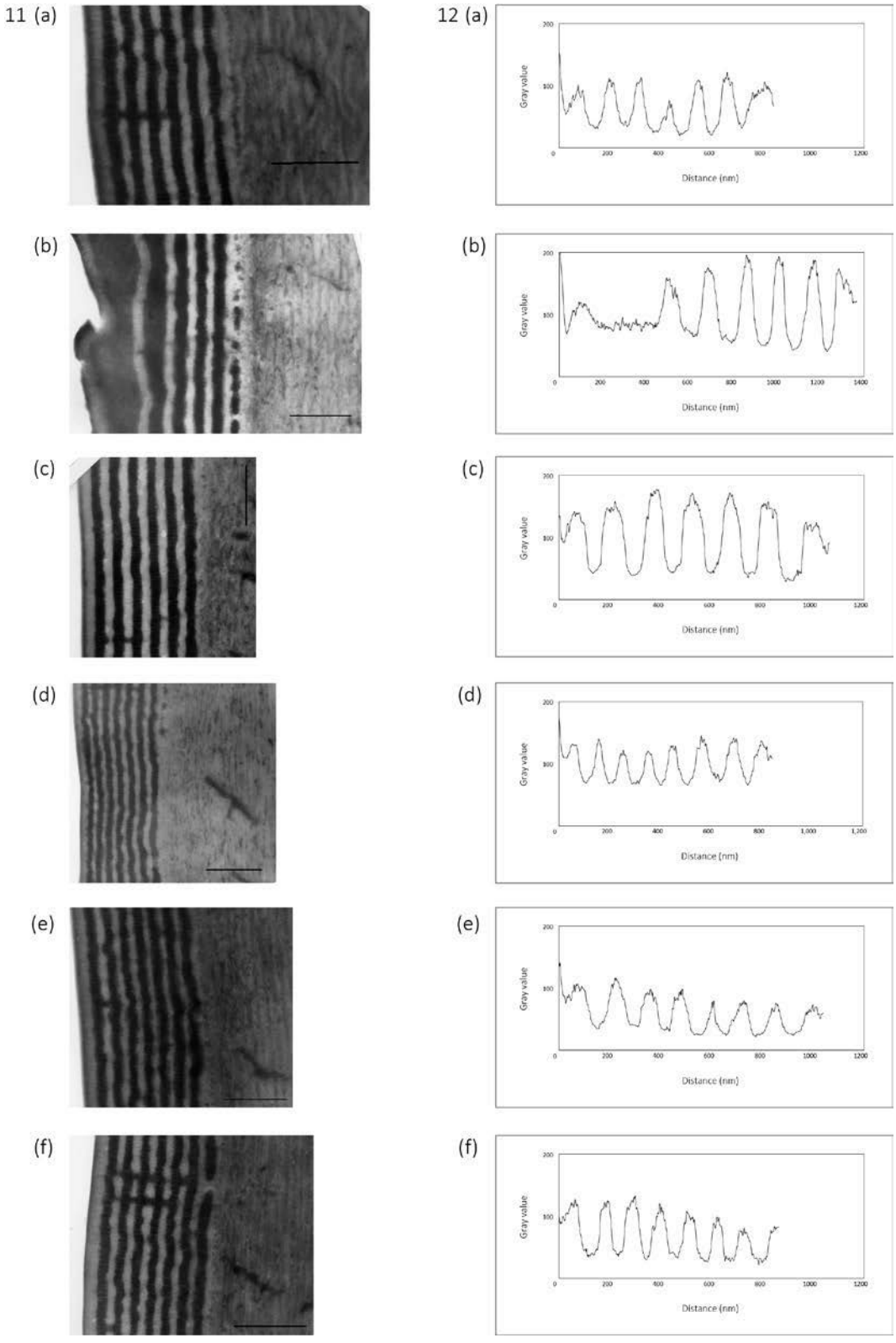

Figure 11. Overview of the TEM pictures of the six colour morphs (a-f). Cross-section 11b shows a slight artefact as a result of hampered preservation. Pore canals, visible as horizontal dark lines in some of the samples provide proof of the perpendicular nature of the cuts. Figure 12. Graphical representation of TEM pictures. The graph displays the relative translucence in relation to the distance from the elytron surface.

Reflectance spectral analysis

The reflectance spectrum analysis of the leaf beetles (see figure 12a-f) showed the following dominant reflectance maxima (see figure 13a-f) of λ= 660, 608, 574, 537, 512 and 495nm. The strong convex nature of the dorsal abdominal cuticle prevented measurements under 45° and 60° incidence. The 'blue shift' of the elytron that can be observed with the naked eye under increasing angle of incidence [342], and which is due to shortening of the travel distance through each layer [139], could unfortunately not be verified with the spectrophotometer.

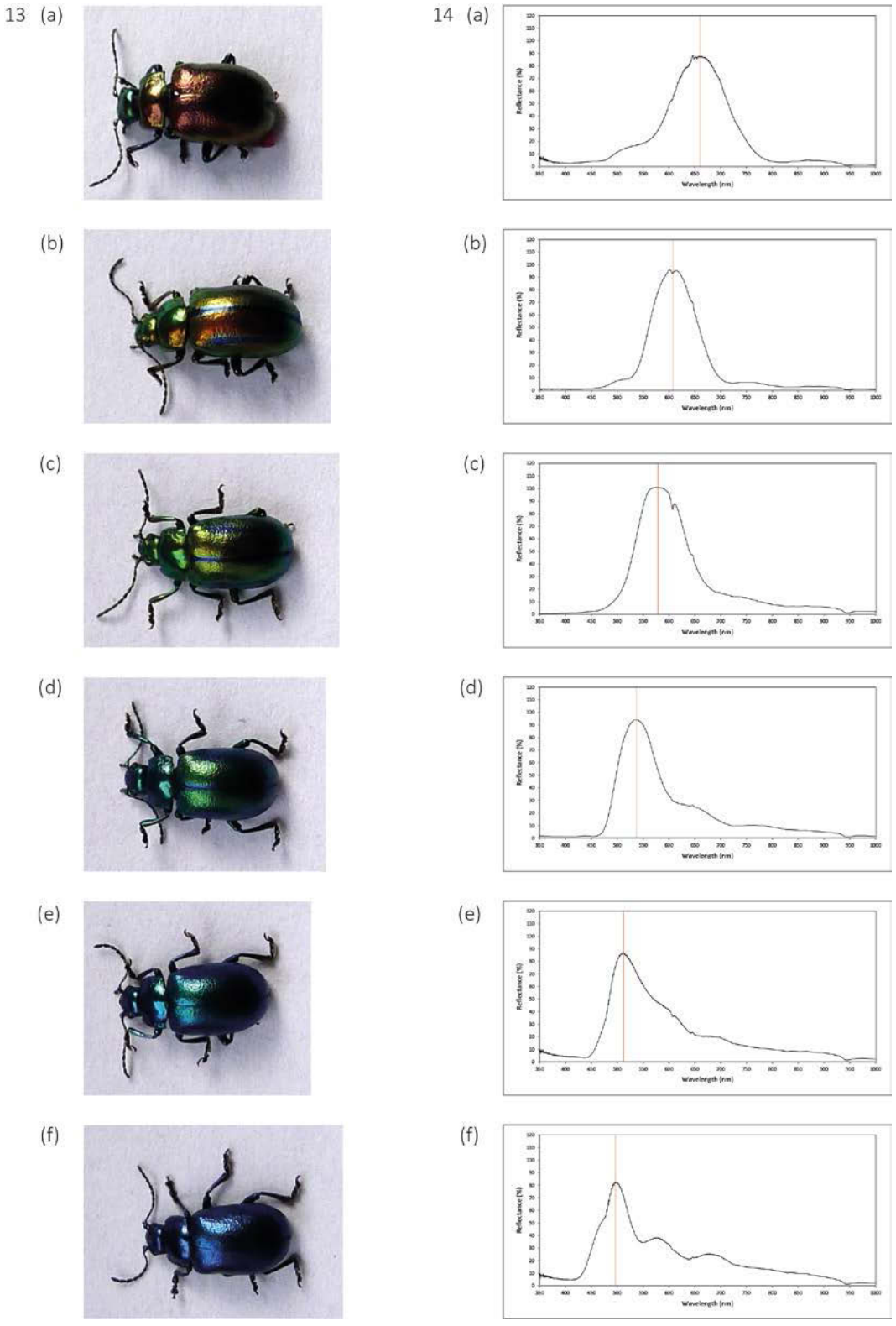

Figure 13. Overview of the different colour morphs of *O. gloriosa.* Figure 14. Spectral reflectance of the 'background colour' of the respective beetles. Measurement of the blue dorsal striping, present in some of the beetles was avoided as much as possible. Red line = peak reflectance.

Discussion

The series of TEM micrographs confirmed the structural basis of colour in *O. gloriosa* and the presence of an epicutical multilayer reflector in the elytra of the leaf beetle. This multilayer consists of 5-7 lamellae with a thickness between 109-211 nm. What we have here is a slightly modified multilayer reflector that differs from the most common type typically found in other beetles [343]. Layers in *O. gloriosa* differ in thickness that decreases with depth. We therefore speak of a chirped reflector. The colour that is reflected by a multilayer depends on the refractive index of the component layers, their thickness, and their spacing. High density layers reflect longer wavelengths than low density ones. Reflectors with thin low density (L) layers produce blue colours.

As shown for the polymorphic leaf beetle *Plateumaris sericea* by Kurachi and co-authors (2002), the proximate cause for colour polymorphism in *O. gloriosa* lies in the differences in the periodicity of reflecting layers [324]. The lack of congruence between the measured and computed reflectance spectrum raises the questions concerning the interpretation of the TEM pictures and the parameter values used. Further study is needed to resolve this discrepancy and explore the structural colour mechanisms discussed in [139].

The ultimate causes for the colouration of *Oreina* leaf beetles remain unclear although we know it serves a purpose in predator-prey interactions [26] and possibly homeostasis. Colour does not seem to be important in mate-recognition [344]. Colour might even be a by-product of the ontogenetic process that maximized integument strength in response to predation as suggested by [334]. The observed differences leave room for non-genetic components to colouration as a result of e.g. conditions with which the larvae have grown up, which might differ across host plans or micro climatic circumstances.

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six.

6. Conclusions and Outlook

Conclusions

The primary goal of this dissertation was to understand the maintenance of colour polymorphism in the leaf beetle genus *Oreina*. Since we hypothesized colour not to be a neutral trait, understanding the maintenance of colour polymorphism might be achieved by identifying the major selective pressures. After having introduced the research system in **chapter 1**, I dedicated the numerous pages of **chapter 2** to the search of the key evolutionary forces. We argue that there isn't just one selective force of paramount importance but a multitude of factors in a heterogeneous landscape producing a variable co-evolutionary mosaic in space and time. Although evidence for this supposition comes from studies focussing on other (chemically defended) insects, its persistent and frequent appearance in the discussion sections of many studies within the evolutionary biology realm, make me confident of its universal character. Despite the current lack of empirical evidence, I have no reason to believe the genus *Oreina* represents an exception to this rule. A second conclusion that can be drawn from chapter 2 is less applicable to the *Oreina* case. It entails the observation that frequency dependent selection in the form of male-harassment is a prevalent evolutionary force shaping intra-specific relationships in female-limited colour polymorphic species. The third conclusion of chapter 2 concerns the genetic basis of colour polymorphisms. Colour polymorphism seems to be largely controlled by single locus autosomal genes. Major genes, coding regions with large effect, are accompanied by closely linked modifier genes that regulate eco-physiological traits. The formation of these co-adapted gene complexes facilitates local adaptation. The genetic basis of colour polymorphism in *Oreina* has not been studied thus far. Four: the seemingly paradoxical colour polymorphism of chemically defended insects can be explained in the light of the first conclusion of chapter 2. The fact that it is unlikely that a single factor is solely responsible for the frequency of a particular colour morph explains the deviation from the expected monomorphism. **Chapter 3** deals with genetic structure within *Oreina speciosissima*. Two colour morphs seem to separate as a function of the plant community in which the beetles develop although non-adaptive processes such as genetic drift, founder events and population bottlenecks might also be responsible for the observed pattern. In order to solve this question fine scale studies following a population genomics approach [138], already advocated in chapter 2, and targeting populations with a patchy distribution of the two ecotypes could provide a powerful framework for detecting adaptive signatures associated with ecological speciation. In **chapter 4** we show that colour has a strong effect on survival in *Oreina gloriosa* beetles.

Introduced beetles that match the locally predominant beetle colour have a significantly higher survival than those that do not match the predominant colour. Learned avoidance by visual predators is therefore an important factor in the survival of *O. gloriosa* beetles (and probably is across the entire genus *Oreina*). Since experimental sites were dominated by *O. gloriosa* this experimental result represents an example of positive frequency-dependent selection on colour. This form of purifying selection is an excellent example of the many (sometimes opposing) selective forces that are at work and combine to form the dynamic equilibrium as predicted by Thompson [22,23] and known as the geographic mosaic of coevolution. **Chapter 5** describes the proximate causes of colour polymorphism. In this chapter we show that *Oreina* colour is structural and that minute changes in the thickness of electron lucent layers in the epicuticle are responsible for the differences in colour of *O. gloriosa*. Through this project, we have gained more insight in the function of colour and the selection pressures that influence it within the genus *Oreina*. Yet, we have far from a complete picture and did not even start to understand the relative importance of the individual selective forces. Although my time with *Oreina* is up I hope that this dissertation might inspire future students to continue working with this interesting genus. In the remainder of this **chapter 6**, I will provide a further outlook upon subjects I have briefly touched upon during my research project but that did, for various reasons, not develop into full-grown chapters. Still, I think they are worth further investigation. Suggestions for further research, already mentioned at the end of the chapters 2-5 will not be repeated here.

Outlook

Predation

Notwithstanding the combined efforts of the many predecessors who focussed their respective dissertations, master theses or post-doctoral research projects on the leaf beetle genus *Oreina* and populated the Evolutionary Entomology Laboratory at the University of Neuchâtel in the last thirty years, not much is known about predation, or the predators of *Oreina* beetles. Asking ourselves who these *Oreina* predators are might seem rather trivial and hardly interesting at first glance. However, one must realize that these beetles are thought to show aposematic colouration and additional chemical defence as an adaptation against predation. In order for both these assumptions to hold the beetles: a) must be predated; b) important predators must be able to receive the colourful signals bouncing off the beetle's back; c) at least one of the more influential predators should be able to associate colour with the chemicals and learn from its experience. Within this context, predation and predators become a pivotal factor that asks for clarification.

As stated above, only a limited number of records of *Oreina* predation exist. The only birds that have been observed to feed on Oreina are white-winged snowfinch (*Montifringilla nivalis*) [personal communication Laurent Juillerat] and dunnock (*Prunella modularis*) (fig 16c). The latter during a video experiment using tethered beetles. Damaged beetles (fig 16a), most likely escapees of avian predation, were found on a regular basis during field work. Accounts of predation by other arthropods include several spider silk covered beetles (fig 16b) and piles of *Oreina speciosissima* elytra inside nests of actively hunting wolf spiders at the stone run habitats described in chapter 3 and the *Oreina cacaliae* beetle (fig.16d) harassed by *Formica sp.* ants around La Fouly, Switzerland. Field experiments at Col du Petit-Saint-Bernard (FR) revealed harvestmen (*Mitopus morio*) as important predators of *Oreina elongata* larva [345]. A 2006 video surveillance at the same site by Gilles Aerni confirmed those results. Hoverfly larvae (*Syrphidae*) and sawflies (*Symphyta*) were also among observed predators [personal observation GA].

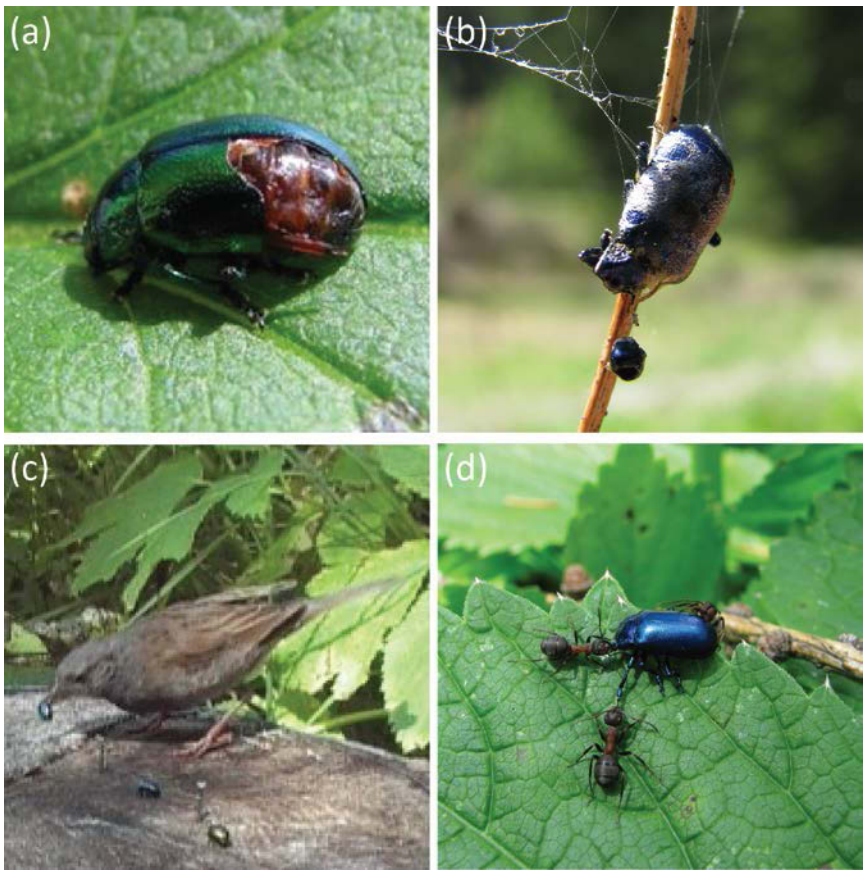

Figure 16. Examples of direct and indirect observations of predation. (a) *Oreina gloriosa* with a partially fractured left elytron. (b) *Oreina cacaliae* covered in spider silk. (c) Video still of dunnock (*Prunella modularis*) feeding on tethered *Oreina gloriosa* beetles. (d) *Oreina cacaliae* harassed by *Formica sp.* ants.

Predator-prey interactions are among the most important natural processes in shaping populations, bringing about evolutionary change and promoting adaptive radiation, yet the truth about these multitrophic interactions often remains obscured due to the inability to observe irregular predation events in the wild. Getting insight in the diet of large predators that feed every few days might be relatively easy but in many instances however, data concerning predator menu is very hard to obtain under field conditions. Predators might be small and secretive and either forage at night or under the cover of a closed canopy of vegetation. Any attempts to facilitate direct observations of predation by tethering potential prey, clearing vegetation or use of artificial light sources and camera's will result in the disturbance of the natural situation and hamper quantification of prey choice and predation frequency (reviewed by Symondson [346]). Reasons that lie at the base of the problems surrounding the quantitative and qualitative estimates of predation under field conditions also strongly influence the outcome of laboratory experiments that try to model predator prey interactions. To circumvent these problems, predator gut content analysis might offer a solution, but a less invasive and more elegant solution might be found in the microscopic examination of bird and mammal faeces, especially when investigating arthropod predators [347]. Since arthropods are likely to leave indigestible remains such as cuticle, head capsules and tarsi, information derived from these samples might be quantifiable. If however, one aims to not only uncover the prey spectrum of known predators but predator spectrum of known prey, things become somewhat more complicated. DNA extraction from faecal pellets, a technique often used in conservation research can offer a solution [348,349,350] and might be worth exploring.

Variation in predation

Under natural conditions defence levels of chemically defended prey animals are subject to variation in their dietary choices and those of their parents, the amount of (recent) narrow escapes and their physical condition. Hence, roles and ratios of models and mimics are subject to constant change. Extra dynamics are added by predators that pick and choose according to their physical state and the availability of undefended alternative prey. Srygley and Kingsolver [48] suggest that "*bird predators may demonstrate seasonal variation in their tolerance of distasteful species. Tolerance of an individual may depend on resource demands that vary seasonally or with breeding status*". They conclude that increased tolerance of distasteful prey is most probably linked to fledgling growth during the peak of the breeding season and the raised demand for food as a consequence of that. Whether distasteful butterflies used in their bioassays served as food items for fledglings or parents is not clear. Both Hileman, Brodie & Formanowicz [351] and

Srygley & Kinsolver [48] suggest that 'hunger level' is more important than experience as a predictor for acceptance or avoidance of defended prey. European starling (*Sturnus vulgaris*) seem to be able to weigh nutritional benefits against toxic effects of defended prey and make decisions according to its energetic needs [352]. Another example of 'indiscriminate predation' of chemically defended insects by avian predators comes from a study focussing on butterflies. The monarch butterfly (*Danaus plexipus*), undoubtedly one of the most famous chemically defended animals in the world, was observed to be killed in vast numbers by avian predators at its wintering site in Central Mexico [353]. Flocks of black-backed orioles (*Icterus abeillei*), and black-headed grosbeaks (*Pheucticus melanocephalus*) predated butterflies irrespective of the cardenolide concentration in their wings [353]. During the subsequent phase black-backed orioles ate significantly less from the more defended butterflies whereas consumption of the butterflies by black-headed grosbeaks proved unrelated with thoracic cardenolide content of the butterflies. As a result the authors state: *"Our finding that individual monarch butterflies containing high concentrations of cardenolides are not protected against two major bird predators raises questions about the part played by these compounds in the butterflies' defence"* [353]. At first glance this seemingly uncritical avian predation might be a bit worrisome for chemical defence concept as a whole. However, the black-headed grosbeaks are regarded as monarch-specialists [354] and black-backed orioles strip and eat only the soft parts of the monarchs that contain lower concentrations of cardenolides [353].24 wild caught black-headed grosbeaks all had monarch parts in their stomachswhile only 6-15 of the 35 black-backed orioles did. Cardenolide content per stomach ranged between 6 and 259µg (mean = 68µg) for black-headed grosbeaks and 0-15µg (mean=7µg) for black-backed orioles. The same study uncovered a few other bird species that hadn't been observed attacking monarchs in the field but did eat them while caged (no-choice experiment). Subsequent force-feeding of 146-157µg cardenolide containing capsules led to emesis in all species except black-headed grosbeak and eastern towhee (*Pipilio erythrophthalmus*). The question how the concepts of aposematism and mimicry are able to prove themselves, even in the light of this avian predation can be answered as follows. Alcock and Brower suggested that intra- and interspecific variation in a predator community can permit gradual evolution of mimicry when more conservative predators generalize among imperfect mimics thereby causing a selective advantage for (weak) mimetics [355,356]. More exploratory species or individual predators subsequently sample imperfect mimics and while doing so, select for the gradual improvement of mimicry. Ruxton, Sherratt and Speed [53] state: "*Complete avoidance is not therefore necessary for aposematism to work; aposematism merely has to provide lower mortality than crypsis for the warning signal to be*

beneficial". The monarch butterfly case clearly shows that 'unprofitability' as displayed by the aposematically coloured, and chemically defended insect is a relative concept. Specialists can deal with defended prey better than generalists. Chemically- or otherwise defended animals might thus be unprofitable for the unadapted generalist but acceptable food items for specialist predators. In certain cases food deprived predators, predators with hungry young, or predators in no-choice or minimal-choice situations (no palatable alternative prey available) will more readily accept otherwise unprofitable prey items.

A follow-up experiment of the research project described in chapter 4 suggested that the *Oreina* community might also be subject to seasonal variation in avian predation as a function of breeding status. I feel that our small sample size has prevented us from formulating definite conclusions. An experiment, possibly using our leash-system, and carried out before, during and after the avian breeding peak might provide insights in *Oreina* population dynamics and the influence of avian predation.

Predator vision

Reliable predictors that offer information about the profitability of a potential food item can aid optimal foraging and indirectly enhance a predator's fitness. Visual cues can offer such information. Skelhorn and Rowe [357] present data that suggests domestic chicks (*Gallus gallus domesticus*) are able to taste-reject dry, identically coloured, food items on the basis of differences in their quinine sulphate content. However, the fluorescent quinine sulphate, or more complete quinine sulphate dehydrate, is also used in standard reference solutions to calibrate spectrofluorometers [358]. Both in solution and in a crystallized state quinine sulphate dehydrate demonstrates these fluorescent qualities [359]. And thus, given the results of [360,361,362] it seems very likely that the experimental animals used by Skelhorn and Rowe and subject to *"14L: 10D cycle using fluorescent lights*" treatment [357], have been able to distinguish between moderately and highly defended starter crumbs by use of visual cues. In case the reasoning above is valid, theory based on hundreds of studies using avian predators feeding on quinine treated food items should be re-thought! Whereas the example above deals with visual cues to unprofitability under lab conditions Srygley and co-authors published a number of papers discussing visual cues that help to predict unprofitability under natural circumstances. They showed that behavioural and morphological differences can help to reliably classify butterflies either as 'palatable' or 'toxic/distasteful' [48,363,364]. According to Srygley & Kingsolver *"mass allocation to the thorax (FMR)* [TVN: Flight Muscle Ratio; thoracic mass/body

mass] *is greater in the more palatable species"* [48]. The allocation of resources towards the thorax should provide more power for acceleration and high speed flight [48]. Additionally they state that: "Fast erratic flight is common in more palatable species , enabling these butterflies to escape from predators" [364].

Although ideas presented above might seem somewhat farfetched given the general topic of this dissertation, they are exemplary for the complex nature of research focussing on predator-prey interactions. Due to the differences between human eyesight and that of avian, reptilian or insect predators these interactions are often invisible to us and definitely worth further investigation.

Acknowledgements

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Thanks to my committee members **Betty Benrey**, **Peter de Jong**, **Jeremy McNeil**, **Martine Rahier** and **Yvonne Willi** for their time and energy to read my work providing me with valuable comments and new insights (and above all for letting me pass).

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thrift shop passion with me. **Gaylord** and **Georg**, although you don't know each other you fall a bit into the same category. If I wouldn't know better I would lump you into one genus. Sociable, sportive, humoristic and irresponsible drinkers who are often absent (contradicting the sociable) in order to attend some far away congress or other unknown destination (and being sociable over there). There are some traits that differ considerably though: Georg's hair is more tameable but his bones are far less flexible than those of Gaylord 'MJ' Desurmont. Too bad we didn't see more of each other but I fully enjoyed the running and drinking events that we did share! **Elvira**, thanks for the kilos of Dutch candy you transported to Neuchâtel around Sinterklaas and Kerstmis through the years. We started together at the Jardin Botanique and I finished my dissertation with my private defence while staying at your place. I hope you will finish your project soon as well! **Matthias Erb**, thanks for all the weirdness, the great evenings at the Dutch consulate, shady places like Highlander, Le Faucon, Las Vegas Café, the co-organized movie nights at the UniMail, the treasure hunt, the luxury dinner at the Hotel Du Peyrou. It was great while it lasted. **Christophe** and **Jesse**, thanks for your hospitality. Thanks for the nice dinner evenings and the magical weekend in your cabin! **Jesse**, thanks for the nice talks we had and your help with my BMC paper. Thanks to all the Tuesday evening (indoor) soccer team mates: **Anca**, **Florian**, **Luc**, **Mickaël (2x)**, **Piero**, **Rob**, **Damien**, **Laura**, **Georg**, **Matthias Erb**, **Gaëtan Islam**, **Gaylord**, **Jerôme Kevin**, **Julien** (2x), **Helena**! I liked it a lot! Equally big thanks to my badminton playing colleagues **Gaylord**, **Johnattan**, **Jerôme**, **Matthias Erb**, **Kevin**, **Gaëtan** and **Greg**. Getting beaten time and again wasn't so much fun but the game itself, together with you, kept me coming back. **Georg**, **Matthias Erb** and **Islam**, CEOs of the ISKE, and all others that joined us one or more times, thanks for the many kebab/falafel *avec toutes* dinners. Unfortunately we never finished our tour around the town's finest fluorescent lamp-lid restaurants. **Jerôme**, after the very successful organisation of the annual PhD meeting, we drank lots of cups of coffee through the years and had wonderful discussions and Speedminton-evenings *au bord du lac* together! Thanks, I liked it a lot! I hope that you will, just like I just did, be able to finish your dissertation while having a full-time job on the side. Good luck, you'll be relieved! **Teresa**, **Albert** and **Luis**, thanks for the many nice days and evenings we spent together with activities ranging from hiking, cycling, playing board games, moving to St. Blaise, fighting weeds in your front yard, sledding, mineral shopping, having dinner together. Thanks to my Neuchâtel visitors: **Raoul** & **Marije**, **Marcel** & **Loes**, **Klaas**, **Franciska**, **Victor**, **Bas**, **Floor**, **Sanne**, **Jeroen**, **Gabry**, **Chris**, **Merijn**, **Reinout**, **Herman**, **Ties**, **Mara**, **Pieter**, **Truus**, **Judith**, **Morteza**, **Cees**, **Gonnie** & **Jan**, **Leny**, **Inge**, **Jeroen** and the children. It was fun sharing and showing part of my Swiss world with- and to you.

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Annexes

Annex I: Supplement to Acknowledgements

Table 3. List of bird species observed around UniMail, Neuchâtel between 12-01-2008 and 27-02-2012 (shameless copy of concept from [365])

No.	Species	Latin name	Record	Daily maximum	Total Number
1	Greylag Goose	*Anser anser*	1	4	4
2	Mallard	*Anas platyrhynchos*	17	5	37
3	Northern Shoveler	*Anas clypeata*	1	1	1
4	Great Cormorant	*Phalacrocorax carbo*	19	60	348
5	Grey Heron	*Ardea cinerea*	19	8	35
6	White Stork	*Ciconia ciconia*	3	7	9
7	European Honey Buzzard	*Pernis apivorus*	10	20	31
8	Black Kite	*Milvus migrans*	101	16	174
9	Red Kite	*Milvus milvus*	63	30	190
10	Griffon Vulture	*Gyps fulvus*	1	1	1
11	Western Marsh Harrier	*Circus aeruginosus*	5	1	5
12	Northern Harrier	*Circus cyaneus*	3	1	3
13	Northern Goshawk	*Accipiter gentilis*	4	1	4
14	Eurasian Sparrowhawk	*Accipiter nisus*	51	4	57
15	Common Buzzard	*Buteo buteo*	144	55	425
16	Booted Eagle	*Aquila pennata*	1	1	1
17	Osprey	*Pandion haliaetus*	1	1	1
18	Common Kestrel	*Falco tinnunculus*	17	2	18
19	Merlin	*Falco columbarius*	1	1	1
20	Eurasian Hobby	*Falco subbuteo*	16	2	19
21	Peregrine Falcon	*Falco peregrinus*	19	1	19
22	Common Crane	*Grus grus*	1	2	2
23	Common Black-headed Gull	*Larus ridibundus*	1	1	1
24	Yellow-legged Gull	*Larus michahellis*	156	16	352
25	Feral Pigeon	*Columba livia domestica*	10	2	16
26	Stock Dove	*Columba oenas*	5	19	50
27	Common Wood Pigeon	*Columba palumbus*	30	3665	19828
28	Eurasian Collared Dove	*Streptopelia decaocto*	104	3	145
29	European Turtle Dove	*Streptopelia turtur*	1	4	4
30	Common Swift	*Apus apus*	101	100	2423
31	Alpine Swift	*Apus melba*	1	2	2
32	European Green Woodpecker	*Picus viridis*	19	1	19
33	Black Woodpecker	*Dryocopus martius*	1	1	1
34	Great Spotted Woodpecker	*Dendrocopos major*	209	2	240
35	Middle Spotted Woodpecker	*Dendrocopos medius*	17	1	17
36	Lesser Spotted Woodpecker	*Dendrocopos minor*	2	1	2
37	Eurasian Skylark	*Alauda arvensis*	6	60	101
38	Sand Martin	*Riparia riparia*	6	15	35
39	Eurasian Crag Martin	*Ptyonoprogne rupestris*	1	1	1
40	Barn Swallow	*Hirundo rustica*	61	100	416
41	Common House Martin	*Delichon urbicum*	146	200	2709
42	Meadow Pipit	*Anthus pratensis*	3	50	94
43	Grey Wagtail	*Motacilla cinerea*	1	2	2
44	White Wagtail	*Motacilla alba*	29	7	48

45	Winter Wren	*Troglodytes troglodytes*	7	1	7
46	Dunnock	*Prunella modularis*	1	1	1
47	European Robin	*Erithacus rubecula*	103	3	109
48	Black Redstart	*Phoenicurus ochruros*	126	6	145
49	Common Redstart	*Phoenicurus phoenicurus*	6	1	6
50	Common Blackbird	*Turdus merula*	266	10	474
51	Fieldfare	*Turdus pilaris*	6	30	105
52	Song Thrush	*Turdus philomelos*	17	33	51
53	Redwing	*Turdus iliacus*	2	1	2
54	Mistle Thrush	*Turdus viscivorus*	8	5	14
55	Eurasian Blackcap	*Sylvia atricapilla*	71	3	82
56	Garden Warbler	*Sylvia borin*	1	1	1
57	Common Chiffchaff	*Phylloscopus collybita*	51	5	79
58	Willow Warbler	*Phylloscopus trochilus*	3	1	3
59	Goldcrest	*Regulus regulus*	9	3	15
60	Firecrest	*Regulus ignicapilla*	1	2	2
61	Spotted Flycatcher	*Muscicapa striata*	3	3	5
62	European Pied Flycatcher	*Ficedula hypoleuca*	6	1	6
63	Long-tailed Bushtit	*Aegithalos caudatus*	28	8	73
64	White-headed Long-tailed Tit	*Aegithalos caudatus caudatus*	2	1	2
65	Marsh Tit	*Parus palustris*	27	2	28
66	Willow Tit	*Parus montanus*	1	1	1
67	European Crested Tit	*Parus cristatus*	3	1	3
68	Coal Tit	*Periparus ater*	7	2	9
69	Blue Tit	*Parus caeruleus*	353	16	763
70	Great Tit	*Parus major*	274	10	538
71	Eurasian Nuthatch	*Sitta europaea*	188	3	213
72	Eurasian Treecreeper	*Certhia familiaris*	15	2	16
73	Short-toed Treecreeper	*Certhia brachydactyla*	26	2	28
74	Eurasian Jay	*Garrulus glandarius*	126	3	153
75	Eurasian Magpie	*Pica pica*	40	8	66
76	Western Jackdaw	*Corvus monedula*	4	55	110
77	Rook	*Corvus frugilegus*	46	21	102
78	Carrion Crow	*Corvus corone corone*	442	53	1559
79	Northern Raven	*Corvus corax*	32	3	46
80	Common Starling	*Sturnus vulgaris*	228	300	2302
81	House Sparrow	*Passer domesticus*	284	50	1574
82	Common Chaffinch	*Fringilla coelebs*	297	1000	4275
83	Brambling	*Fringilla montifringilla*	12	1000	1432
84	European Serin	*Serinus serinus*	1	5	5
85	European Greenfinch	*Carduelis chloris*	156	15	424
86	European Goldfinch	*Carduelis carduelis*	16	4	31
87	Eurasian Siskin	*Carduelis spinus*	56	25	315
88	Hawfinch	*Coccothraustes coccothraustes*	47	4	85
	TOTAL		**4806**	**7181**	**43151**

Annex II: Curriculum Vitae

Tom van Noort

Groen van Prinstererlaan 44[C]

3818 JR Amersfoort, The Netherlands
tvannoort@hotmail.com

0031 657535836

Education

2007-2013	**PhD Researcher Evolutionary Entomology**, University of Neuchâtel, Neuchâtel, Switzerland
2011	**Summerschool** Nature conservation, Swiss Plant Science Web, Ben Eighe, Scotland
2010	**Summerschool** World Food Crisis: how can plant science contribute? Swiss Plant Science Web, Mürren
2005	**MSc Animal Ecology/ Entomology**
2004	**Summerschool** Parasites, Pathogens and their Hosts; Ecology, Molecular Interactions and Evolution, University of Hohenheim, Germany
2004	**BSc Ecosystem Biology** Wageningen University, Wageningen, The Netherlands
2000	**BSc Aquatic Eco Technology** HZ University of Applied Sciences, Flushing (Vlissingen), The Netherlands
	MAVO, HAVO, VWOa

Work experience

2012-now	**Scientific collaborator Research, Development and Quality control** De Groene Vlieg BV, Dronten, The Netherlands
2007-2013	**PhD Researcher Evolutionary Entomology**, University of Neuchâtel, Neuchâtel, Switzerland
2007	**Field Analyst** European Groundwater Quality Monitoring Project, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
2005	**Junior researcher (INVITED)** Department of Entomology, University of California, Riverside, United States of America

Publications

Matthias Borer, Tom van Noort, Nils Arrigo, Sven Buerki, Nadir Alvarez, Does a shift in host plants trigger speciation in the Alpine leaf beetle Oreina speciosissima (Coleoptera, Chrysomelidae)? **BMC Evolutionary Biology 11:** 310 2011.

Matthias Borer, Tom van Noort, Martine Rahier, and Russell E. Naisbit, Positive Frequency dependent selection on warning color in Alpine leaf beetles. **Evolution**, Volume 64 (12) 2010, Pages 3629–3633.

Rugman-Jones, Paul F., Robert Wharton, Tom van Noort, Richard Stouthamer, *Molecular differentiation of the* Psyttalia concolor *(Szépligeti) species complex (Hymenoptera: Braconidae) associated with olive fly,* Bactrocera oleae *(Rossi) (Diptera: Tephritidae), in Africa***. Biological Control,** Volume 49 (1) 2009, Pages 17-26.

Fivat, Jean-Marc, Fabrice Ducloux, Georges Gilliéron, Tom van Noort, René Voisin & Bertrand Posse, *Un groupe exceptionnel de cigognes blanches* Ciconia ciconia *dans les Alpes.* **Nos Oiseaux**, Volume 56, 2009, Pages 31-35.

van Oosten, H.H., Beunen, R., van de Meulengraaf, B. & van Noort, T. An observation of a White-masked antbird *Pithys castaneus* and Orange-throated tanager *Wetmorethraupis sterrhopteron* at a new location in Amazonas, Peru. **Cotinga**, Volume 28, 2007, Pages 79-81.

Annex III: Education Certificate

UNIVERSITÉ DE NEUCHÂTEL
Institut de biologie

Interuniversity Doctoral Program in Organismal Biology

Certificate of Completion

From October 2007 to January 2012 **Tom van NOORT** obtained 14.0 credit points within the Doctoral Program (DP) and 10.5 credit points outside the Doctoral Program (EX) with the following activities:

Communication activities:

DP	How to "sell science" to good journals	December 2008	1.0
DP	How to make scientific presentations and posters interesting	May 2009	1.5
EX	Open doors, University of Neuchâtel (organization)	May 2009	0.5
DP	25th meeting of the International Society of Chemical Ecology, ISCE 2009, Neuchâtel, Switzerland (participation in the organization)	August 2009	1.0
EX	Biology10, Neuchâtel (organization)	February 2010	1.0
DP	Peer review and writing manuscripts	March-April 2010	2.5
EX	Faunistic excursions (teaching, birds expertise)	2009 and 2010	0.5
DP	Planning a career strategy - Part 2	November 2010	1.0

Research tools activities:

DP	Analyzing and attributing natural selection to particular sources	January 2009	1.5
DP	Scanning electronic microscopy	December 2009	1.0
EX	Field course "Conservation and development of the Beinn Eighe area", Scotland (U.K.)	July 2011	3.0

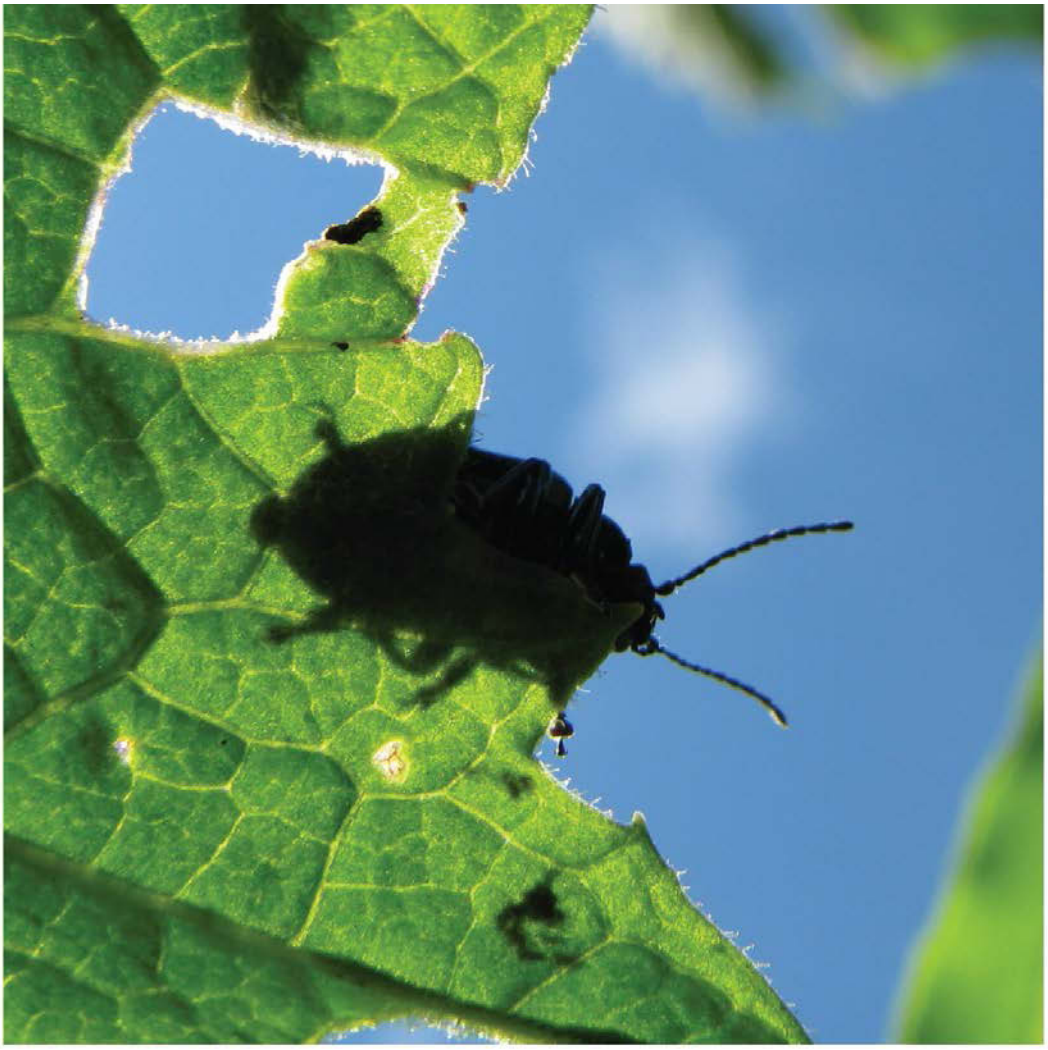
Scientific activities:

DP	Annual Ph.D. students meeting 2008 (attendance)	April 2008	0.5
EX	Biology09, Bern (poster)	February 2009	0.5
EX	International workshop in evolutionary biology, Guarda, Switzerland	June 2008	2.0
DP	Annual Ph.D. students meeting 2009 (poster)	April 2009	1.0
DP	Social evolution: from theory to data (and back again). Joint workshop with 3e cycle romand en sciences biologiques	June 2009	1.0
EX	21st meeting of the Dutch entomological society, Ede, The Netherlands (poster)	December 2009	0.5
EX	Biology10, Neuchâtel (poster)	February 2010	0.5
DP	Annual Ph.D. students meeting 2010 (organization of the meeting)	April 2010	1.0
EX	SPSW summer school: the global food crisis - how can plant sciences contribute? Mürren	June 2010	2.0
DP	Avoiding tragedies of the commons: an evolutionary approach to human cooperation	September 2010	1.0

Professor Ted Turlings
Director

Dr Christiane Bobillier
Coordinator

Neuchâtel, 15.5.2013 - Page 1/1



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