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Adaptation or chance?
Ecology and evolution of colour polymorphism in reptile species with contrasting biological traits (*Natrix natrix* and *Vipera berus*)

-SUMMARY OF THE DOCTORAL THESIS-

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CONTENT

Introduction

Coloration is a central topic in evolutionary biology, being associated since early studies of evolution with fundamental processes such as natural selection and adaptation. Today, the study of coloration has become a complex interdisciplinary field, with applications in biology, physics, chemistry, materials science, and medicine (Cuthill *et al.*, 2017). Animal colors may have a pigmentary origin (melanins, carotenoids, pteridines), structural origin (reflection and refraction of light by nanostructures), chemical origin, or may result from a combination of these mechanisms (Shawkey & D'Alba, 2017).

The functions of coloration are diverse, the most important being camouflage, intra- and interspecific communication, thermoregulation, and protection against ultraviolet radiation (Cott, 1940; Cuthill *et al.*, 2017). These functions may act simultaneously and vary in importance depending on the ecological context, season, sex, or life stage of the individual (Postema *et al.*, 2023). Thus, understanding the role of coloration requires considering a complex ecological and evolutionary system in which chromatic traits interact with a variety of selective factors.

In addition, intra-population chromatic variation has been correlated with differences in other morphological, physiological, and behavioral traits (Gray & McKinnon, 2007; Mckinnon & Pierotti, 2010), making animal coloration—and particularly color polymorphism—an excellent model for investigating multiple evolutionary mechanisms and theories (Endler & Mappes, 2017). Color polymorphism refers to the presence of several distinct color forms within the same population and is maintained over time by a balance between the advantages and disadvantages of each phenotype, depending on living conditions, predation pressure, or reproductive success (Roulin, 2004; Bond, 2007).

Melanism is the most common form of color polymorphism studied among vertebrates and invertebrates (Kettlewell, 1973; Clusella Trullas *et al.*, 2007). It is defined by the presence of individuals with dark coloration, caused by an increased amount of melanin in the tissues (True, 2003). In reptiles, especially among squamates, melanism is relatively frequent and has been documented in several families, including Colubridae, Lacertidae, and Viperidae (Davenport & Dellinger, 1995; Sahlean *et al.*, 2024). According to the thermal melanism hypothesis (Bogert, 1949), melanistic individuals may benefit from faster and more efficient body heating, which provides them with an advantage under low-temperature conditions,

through longer activity periods, higher growth rates, increased survival, and greater reproductive success (Clusella Trullas *et al.*, 2007). Other potential advantages include increased protection against UV radiation and greater immune resistance (Dubey & Roulin, 2014; Baeckens & Damme, 2018). Nevertheless, the presence of melanism may also be associated with costs, such as a higher risk of detection by predators in certain habitats or increased susceptibility to thermal stress under high-temperature conditions (Andrén & Nilson, 1981; Clusella Trullas *et al.*, 2007).

Considering the complexity and variability of melanism as an integral part of a polymorphic system, this thesis aimed to document and investigate the phenomenon in snakes, from both a general and an applied perspective.

Objectives

This doctoral thesis investigates the ecological and evolutionary significance of melanism in polymorphic terrestrial snakes, focusing on two representative species: *Vipera berus* (Linnaeus, 1758) and *Natrix natrix* (Linnaeus, 1758). Given that approximately 20–30% of snake species exhibit color polymorphism (Pizzatto & Dubey, 2012; Davis Rabosky *et al.*, 2016), and that melanism is frequently encountered, this study aims to make a significant contribution to the understanding of this phenomenon by doing:

1. A meta-analysis of the effects of melanism among polymorphic snake species, addressing its prevalence, sex differences, and potential advantages;
2. Analysis of the spatial distribution and climatic niche differentiation between melanistic and non-melanistic individuals of *Vipera berus*;
3. Investigation of the natural selection pressure exerted by predators on melanism frequency and evaluation of the aposematic role of coloration;
4. Determination of melanism frequency and ecological preferences of three color forms identified within a polymorphic population of *Natrix natrix*, as well as assessment of the influence of melanism on individual body size;
5. Investigation of the relationship between color form and reproductive success in a polymorphic population of *Natrix natrix*;
6. Documentation of nocturnal activity in *Natrix natrix*, with emphasis on differences among color forms in relation to weather conditions.

1. The current state of knowledge

1.1. Snake coloration

Snake coloration is a trait with remarkable intra- and interspecific variability. Most species display cryptic coloration with dull shades (brown, gray, green, black), adapted to the habitats in which they live, providing effective camouflage (Cox & Davis Rabosky, 2023). At the same time, some species exhibit combinations of bright, contrasting colors, serving as warning signals, such as red, yellow, or blue (Davis Rabosky *et al.*, 2016). Color patterns can be simple (differences between the head and body) or complex (bands, stripes, zigzags, patterns), and their diversity reflects both selective pressures and the phylogenetic history of the taxonomic group (Cox & Davis Rabosky, 2023).

Color polymorphism, defined as the presence of several distinct color forms within the same population, is found in approximately 20–30% of snake species (Pizzatto & Dubey, 2012; Davis Rabosky *et al.*, 2016). The existence of color polymorphism may lead to a higher probability of survival in the event of rapid environmental changes, as polymorphic species or populations can exploit a wider range of ecological niches (Castella *et al.*, 2013). Thus, different color forms may utilize diverse microhabitats, each achieving greater success within its respective habitat (Shine *et al.*, 2003).

A large part of the literature dedicated to color variation in snakes has focused on understanding the functions that coloration fulfills. Thus, coloration serves multiple functions, which can be grouped into three main categories (Cox & Davis Rabosky, 2023):

- Physiological function, particularly in thermoregulation, is well documented. Dark-colored individuals heat up more quickly, a significant advantage for reptiles (Gibson & Falls, 1979; Clusella Trullas *et al.*, 2007; Muri *et al.*, 2015). Moreover, dark coloration provides better protection against UV radiation (Goldenberg *et al.*, 2024) and may influence resistance to parasites or venom degradation (Pough *et al.*, 1978; Shine *et al.*, 1998).
- Informational function includes camouflage and aposematism. Camouflage aids both in avoiding predators and in capturing prey (Cox & Davis Rabosky, 2023). Color patterns such as zigzags or bands can induce optical effects like “flicker-fusion,” reducing predator efficiency (Adams *et al.*, 2019; Valkonen *et al.*, 2020). At the same time, aposematic colors warn predators of an individual’s toxicity, reducing predation rates (Brodie III, 1993; Pfennig *et al.*, 2001).

- Correlative function refers to the association of coloration with habitat or local climatic conditions, possibly reflecting selective adaptations at the regional level (Cox & Davis Rabosky, 2023).

1.2. Study organisms

1.2.1. Vipera berus

The common adder (*Vipera berus*) is a terrestrial snake species with a wide geographic distribution across Europe and Asia, adapted to a broad range of climatic conditions (Geniez, 2018). The color polymorphism of this species is represented by two main color morphs: the typical morph, with a dorsal zigzag pattern on a brown dorsal background (in females) or silvery-gray (in males), and the melanistic morph, which is entirely black (Geniez, 2018). Melanism has been documented across almost the entire species range and is influenced by ecological and evolutionary factors (Jansen *et al.*, 2024).

1.2.2. Natrix natrix

The grass snake (*Natrix natrix*) is a semi-aquatic, oviparous species with a wide distribution across Europe and Asia (Geniez, 2018; Asztalos *et al.*, 2021). The color polymorphism of this species is represented by three main color morphs: the typical morph, with an olive, brown, or gray dorsal coloration and black spots; the bilineata morph, with two light longitudinal stripes; and the melanistic morph (Geniez, 2018), which occurs sporadically across the species' range but is common in some areas (Bury *et al.*, 2020), such as the Danube Delta (Fănaru *et al.*, 2022).

2. Melanism in polymorphic terrestrial snakes

2.1. Background

Coloration plays a crucial role in fundamental aspects of animal biology (Endler & Mappes, 2017) and is subject to diverse selective pressures (Bond, 2007; Protas & Patel, 2008). Color polymorphism—the presence of multiple color phenotypes within a single population—constitutes a valuable model in ecological and evolutionary biology studies, reflecting adaptations to varying environmental conditions (Forsman *et al.*, 2008; McLean & Stuart-Fox, 2014). Among polymorphic morphs, melanism is the most frequent and conspicuous, often observed in snakes (Lorioux *et al.*, 2008).

Melanism has been explained by two ecogeographic hypotheses: Gloger's rule (Delhey, 2017), which predicts that melanism is more frequent in warm and humid regions, and the thermal melanism hypothesis (Bogert, 1949), which predicts higher frequency in cold regions.

The aim of this study is to synthesize the current literature on the role of melanism in color-polymorphic terrestrial snake species and to address four key questions: i) how widespread is melanism among these species, ii) which environmental variables can explain the proportion of melanistic individuals in populations, iii) whether the proportion of melanistic individuals differs between sexes, and iv) whether melanistic individuals have greater body size compared to standard-colored individuals.

2.2. Material and methods

For the systematic search, we followed the PRISMA guidelines (Page *et al.*, 2021), using the Google Scholar and Web of Science databases up to 30 June 2023. After removing duplicates and irrelevant or incomplete articles, 57 articles were retained for the meta-analyses. Meta-analyses included only species investigated in at least two studies, whereas meta-regressions included species present in a minimum of four studies.

Melanism frequency was expressed as log-transformed proportions (log-odds), and sex ratios as log odds ratios (lnOR). Differences in body size were analyzed using the log response ratio (lnRR), a unitless measure allowing comparison across studies with different scales. Positive lnRR values indicate larger body size in melanistic individuals compared to standard-colored individuals. Based on geographic coordinates, seven ecological variables

were extracted for meta-regression. Analyses were conducted following the recommendations detailed in Nakagawa *et al.* (2022, 2023). Specifically, we conducted:

- A meta-analysis of proportions to obtain the global mean prevalence of melanism in the analyzed polymorphic terrestrial snake species;
- A meta-regression to test variables that could explain variation in observed prevalence;
- A meta-regression of odds ratios to evaluate whether melanism is more likely to occur in one sex;
- Two meta-regressions of the log response ratio (ratio of means): the first to test whether melanistic individuals have larger body size compared to standard-colored individuals, and the second applied exclusively to the common adder (*Vipera berus*) to investigate whether melanistic individuals differ in size from those with the zigzag pattern.

2.3. Results

A review of the literature identified studies on 12 snake species exhibiting color polymorphism, in which one of the color morphs is melanistic. Among these, nine species reported data on the frequency of color morphs within studied populations: *Elaphe quadrivirgata*, *Heterodon platirhinos*, *Hierophis viridiflavus*, *Natrix natrix*, *Natrix tessellata*, *Thamnophis sirtalis*, *Vipera aspis*, *Vipera berus*, and *Vipera renardi*.

The mean prevalence of melanism across the analyzed species was 31% (95% CI: 9–68%), with no statistically significant interspecific differences (p = 0.06). Among climatic variables, annual precipitation (BIO 12) emerged as the most influential factor associated with melanism frequency, with a relative importance of 66%. Increasing annual precipitation was associated with a 4.5-fold higher probability of snakes exhibiting melanism (95% CI: 1.7–11.7). In contrast, the remaining climatic variables had weak, non-significant effects on melanism prevalence, with confidence intervals overlapping zero.

The odds ratio for the occurrence of melanism between sexes was low and not statistically significant, with confidence intervals overlapping zero. The number of studies from which body size data could be extracted was relatively small (N = 13), most of which (N = 8) involved *Vipera berus*, while the remaining five were distributed among three other species (*Natrix natrix*, *Hierophis viridiflavus*, and *Elaphe quadrivirgata*). Results indicated no

significant differences in body size between melanistic and typical-colored individuals [F(df1 = 5, df2 = 1) = 3.580, p = 0.38].

In the meta-analysis conducted exclusively on the common adder (*V. berus*), the largest number of effect sizes was obtained (k = 17) from eight studies. Similarly, no significant differences in body size were observed between melanistic and zigzag-patterned individuals, although melanistic snakes were on average 4% larger (not statistically significant).

2.4. Discussion

A considerable number of studies have investigated the role of melanism in polymorphic snake species; however, surprisingly few syntheses have addressed this topic in reptiles more broadly (Clusella Trullas *et al.*, 2007; Olsson *et al.*, 2013), and to date, no quantitative analyses have been conducted.

Examination of the literature revealed a concentration of studies on a limited number of species and geographic regions in the Northern Hemisphere. The precise cause of this bias is difficult to determine. Possible explanations include the relatively small land area of the Southern Hemisphere and the limited occurrence of cold regions, which may reduce the prevalence of melanism in terrestrial snakes in these areas. The total number of polymorphic snake species exhibiting melanism remains unknown. Data were obtained for 12 species, but only six had sufficient information for a qualitative synthesis.

The results indicate that the mean prevalence of melanism in polymorphic terrestrial snakes is 31%, with considerable variation between 9% and 68%. Although independent studies on *V. berus* (Forsman, 1995) and *T. sirtalis* (Lawson & King, 1996; Bittner & King, 2003) concluded that melanistic proportions are maintained by selective processes, the meta-analysis did not detect significant interspecific differences. This suggests that melanism is not solely the result of selective or non-selective forces (e.g., random genetic drift).

The most notable finding of this study is the significant positive influence of annual precipitation on melanism frequency in polymorphic terrestrial snakes. This suggests that, for the species analyzed here, melanism may be explained by Gloger's rule (Delhey, 2017), contrary to the prevailing view that reptile melanism is primarily driven by Bogert's rule (Clusella Trullas *et al.*, 2007). Several studies have documented positive correlations between high humidity and increased melanism in snakes. For instance, the probability of melanistic individuals was higher in regions with abundant precipitation in *Pantherophis obsoletus* (Hantak *et al.*, 2022), and a similar pattern was observed in *Hierophis viridiflavus*

(Goldenberg *et al.*, 2024). Martínez-Freiría *et al.* (2009) also reported a positive relationship between the number of dorsal markings in two viper species (*Vipera aspis* and *V. latastei*) and precipitation levels, with more markings observed in northern regions characterized by high rainfall. However, numerous other studies have highlighted complex relationships between climatic factors and melanism prevalence. Therefore, the results of the present study should be interpreted with caution and viewed as broad-scale patterns reflecting the number of species and the available usable data.

The thermal melanism hypothesis predicts that melanistic individuals have an advantage over typical-colored individuals, potentially leading to higher melanism frequency in females, which achieve larger body sizes that allow higher reproductive frequency and more offspring (Andrén & Nilson, 1981; Luiselli, 1992). Conversely, higher melanism in males could increase reproductive success through larger body size and better physical condition (Luiselli, 1993). However, we found no support for either prediction; our results are consistent with other studies showing no significant sex differences in melanism frequency (Forsman & Ås, 1987; Zuffi, 2008; Strugariu & Zamfirescu, 2011).

Melanism also did not have a significant effect on body size, consistent with previous reports (Forsman & Ås, 1987; Gibson & Falls, 1988; King, 1988; Strugariu & Zamfirescu, 2011). Additional analyses restricted to *V. berus* likewise revealed no significant differences. Nonetheless, conclusions regarding the relationship between melanism and body size should be interpreted with caution due to the limited number of studies available.

3. Color polymorphism in the common adder (*Vipera berus*): spatial segregation and climatic niche differences

3.1. Background

Color polymorphism, characterized by the coexistence of multiple color morphs within a species, is a central topic in ecological and evolutionary studies (Gray & McKinnon, 2007). The distribution and frequency of morphs can vary geographically, sometimes following climatic gradients, suggesting adaptations to different ecological niches. Two ecogeographic rules may explain such variation: Gloger's rule (Delhey, 2017), which predicts darker morphs in humid regions, and Bogert's rule (Bogert, 1949), which predicts higher melanism frequency in colder regions as a thermal adaptation.

Vipera berus, a widely distributed species with two main color morphs (zigzag and melanistic), represents an excellent model for testing geographic variation in polymorphism. Previous studies have documented differences in frequency, morphology, and ecology between these morphs, but these studies were conducted over restricted geographic areas (Andrén & Nilson, 1981; Monney *et al.*, 1995). Although melanism is generally associated with cold climates according to Bogert's rule (Jansen *et al.*, 2024), exceptions exist, suggesting the influence of local factors and complex adaptations.

The working hypothesis of this study is that the color morphs of *V. berus* (zigzag and melanic) exhibit non-random spatial variation across their geographic range, occupying distinct climatic niches. To test this hypothesis, presence data for each morph were obtained from open-science databases to determine the geographic context of color polymorphism in *V. berus*. Specifically, we tested for geographic segregation of color morphs and evaluated the equivalency and similarity of their climatic niches to highlight differences and identify climatic factors associated with these variations.

3.2. Material and methods

The initial database consisted of *V. berus* observations available from the Global Biodiversity Information Facility (GBIF) that included at least one attached photograph. Each observation was classified as either melanistic or zigzag, and records with aberrant phenotypes were removed. Observations of non-adult individuals, duplicates, and records with high geographic uncertainty were also excluded. The final database included 1,512 observations, of which 1,142 (75.5%) were zigzag vipers and 370 (24.5%) were melanistic vipers.

Environmental data were downloaded from two climatic databases: WorldClim v.2 (Fick & Hijmans, 2017) and ENVIREM (Title & Bemmels, 2018). The environmental variables used in the statistical analyses were: mean annual temperature – BIO1 (°C), mean diurnal range – BIO2 (°C), isothermality – BIO3 (°C), annual precipitation – BIO12 (mm), precipitation seasonality – BIO15 (mm), potential annual evapotranspiration – PET (mm/year), Thornthwaite aridity index – Ar, and mean solar radiation – SR (kJ/m²/day).

An exploratory spatial analysis was performed to study the geographic segregation of the two color morphs, using the Point Pattern Process (PPP) method proposed by Farquhar *et al.* (2023). Observations of the zigzag and melanistic morphs were treated as points in a two-dimensional space, and the study area was defined as a 200 km circular buffer around all observations. This area was divided into 5×5 km grid cells, and the randomness of the observation distributions was tested using a chi-squared test.

Intensity maps of each morph were produced using a Gaussian smoothing process with a 200 km radius. These maps highlighted the geographic segregation of the two color morphs, and a Kolmogorov-Smirnov test was applied to confirm the covariation of their distributions.

Climatic niche similarities and differences between the two morphs were analyzed using both univariate and multivariate methods. For univariate analyses, density profiles of each environmental variable were calculated using kernel density estimates based on the environmental values at the geographic coordinates of the observations. Multivariate analyses included: COUE analysis (Guisan *et al.*, 2014), which examines centroid shift, overlap, unfilling, and expansion, and *n*-dimensional hypervolume analysis (Blonder *et al.*, 2014, 2018). Additionally, measures of niche overlap, equivalency, and similarity were calculated to quantify the degree of niche differentiation between morphs.

3.3. Results

The geographic distributions of melanistic and zigzag vipers largely overlap across most of the species' range. With the exception of the easternmost regions, the melanistic morph occurs throughout the entire range, exhibiting variable frequencies in different populations. Most observations of the melanistic vipers are concentrated in Ukraine, southwestern Russia, and the Alpine region. However, in the Alps, zigzag vipers predominate over melanistic ones. In contrast, zigzag vipers occupy extensive portions of the western part of the range, particularly in the United Kingdom, Denmark, the Netherlands, and the Scandinavian Peninsula.

The chi-squared test conducted as part of the PPP analysis indicated that the two color morphs are spatially clustered, with non-homogeneous distributions ($\chi^2 = 2056.2$, df = 40, $p < 0.001$). Based on visual inspection of the panels resulting from the test for spatial independence and randomness, the null hypothesis (H_0) was rejected, showing that the distributions of the two morphs are neither independent nor identical. Furthermore, visual inspection of the generated maps confirmed significant geographic segregation between the two morphs, which was supported by the results of the Kolmogorov-Smirnov test ($p < 0.001$).

Comparisons between the available climatic conditions and those actually used by the two color morphs revealed that the climatic niche of the melanistic morph is largely nested within the conditions available to the zigzag morph. Within the two-dimensional space defined by the PCA analysis, the climatic niche of the melanistic morph is nearly a subset of the zigzag morph's niche, with almost complete overlap. This is further supported by the niche decomposition results: stability at 99.34%, low unfilling at 2.23%, and virtually no expansion at 0.66%.

The climatic niches of the two morphs exhibit a moderate-to-high degree of overlap ($D = 0.69$, $I = 0.88$). Niche tests revealed that the climatic niches of melanistic and zigzag vipers are not equivalent ($D - p = 1$; $I - p = 0.988$). Nevertheless, the niches of the two morphs are more similar than would be expected by chance ($p < 0.05$).

Examination of the niche hypervolumes in multidimensional climatic space yielded results consistent with the COUE analysis. The melanistic vipers' climatic niche partially overlaps with that of the zigzag vipers (68.98%), and the degree of similarity is moderate-to-high, with a Jaccard index of 0.61 and a Sørensen index of 0.76. The unique fraction of the melanistic niche (13.54%) corresponds to drier areas with relatively stable day–night temperatures and lower precipitation, whereas the unique fraction of the zigzag niche (34.55%) occurs in warmer and wetter regions.

3.4. Discussion

For many polymorphic species, the geographic distribution and frequency of phenotypes remain poorly documented, even though polymorphism can play a key role in the process of speciation (McLean & Stuart-Fox, 2014). Our results indicate that zigzag and melanistic vipers are largely sympatric across much of the species' range but exhibit geographic segregation, with distinct areas where one morph predominates, as revealed by the Point

Pattern Process (PPP) analysis. Previous research has demonstrated the major influence of climate on color variation in ectothermic species (McLean & Stuart-Fox, 2014; Moore *et al.*, 2019; Farquhar *et al.*, 2023; Nicola *et al.*, 2024).

Univariate and multivariate analyses (i.e., COUE and *n*-dimensional hypervolume) revealed differences in the realized climatic niches of the two morphs. Equivalency and similarity tests further indicated that while melanistic and zigzag vipers occupy broadly similar climatic niches, these niches are distinct. Specifically, melanistic vipers tend to inhabit cooler and more arid areas, characterized by lower precipitation and higher mean diurnal temperature ranges. These findings support our initial hypothesis that the distribution of the two color morphs is non-random and shaped by climatic factors. They are consistent with a body of research demonstrating the direct influence of climatic variables, such as temperature and precipitation, on color variation in ectotherms (McLean *et al.*, 2015; Hantak *et al.*, 2022; Farquhar *et al.*, 2023), and particularly in viper species (Jansen *et al.*, 2024; Nicola *et al.*, 2024).

Local-scale evolutionary processes may explain variation in color morphs within *V. berus*. Each population experiences highly variable levels of factors critical for the expression of a given morph, including climate, prey availability, and predation pressure (McLean & Stuart-Fox, 2014; Jansen *et al.*, 2024). Among these factors, climate is undoubtedly the most influential, as it can directly shape the foundational structure of an ecosystem (Goldenberg *et al.*, 2024). Because color morphs are favored under conditions that best match their ecological requirements, climate acts as a primary selective force driving the geographic variation of color polymorphism. Color variation associated with local climatic conditions may thus reflect adaptive responses of populations, highlighting distinct evolutionary strategies within a species (Goldenberg *et al.*, 2024).

4. Testing predation rates on the common adder's color morphs using plasticine models

4.1. Background

Animal coloration serves essential functions, including protection against predators (Cott, 1940). Protective coloration can be broadly divided into two non-exclusive main categories: warning coloration and cryptic coloration or camouflage (Endler & Mappes, 2017; Postema *et al.*, 2023).

In the case of the common adder (*Vipera berus*), the dorsal zigzag pattern may serve both as cryptic protection (camouflage) (Andrén & Nilson, 1981) and as a warning signal (aposematism) (Wüster *et al.*, 2004). Melanistic vipers, with their darker coloration, are more frequent in colder regions and enjoy advantages such as higher growth rates and longer activity periods, but they may be more exposed to predation due to the loss of the zigzag's cryptic and aposematic function (Jansen *et al.*, 2024).

In recent years, experiments using plasticine/clay models have become standard in studies on aposematic coloration in snakes (Brodie III, 1993), and have been widely used to study the role of the zigzag pattern in European vipers (Wüster *et al.*, 2004; Niskanen & Mappes, 2005; Valkonen *et al.*, 2011).

Following this approach, we conducted experiments using plasticine models representing the two color morphs (zigzag and melanistic) to quantify predation attempts. The objectives of these experiments were to test the following hypotheses: (i) the high frequency of melanistic individuals is maintained by lower predation pressure on this color morph; (ii) the zigzag pattern maintains its aposematic function even in areas where only melanistic vipers occur; and (iii) in populations composed exclusively of melanistic vipers, this coloration functions as an aposematic signal.

4.2. Material and methods

We conducted experiments using non-toxic plasticine models (Fimo Soft, Staedler) replicating the size of an adult *Vipera berus* in two color morphs: melanistic (black) and the characteristic zigzag pattern (light gray). The models were deployed in two study sites—Bârnova Forest (polymorph populations) and Gura Crivățului (melanistic-only populations)—on both natural substrates and white cardboard to eliminate the potential cryptic effect of the zigzag pattern.

Experiments were carried out between 2022 and 2023, with models placed randomly along transects. Each model was anchored to prevent displacement and inspected periodically for predator attack marks. Predation attempts were identified based on traces left by predators, including birds, carnivorous mammals, and rodents. Data were analyzed using chi-square tests to assess differences in attack rates between color morphs and substrates.

4.3. Results

After inspecting all models (n = 596), a total of 58 potential predation attempts were recorded, attributed to birds (n = 31) and carnivorous mammals (n = 27). The overall predation rate, combining attacks from both birds and mammals, was 9.73%. Models deployed in the melanistic population in Prahova experienced a significantly higher predation rate compared to those in Iași (*z*-test for proportions: $z = 2.114$, $p = 0.035$).

No significant differences were observed in predation rates between the two background types (white background vs. natural substrate) ($\chi^2 = 0.401$, df = 1, $p = 0.526$). Similarly, there were no significant differences in attacks when considering the interaction between model type and background ($\chi^2 = 1.83$, df = 3, $p = 0.608$).

Focusing on bird attacks, the overall predation rate was 5.2%. In Iași, 12 of the models were attacked by birds (4.56%), while in Prahova, 19 attacks were recorded (5.71%). A *z*-test for proportions indicated no significant difference between the two populations ($z = 0.624$, $p = 0.533$). No significant differences were observed in bird attacks between background types ($\chi^2 = 0.016$, df = 1, $p = 0.899$), nor for the interaction between model and background ($\chi^2 = 4.09$, df = 3, $p = 0.252$). Across all experiments, birds attacked melanic models more frequently ($\chi^2 = 4$, df = 1, $p = 0.045$).

4.4. Discussion

The overall predation rate, as well as the rate of attacks exclusively by birds, was similar to those reported in other studies (Wüster *et al.*, 2004; Valkonen *et al.*, 2011). In our study areas, models in Prahova experienced higher predation than those in Iași, primarily due to more frequent attacks by mammals, including badgers. Bird attacks were more common in spring, when avian predators were more active.

Melanistic models were attacked more frequently by birds than zigzag models, contradicting the hypothesis that melanism is maintained through reduced visual predation pressure. Although some studies suggest that the zigzag pattern provides predator

avoidance via crypsis (Andrén & Nilson, 1981), our results support the hypothesis that differences in attack rates between the two color morphs are more likely due to predators avoiding the zigzag pattern (Wüster *et al.*, 2004; Valkonen *et al.*, 2011), further supported by the absence of significant differences in attacks on models placed on a contrasting background versus natural substrate.

In exclusively melanistic populations, no significant differences in attack frequency were observed between the two color morphs, which does not support either the hypothesis that the zigzag maintains its aposematic function or that melanism becomes aposematic in these populations. However, melanistic models were attacked by birds twice as often as zigzag models (13 versus 6 attacks), suggesting that the zigzag pattern retains its aposematic function even in populations where the melanistic morph is exclusive. Although differences were not statistically significant in the polymorphic population, the *p*-value was close to the 0.05 threshold, indicating a possible trend.

5. Activity patterns and body size differences between color morphs in a polymorph population of *Natrix natrix*

5.1. Background

In snakes, melanism is relatively common and has been studied across various species, with most research focusing on viviparous species (Sahlean *et al.*, 2024). In oviparous snakes, melanism appears to have a negative effect on body size, though this phenomenon is not yet fully understood (Zuffi, 2008; Bury *et al.*, 2020). In *Natrix natrix*, a species exhibiting pronounced color polymorphism, melanism occurs sporadically, and in some areas, such as the Danube Delta Biosphere Reserve, all color morphs can coexist within a single population (Fănaru *et al.*, 2022). Although melanism may increase individual vulnerability to predation, this phenotype persists, suggesting that its benefits outweigh the associated risks (Madsen, 1987a).

This study investigated a polymorphic population of *Natrix natrix* in southeastern Romania, aiming to determine the frequency of melanism, the environmental preferences of the different color morphs, and the effects of melanism on the body size of individuals.

5.2. Material and methods

The investigations were carried out in the locality of Sfântu Gheorghe, within the Danube Delta Biosphere Reserve, an area characterized by saline sandy soils and sub-Mediterranean influences. The total study area exceeded 100 ha, but research focused on approximately 20 ha, with wetlands and canals serving as preferred habitats for *Natrix natrix*. Ecological and biological variables were recorded, including geographic coordinates, cloud cover, sex and age of individuals, color morph, substrate type, and observed behavior. Environmental data included ground temperature and relative humidity, while for captured individuals, body length and mass were measured.

To analyze differences between color morphs and ecological and biological variables, chi-square tests (χ^2) were applied. Morph preferences in relation to the environment were assessed using Multiple Correspondence Analysis (MCA). Furthermore, to evaluate the effects of color morph and sex on body size and weight, linear mixed models were used, with year of data collection included as a random variable. Additional statistical analyses were performed using ANOVA and the Kruskal-Wallis test for supplementary comparisons.

5.3. Results

A total of 148 individuals were recorded during the field surveys. Of these, 16 were classified as immature and excluded from the analyses, leaving 132 considered adults. Regarding the color morphs of the adult individuals, 81 displayed the classic morph, 24 the bilineata morph, and 27 were melanistic. Chi-square tests revealed no significant differences between color morphs and the environmental variables analyzed.

The results of the Multiple Correspondence Analysis (MCA), performed with sexes pooled, confirmed the conclusions of the chi-square tests. The three color morphs were positioned close to the zero values of the main correspondence axes, suggesting no significant differences in their preferences for the environmental variables analyzed. The MCA results that included sex separation followed the same general trend, suggesting no clear preferences for specific microhabitats. Nonetheless, females of the bilineata morph were predominantly observed in habitats with low vegetation, sparse cover, and low to moderate temperatures. In contrast, males of the classic morph showed a certain preference for microhabitats with taller vegetation, higher temperatures, and moderate relative humidity, often being observed fully exposed under these conditions.

A total of 87 grass snakes were captured during the study. After filtering the data, 79 observations were included in the linear mixed regression analysis for body length (SVL) and 78 for body mass (BM).

The mean body length of bilineata individuals (n = 13) was 658.46 ± 100.78 mm, that of classic morph individuals (n = 54) was 631.69 ± 90.01 mm, and that of melanistic individuals (n = 11) was 632.55 ± 104.13 mm. When sexes were pooled, differences were not statistically significant ($F(2,74.73) = 0.09$, $p = 0.918$).

SVL did not differ between morphs in males, according to the Kruskal-Wallis test ($H(2) = 0.51$, $p = 0.774$). Same pattern was observed in females ANOVA ($F(2,9.7) = 1.19$, $p = 0.344$). The linear mixed model applied SVL revealed a significant effect of sex ($F(1,59.77) = 90.46$, $p < 0.05$) on SVL, while color morph ($F(2,71.97) = 0.18$, $p = 0.84$) and the interaction between morph and sex ($F(2,72.38) = 0.94$, $p = 0.394$) showed no statistically significant effects. On average, females were about 20% longer than males, regardless of color morph.

The mean body mass of bilineata individuals (n = 13) was 89.45 ± 39.09 g, that of classic morph individuals (n = 53) was 80.1 ± 38.34 g, and that of melanistic individuals (n = 11)

was 77.35 ± 37.9 g. Here too, no statistically significant differences were recorded between color morphs ($F(2,74.26) = 0.17$, $p = 0.846$).

Body mass did not differ between morphs in males, according to ANOVA ($F(2,10.12) = 0.16$, $p = 0.852$). Again, no statistically significant differences between groups were recorded in females, according to ANOVA ($F(2,10.09) = 0.17$, $p = 0.847$). The linear mixed model revealed a significant effect of sex ($F(1,63.27) = 85.32$, $p < 0.05$) on BM, while color morph ($F(2,71.38) = 0.91$, $p = 0.91$) and the interaction between morph and sex ($F(2,71.3) = 0.21$, $p = 0.81$) showed no statistically significant effects. On average, females were about 120% heavier than males, regardless of color morph.

5.4. Discussion

The results of this study suggest that the color morphs of *Natrix natrix* do not significantly influence habitat selection, with most individuals being observed in microhabitats characterized by low vegetation, moderate temperatures, and high humidity. This indicates that melanism does not appear to provide an ecological advantage within this population. Although melanism has been evolutionarily associated with thermoregulatory benefits in cold environments (Clusella Trullas *et al.*, 2007), the warm and humid conditions of the Danube Delta may offset these potential benefits. Instead, the presence of melanism may be linked to Gloger's rule, which predicts that darker forms are more common in warm and humid climates (Delhey, 2017), a pattern also confirmed in snakes from the Northern Hemisphere (Sahlean *et al.*, 2024).

With regard to body size, no significant differences were detected among the color morphs, although individuals with the classic morph tended to be larger than melanistic ones. This finding contradicts the hypothesis that melanism facilitates more efficient thermoregulation and larger body sizes (Andrén & Nilson, 1981; Clusella Trullas *et al.*, 2007). Instead, sex appears to be the key factor influencing body size: females are considerably larger than males (Geniez, 2018), likely reflecting their increased reproductive capacity..

Although melanistic individuals were not larger, the persistence of this trait suggests that it may provide other benefits, such as protection against UV radiation or enhanced resistance to infections (Baeckens & Damme, 2018; Goldenberg *et al.*, 2024). Future research should further investigate the impact of predation and include analyses of a broader range of biological traits to better understand the role of melanism in *Natrix natrix* populations.

6. The effects of coloration on reproductive success in *Natrix natrix*

6.1. Background

Color polymorphism, present in approximately 20–30% of snake species (Pizzatto & Dubey, 2012; Davis Rabosky *et al.*, 2016), may confer evolutionary advantages by allowing species to exploit diverse ecological niches (Forsman *et al.*, 2008). Some polymorphic species, such as *Vipera berus*, include melanistic morphs, yet the role and drivers of melanism remain subjects of debate (Jansen *et al.*, 2024; Sahlean *et al.*, 2024). Melanism can provide thermoregulatory benefits, enabling snakes to extend their activity periods, grow more rapidly, and reach larger body sizes, which in turn may lead to higher reproductive success (Luiselli, 1992). However, this advantage is not universal, as studies on other species, such as *Elaphe quadrivirgata* and *Thamnophis sirtalis*, have not found consistent relationships (Gibson & Falls, 1988; King, 1988; Tanaka, 2009; Tanaka & Mori, 2010).

In *Natrix natrix*, a polymorphic species with a wide distribution, three color morphs have been identified: classic, bilineata, and melanistic (Geniez, 2018). Although color polymorphism in this species has received relatively little attention, existing research has investigated body size and the frequency of melanistic individuals in relation to sex (Bury *et al.*, 2020; Fănaru *et al.*, 2022). To date, however, no studies have examined the effects of color morphs on reproductive success. Thus, the aim of the present research is to investigate differences in reproductive success among the three color morphs in a population from the Danube Delta.

6.2. Material and methods

Field investigations were conducted in the study area at Sfântu Gheorghe, Tulcea, where female *Natrix natrix* were captured in June of 2023 and 2024. Each female was examined for reproductive status, and gravid individuals were transported to the laboratory, where they were maintained in captivity until oviposition. In the laboratory, females were provided with appropriate conditions for egg laying, and each egg was weighed and placed in an incubator for a separate study. After oviposition, the females were released at their original capture sites.

To compare color morphs in terms of clutch size and egg mass, one-way ANOVAs were performed. In addition, Pearson correlation tests and analyses of covariance (ANCOVA)

were applied to evaluate the relationship between female body length, color morph, clutch size, and egg mass.

6.3. Results

Over the two study years, 28 gravid females of *Natrix natrix* were captured, 15 in 2023 and 13 in 2024. Of these, 16 exhibited the classic color morph, 7 the bilineata morph, and 5 were melanistic. In total, the females laid 410 eggs. Oviposition occurred in both reproductive seasons, from late June to early August, with the majority of clutches deposited in July.

Classic morph females laid on average 14.81 ± 4.15 eggs, with the most prolific female producing 22 eggs. Bilineata females laid on average 15 ± 2.89 eggs, while melanistic females laid 13.6 ± 3.58 eggs. A parametric ANOVA indicated no significant differences among color morphs in clutch size ($F(2,25) = 0.24$, $p = 0.791$).

With respect to clutch mass, one clutch from a melanistic female was excluded from analysis, as half of the eggs were non-viable. After exclusion, the mean clutch mass was 64.47 ± 19.68 g for classic morph females, 74.06 ± 11.98 g for bilineata females, and 64.04 ± 17.08 g for melanistic females. ANOVA revealed no significant differences among color morphs in clutch mass either ($F(2,8.28) = 1.13$, $p = 0.369$).

Clutch size showed a significant positive correlation with female body length when all morphs were analyzed together ($r = 0.64$, ANOVA: mean squares = 149.77, $F(1,25) = 17.75$, $p < 0.001$). Separate analyses by morph revealed a strong and significant correlation between body length and clutch size only in classic morph females ($r = 0.85$, ANOVA: mean squares = 182.63, $F(1,13) = 33.58$, $p < 0.001$). ANCOVA revealed a significant interaction between body length and color morph ($p = 0.042$), indicating that classic morph females exhibit a steeper increase in clutch size with body length compared to bilineata females. No significant differences were found between regression slopes of melanistic and bilineata females ($p = 0.867$).

Clutch mass also showed a significant positive correlation with female body length when all morphs were analyzed together ($r = 0.71$, ANOVA: mean squares = 163.06, $F(1,24) = 22.23$, $p < 0.001$). Separate analyses indicated a strong and significant correlation only in classic morph females ($r = 0.78$, ANOVA: mean squares = 3490.2, $F(1,13) = 20.43$, $p < 0.001$). ANCOVA revealed a significant interaction between SVL and color morph ($p = 0.032$), suggesting that classic morph females display a stronger increase in clutch mass with body

length compared to bilineata females. No significant differences were detected between the regression slopes of melanistic and bilineata females ($p = 0.447$)

6.4. Discussion

The study of reproductive traits across color morphs of the grass snake (*Natrix natrix*) revealed that coloration does not significantly influence clutch size or clutch mass, consistent with findings in other oviparous species, such as *Elaphe quadrivirgata* (Tanaka & Mori, 2010). Nonetheless, melanistic females produced, on average, the fewest eggs and had lighter clutches compared to classic morph females. Bilineata females laid a similar number of eggs as the classic morph, but their clutches were heavier.

Body size significantly influenced reproductive traits, but only in classic morph females, where larger individuals were associated with higher reproductive output. Although no differences in body size among color morphs were observed in the Danube Delta population, studies from Poland reported that melanistic females are smaller and that melanism is more common among males (Bury *et al.*, 2020), suggesting a potential advantage of melanism in males.

Larger female size is typically associated with greater reproductive success but also with a higher risk of predation (Madsen, 1987b). Melanism may provide a thermoregulatory advantage by facilitating faster development, yet the loss of aposematic coloration could increase vulnerability to predators, which may help explain the low frequency of large melanistic females in the population (Andrén & Nilson, 1981; Madsen, 1987a).

7. Nocturnal activity in *Natrix natrix*: geographic variation, climatic drivers and differences among color morphs

7.1. Background

Activity patterns in animals are species-specific and are generally classified as diurnal, crepuscular, or nocturnal (Abom *et al.*, 2012). Nocturnal activity is widespread and shaped by factors such as competition, predation, and temperature, particularly in ectotherms (Levy *et al.*, 2019).

Snakes exhibit diverse activity patterns, driven by thermoregulatory needs and environmental conditions. In arid or tropical regions, nocturnal activity can help individuals avoid extreme daytime heat and reduce water loss (Signore *et al.*, 2022). Some species also show seasonal shifts in activity patterns (Abom *et al.*, 2012).

In Europe, most snake species are diurnal, with a few exceptions (e.g., *Eryx jaculus*, *Telescopus fallax*) that are primarily nocturnal (Geniez, 2018). Nevertheless, nocturnal activity has been documented in certain *Natrix* species (e.g., *N. maura*, *N. tessellata*) (Scali *et al.*, 2001). *Natrix natrix* sensu lato, formerly regarded as a single species, has recently been split into three distinct taxa, with the present study focusing on *N. natrix* sensu stricto. This species shows marked sexual dimorphism (females being larger), a diet dominated by amphibians, and a wide geographic distribution. It also exhibits chromatic polymorphism (classic, bilineata, and melanistic morphs), with potential implications for thermoregulation and activity (Geniez, 2018; Bury *et al.*, 2020). However, research directly linking coloration to behavior remains scarce. Citizen-science databases have proven valuable for studying species' distributions and behavioral traits (Fritz & Ihlow, 2022; Jansen *et al.*, 2024).

The aim of this study is to document crepuscular and nocturnal activity in *N. natrix* across its distribution range, using a combination of citizen-science records and field observations. Specifically, we assess: (1) the frequency of nocturnal activity, (2) geographic variation, (3) the influence of temperature and lunar illumination, and (4) differences among color morphs in nocturnal activity.

7.2. Material and methods

To analyze nocturnal activity in *Natrix natrix*, we downloaded georeferenced observations from iNaturalist and Observation.org, and calculated sunrise and sunset times. Observations were classified as crepuscular or nocturnal following the protocol of Dyugmedzhiev *et al.*

(2020) and were validated both manually and by consensus among three independent observers. We also included our own field data collected between 2010 and 2023. Each record was annotated with information on color morph, age, number of individuals, habitat, lunar illumination, and daily mean temperature (T24h), obtained from Open-Meteo.com. Statistical analyses, including ANOVA, Kruskal–Wallis, Mann–Whitney U, and chi-square tests, were performed to test possible associations.

7.3. Results

From iNaturalist, we extracted 9,745 observations of *Natrix natrix*, of which 93 met the criteria for crepuscular or nocturnal activity. Including our own field observations (n = 22) and records from Observation.org (n = 12), the total number of nocturnal observations reached 127: 33 (25.98%) were classified as crepuscular and 94 (74.02%) as nocturnal. The total number of individuals observed was slightly higher—34 crepuscular and 99 nocturnal snakes—since some observations included multiple individuals.

Nocturnal activity was recorded between 12 March and 5 November, with most observations occurring from May to August. Of the 127 observations, 91 corresponded to the classic color morph, 31 to the bilineata morph, four to the melanistic morph, and for one individual the color morph could not be determined due to low image quality. Among these, 88 were adults and 35 juveniles, while the age class could not be determined for ten individuals.

The daily mean temperature (T24h) at which snakes were active was 20.01 ± 0.48 ºC (range: 4.7–31 ºC; n = 127) when considering all crepuscular and nocturnal observations combined. Analyzed separately, T24h was 19.75 ± 0.96 ºC (4.7–24.7 ºC; n = 33) for crepuscular individuals and 20.1 ± 0.56 ºC (9.2–31 ºC; n = 94) for nocturnal individuals. A Mann–Whitney U test indicated no significant difference in T24h between crepuscular (*Mdn* = 20.1) and nocturnal activity (*Mdn* = 19.9) (U = 1552.5, n1 = 33, n2 = 94, $z = 0.005$, $p = 0.996$).

Considering color morph, T24h was 19.18 ± 0.58 ºC (4.7–30.7 ºC; n = 91) for classic morphs, 21.6 ± 1.92 ºC (17.4–26 ºC; n = 4) for melanistic morphs, and 22.19 ± 0.83 ºC (10.9–31 ºC; n = 31) for bilineata morphs. One-way ANOVA revealed a significant difference between color morphs in T24h ($F(2,123) = 3.94$, $p = 0.022$). Post-hoc analyses indicated that bilineata individuals were active at significantly higher T24h than classic morphs ($p = 0.021$). However, when the analysis was geographically restricted to the latitudinal range 43°–47°, a Kruskal–Wallis test showed no significant differences in T24h between color morphs ($H(2) = 0.13$, $p = 0.94$).

A chi-square test of independence revealed no significant association between moon presence and age class ($\chi^2(1, n = 112) = 0.198$, $p = 0.656$), nor between type of nocturnal activity (crepuscular vs. nocturnal) and moon presence ($\chi^2(1, n = 119) = 0.0006$, $p = 0.981$). Additionally, a Mann–Whitney U test applied only to observations with the moon present (n = 56) showed no significant differences in illumination levels between crepuscular (*Mdn* = 0.45) and nocturnal observations (*Mdn* = 0.7) (U = 252, n1 = 15, n2 = 41, $z = 1.018$, $p = 0.309$).

7.4. Discussion

Although *Natrix natrix* is generally considered a diurnal species, several studies have reported crepuscular and nocturnal activity in members of the subfamily Natricinae (Scali *et al.*, 2001; Mebert *et al.*, 2011). In this study, we provide additional evidence of nocturnal activity in the grass snake, based on observations throughout the active period (May–August), including the reproductive season. Nocturnal activity was recorded across a wide temperature range (4.7–31 °C), with no significant differences between crepuscular and nocturnal observations. Individuals of the striped (bilineata) morph were more frequently observed on warmer days, although this pattern disappeared in regions where multiple morphs coexist. Moon phase did not significantly influence activity. The number of crepuscular and nocturnal observations has increased since 2020, reflecting the growing use of citizen-science platforms, which prove valuable for studying the behavior and distribution of cryptic species like *N. natrix* (Fritz & Ihlow, 2022).

Conclusions

This work addressed various aspects of color polymorphism in snakes, with a focus on melanism, and yielded the following results:

1. The prevalence of melanism in polymorphic terrestrial snake species is approximately 31%. Contrary to classical hypotheses, melanism more closely follows Gloger's rule, being more frequent in humid regions, rather than Bogert's rule (in the species analyzed). No evidence was found for sex-biased frequency or an association between melanism and larger body size.
2. In the common adder (*Vipera berus*), melanism conforms to Bogert's rule, being more frequent in cold and arid areas, where it may provide thermoregulatory advantages. The melanistic morph occupies a narrower climatic niche, adapted to extreme local conditions, compared with the zigzag morph. Although the two morphs coexist in many regions (sympatry), their climatic niches are only partially overlapping, not equivalent.
3. Predation pressure, tested in two populations of *V. berus* (one polymorphic and one exclusively melanistic), showed a slight tendency for melanistic individuals to be attacked more often, though differences were minor. Thus, negative selection by predators does not fully explain the maintenance of high melanism frequencies. The zigzag pattern has a proven aposematic function, likely reducing attacks by visual predators, although its effectiveness may vary depending on the population context.
4. In the polymorphic *Natrix natrix* population at Sfântu Gheorghe, the three color morphs (classic, bilineata, and melanistic) did not show clear preferences for specific habitat types, with minimal differences in relation to environmental variables. Likewise, no significant differences in body size were detected, refuting the hypothesis that melanistic individuals are larger. This lack of differentiation may be associated with increased predation pressure, which could limit the growth of melanistic individuals.
5. Reproductive success in female *N. natrix* was not influenced by color morph. Neither the number nor the weight of eggs varied significantly between morphs. However, body length was positively correlated with reproductive success, particularly in classic-colored females, supporting the idea that larger individuals have higher reproductive potential. These results also confirm the absence of a size advantage for the melanistic morph.

6. The study on nocturnal activity in *N. natrix* demonstrated that the species is more active at night than previously thought, with observations spanning the entire range. The bilineata morph was more active nocturnally after warmer days, particularly in the southern part of the distribution, but in areas of sympatry, no notable differences between morphs were detected.
7. Two of the studies presented in this thesis would not have been possible without the essential contributions of nature enthusiasts who uploaded their observations to open-science platforms such as iNaturalist and Observation.org. These contributions once again highlight the invaluable role of citizen science in ecological and evolutionary research, particularly in providing information on novel behaviors and phenotypic variation, thanks to the broad geographic coverage these platforms offer. This underscores the increasingly evident importance of collaboration between the scientific community and the general public in advancing knowledge of the natural world.

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