



UNIL | Université de Lausanne

Faculté de biologie
et de médecine

Ecole de biologie

**THERMOREGULATION AND MICROHABITAT CHOICE IN THE
POLYMORPHIC ASP VIPER (*Vipera aspis*)**

**Travail de Maîtrise universitaire ès Sciences en comportement, évolution et
conservation**

Master Thesis of Science in Behaviour, Evolution and Conservation

par

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Janvier 2015

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POLYMORPHIC ASP VIPER (*Vipera aspis*)**

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1 **Résumé**

2 Chez les reptiles, la température corporelle dépend fortement de sources externes de chaleur.
3 Cependant, d'autres paramètres peuvent considérablement influencer l'efficacité des échanges
4 thermiques avec l'environnement, et parmi ceux-ci, la couleur de la peau est un des plus
5 importants. En effet, grâce à ses propriétés physiques, la pigmentation noire permet aux
6 morphes mélaniques de profiter d'une meilleure thermorégulation que les non-mélaniques.
7 Cependant, malgré que les bénéfices thermiques aient souvent été démontrés en conditions
8 expérimentales, il est plus difficile de comprendre comment les individus foncés profitent de
9 cette condition biologique dans leur environnement naturel. Cela est dû au fait que les limites du
10 mélanisme, comme la réduction de l'habilité de camouflage, peuvent induire les individus
11 mélaniques à utiliser différemment leur thermorégulation plus efficace. En d'autres termes, les
12 morphes mélaniques peuvent utiliser leur avantage thermique de deux manières différentes; soit
13 pour augmenter et maintenir une température corporelle plus élevée (avec les bénéfices qui en
14 découlent sur leur taille et leur taux de croissance), soit pour diminuer leur temps d'exposition et
15 éviter les micro-habitats ouverts et thermiquement favorables. Dans cette étude nous avons
16 utilisé une population de vipère aspic (*Vipera aspis*) caractérisée par une forte présence de
17 mélanisme dans le but d'étudier l'influence de la coloration de la peau sur la température
18 corporelle. La même analyse a été par la suite faite sur une base de données contenant seulement
19 des femelles gestantes pour pouvoir évaluer l'importance du statut reproductif. Les résultats ont
20 montré une différence seulement au sein des femelles gestantes, indiquant que les individus
21 mélaniques avaient une température interne plus élevée que les individus avec des motifs. Une
22 deuxième analyse réalisée sur le choix du micro-habitat a montré que les morphes mélaniques
23 préfèrent des zones caractérisées par une exposition solaire réduite et par une importante
24 couverture végétale contrairement aux morphes non-mélaniques. Ce résultat est crucial. En
25 effet, en plus de fournir une possible explication pour le manque de différence de température

26 corporelle (trouvé dans l'analyse incluant toutes les vipères), cela confirme que les individus
27 mélaniques peuvent potentiellement utiliser leur thermorégulation plus efficace pour vivre dans
28 des micro-habitats moins exposés et thermiquement moins favorables, dans les quels le risque
29 de prédation est moins important.

Summary

In reptiles, body temperature strongly depends on external heat sources. However, other parameters can considerably influence the efficiency of thermal exchanges with the environment, and among these, skin colour is one of the most relevant. Indeed, due to its physical properties, darker pigmentation allows melanistic morphs to enjoy more efficient thermoregulation compared to non-melanistic ones. However, despite thermal benefits of melanism often having been highlighted under experimental conditions, it is more difficult to understand how darker individuals profit from this biological condition in natural environment. This is because limits of melanism, such as reduced camouflage ability, can push darker individuals to differently manage their efficient thermoregulation. In other words, melanistic morphs can either use their thermal advantage in order to increase and maintain higher body temperature (with consequent benefits on the growth rate and body size), or in order to reduce basking duration and avoid well-exposed and thermally favourable microhabitat (in which they would be easier to capture by predators). In this study we used an asp viper (*Vipera aspis*) population characterized by a strong presence of melanism in order to investigate the influence of skin colour on the internal temperature. The same test was subsequently carried out on a database containing only gravid females on the purpose of assessing the weight of reproductive statute. Results highlighted a difference only within gravid females with melanistic individuals having higher body temperature compared to blotched ones. A second analysis carried out on the microhabitat choice, showed that melanistic vipers prefer zones marked by a scarcer sun exposure and by higher vegetation cover compared to blotched ones. This result is crucial. In fact, besides providing a possible explanation for the lack of difference in body temperature (found for the analysis including all vipers), it confirms that darker individuals can potentially use their efficient thermoregulation in order to inhabit less exposed and thermally unfavourable microhabitats, in which predation risk is reduced.

55 **Key-words**

56 Blotched vipers, body temperature, gravid statute, melanism, melanistic vipers, reptiles, thermal
57 benefits

Introduction

Polymorphism plays a major role in survival and viability both at inter- and intraspecific levels. Genetic, phenotypic and behavioural diversity are the key of evolutionary success, ecological adaptations and ability to deal with environmental changes. Species, respectively populations in which individuals present good genetic and phenotypic variations, are able for example to colonize heterogeneous habitat, to cope with constraints imposed by the environment as well as to better deal with parasite or disease appearance (Wilson *et al.* 2001; Forsman & Åberg 2008a; b; Forsman *et al.* 2008; Pizzatto & Dubey 2012).

Melanism corresponds to a particular phenotype, characterizing individuals darker in pigmentation (Millar, Lambert & Majerus 1999; Clusella Trullas, van Wyk & Spotila 2007). This particular condition has been studied especially in ectotherms because of several hypotheses that may explain its onset and its adaptive function (Clusella Trullas *et al.* 2007; Ducrest *et al.* 2014). Among these there is for example the protection from ultraviolet radiation (Gunn 1998), disease resistance (Wilson *et al.* 2001) as well as sexual selection (Wiernasz 1989). Nevertheless, one of the most plausible and widely studied hypothesis highlights an advantage in terms of thermoregulation associated to the darker pigmentation (Kingsolver & Wiernasz 1991; Forsman 1995, 2011; Clusella Trullas *et al.* 2007; Clusella-Trullas, Van Wyk & Spotila 2009).

In snakes, and more in general in reptiles, body temperature depends on thermal environment (Brattstrom 1965; Huey 1982). Consequently, in these organisms, an effective thermoregulation is carried out through an important behavioural and physiological flexibility (Seebacher 2005). Some examples are given by the equilibrium between basking and shelter seeking, by body movement and arrangement on the exposure surface, by proportion of body kept in the shade and by the regulation of cardiovascular activity (Seebacher 2005; Seebacher & Franklin 2005; Huey 1982). In turn, these behaviours vary in function of biological parameters

83 like sex, body size, health and shedding statute (Huey 1982, Lillywhite 1987, Peterson *et al.*
84 1993). The need of heat also varies depending on the feeding and reproductive statutes and it is
85 greater for individuals during digestive period as well as for gravid females (Shine 2004;
86 Tattersall *et al.* 2004). Environmental conditions are nevertheless among the most important
87 limits to the body temperature (Peterson 1987; Blouin-Demers & Weatherhead 2001) promoting
88 or precluding the presence of particular behaviours or phenotypes in specific microhabitats.
89 Finally, as indicated above, skin colour also has an important influence on body temperature,
90 with darker morphs that enjoy thermal advantage due to their greater thermoregulatory abilities.
91 Such advantage arise from the fact that black colour has a lower reflectance (Brakefield &
92 Willmer 1985; Jong, Gussekloo & Brakefield 1996). This allows melanistic individuals (under
93 equal environmental conditions like air temperature, solar radiation, wind speed and soil
94 structure) to heat faster, reach a higher optimal temperature and maintain the latter for longer
95 time compared to non-melanistic ones, as suggested by various experimental studies (Crisp,
96 Cook & Hereward 1979; Forsman 1995; Tanaka 2005, 2007). In turn, a thermal advantage can
97 have a positive impact on several ecological parameters. For example, some studies conducted
98 on different snakes species such as *Vipera aspis*, *Vipera berus* and *Hierophis viridiflavus*
99 highlighted a higher growth rate and/or body size in melanistic individuals (Andr n & Nilson
100 1981; Luiselli 1995; Monney, Luiselli & Capula 1996). Another benefit may be related to a
101 major daily and seasonal activity, overall in cooler regions. In this regard, in a study performed
102 on the Japanese striped snake (*Elaphe quadrivirgata*), it has been shown that only melanistic
103 morphs of exposed themselves during the early winter (Ota & Tanaka 2002). However, in most of
104 cases, any difference in body temperature was detected between the two morphs in free ranging
105 individuals (Crisp *et al.* 1979; Forsman 1995; Tanaka 2005, 2007; Clusella-Trullas *et al.* 2009;
106 Geen & Johnston 2014). This is understandable considering that, in natural conditions, benefits
107 of a better thermoregulation may be reduced or differently used because of the presence of some

limits linked to the melanism. Among these, especially emerges the fact that darker morphs are less cryptic compared to patterned ones and consequently easier to flush out by predators (Andrén & Nilson 1981; Forsman 1995; Wüster *et al.* 2004; Niskanen & Mappes 2005). This constraint could in fact push melanistic individuals to adapt their behaviour in order to reduce their exposition or to avoid well exposed and thermally favourable microhabitats (Forsman 1995; Tanaka 2009). Thus, by considering costs and benefits of melanism, it is possible to imagine two major scenarios that can explain how darker individuals take advantage of their more efficient thermoregulation. First, free ranging darker individuals are characterized by a higher body temperature compared to lighter ones, since they use their more efficient thermoregulation in order to increase activity, growth rate and body size. Second, there are no differences in body temperature between melanistic and patterned morphs, since darker individuals use thermoregulatory advantage in order to minimize their exposition, or, in order to inhabit thermally unfavourable microhabitats (in which patterned morphs could hardly live). A short basking duration as well as the choice of microhabitats marked by scarce sun exposure and by important vegetation cover could in fact allow darker morphs to reduce predation risk.

In this study we tested, on an asp viper (*Vipera aspis*) population characterized by a strong presence of melanism, which of the two scenarios mentioned above proved to be more truthful. More precisely, we checked if melanistic vipers significantly differed from blotched ones in their internal temperature. The same test was performed, in parallel, on a database including only gravid females, with the purpose to evaluate the influence of reproductive statute. In a second time, hypothesizing that the thermoregulation advantage may enable melanistic individuals to better deal with unfavourable environmental conditions, we verified if the two morphs had a tendency to choose different microhabitats. The choice of the asp viper as model for this study is linked to the fact that such species has already been used in other studies and its

biology is well known. Furthermore it is a relatively common snake in the sampling region (western Swiss Alps), besides being quite easy to capture and manipulate.

Materials and Methods

Study species

Vipera aspis is a venomous snake mainly distributed along the Alpine arc, where it can be found in plain as well as at altitudes above 3000 m asl. (Ursenbacher *et al.* 2006). As most of European Viperidae the asp viper is a viviparous reptile. Although some coupling can occur in autumn this snake mainly reproduces in spring (especially in April) and gives birth to the offspring at the end of summer. This species is also characterized by an important polymorphism, which is particularly evident in our studied area, with a 65-70% of melanistic vipers (Castella *et al.* 2013; Schuerch *et al.* submitted). The asp viper habitat is quite heterogeneous, especially in populations characterized by the presence of different morphs (Broennimann *et al.* 2014) and follows a vegetation gradient from patches marked by a high plant density to open area (Appendix 2).

Studied area and sampling

All vipers included in the study were collected and measured in western Swiss Alps, more precisely in the district of Riviera-Pays-d'Enhaut (Canton de Vaud-Switzerland). The sampling sites were located on the southern slope of the valley (46.4913° N, 7.0859° E) between 870 and 1600 m asl. and spread out over a surface of approximately 1800 ha. Sampling was carried out between 2012 and 2014 from April to September and snakes were captured by hand (using a double pair of protective gloves). On a total of 608 captured vipers (including recaptures) 233 were found in open areas, 181 inside a belt of 4 meters representing the border between

vegetation patches and open area, and 194 vipers were finally found inside vegetation. For each individual sex and colour (melanistic or blotched) were determined and the following measures were recorded: time of capture (hour and minutes), altitude (m asl.), Swiss coordinates, air temperature (°C), internal temperature (°C) measured with a cloacal probe, and snout-vent length (SVL) (mm). For females whose length exceeded 40 cm (Bonnet & Naulleau 1996; Castella *et al.* 2013; Monney, Luiselli & Capula 1996) the reproductive statute (gravid or not gravid) was verified.

Computed environmental predictors

With the purpose to define the asp viper microhabitat in the studied region, three types of environmental predictors were considered: topographic (slope in degrees), bioclimatic (solar radiation in KWh/m²), and the Normalized Difference Vegetation Index (NDVI). The fourth environmental predictor corresponded to the altitude and was calculated on the field. In order to spatially compute slope and solar radiation, digital elevation models (DEMs) with a resolution of 2 meters were used. These DEMs were subsequently computed by aggregating a 1 meter DEM from Swisstopo (Pradervand *et al.* 2013). Slope (2 meters) was calculated using the spatial analyst tool in ArcGIS 10 with a 3 x 3 pixel moving window (Pradervand *et al.* 2013). Solar radiation (2 meters) was calculated for each pixel and every day of year. The entire area has been used as input in order to compute, using DEM, the direct diffused and reflected solar radiation. Local exposure and shading topography have also been considered for computing, using the spatial analyst tool in ArcGIS 10 (Pradervand *et al.* 2013). Solar radiation and slope values for each pixel (corresponding to the coordinates of a capture event) were obtained from ArcGIS raster data. In the statistical analysis, solar radiation was used as the average of days included in the period between April and September. Finally, it is important to remember that solar radiation is a potential value, which, in the case of a forest or a surface with strong plant

productivity, is calculated at the canopy level and not on the soil as in open area. This means that there is a slight overestimation of the solar radiation for captures carried out in points marked by a strong vegetation density. Finally, NDVI corresponds to an annual average of the vegetal biomass productivity and its index ranges from -1 to +1. Highly positive values (> 0.5) represent surfaces with important vegetation density. Low positive values represent shrub and grassland (approximately 0.2 to 0.4). Values close to zero (-0.1 to 0.1) mainly highlight barren or rocky areas, while values lower than -0.1 characterizes a complete lack of vegetation (Carlson & Ripley 1997). NDVI rasters with a resolution of 2 x 2 meters were obtained from red and infrared aerial photographs (Swissimage FCIR, Swisstopo). Values were subsequently calculated using the following formula (Wang, Rich & Price 2003), implemented in ArcGIS 10:

$$NDVI = \frac{(NIR-RED)}{(NIR+RED)}.$$

Statistical analysis

PCA analysis for environmental predictors

Environmental predictors selected for both thermoregulation and microhabitat models (NDVI, altitude, solar radiation and slope) showed important correlations between themselves that could cause some collinearity problems. Thus, in order to summarize the information contained in these variables, a principal component analysis (PCA) has been performed using R software (version 3.1.1). The PCA axes, used later in both analyses, were selected based on two criteria: eigenvalues higher than 1 and proportion of explained variance exceeding 10% (Quinn & Keough 2002) (Appendix 3 and 4).

Generalized linear mixed model (GLMM) selection

All models used were characterized by continuous response variables and the backward selection was entrusted to a F-statistic with p-values associated. When necessary, one or more

variance structures were added to the model in order to deal with heterogeneity problems (Zuur *et al.* 2009). Also, some outliers (probably obtained because of measurement errors or corresponding to juveniles being under one year old) have been removed when the assumptions were not complied (Zuur *et al.* 2009).

Thermoregulation

The main idea of this analysis was to understand if melanism and other variables had an influence on the internal temperature of vipers. In this regard models were built based on the 2014 database (only during this year the internal temperature was measured). For this analysis Generalized Linear Mixed Models (GLMM) were performed using R software (version 3.1.1) with package "nlme". The first model was tested on the set of vipers captured during 2014 (318 captures). The following explanatory variables were added to the model and all pairwise interactions tested: sex, melanism (melanistic or blotched), SVL, capture hour, month, PC1 (first PCA axis) and PC2 (second PCA axis). SVL was log-transformed and, because of bias due to the sampling dates, air temperature was added to the model as offset variable (Zuur *et al.* 2009). Finally, in order to take into account the recapture "weight", the identity of the individual was added to the model as random factor. The second model was built only on the database of gravid females (96 captures). Variables and pairwise interactions were the same as in the first model, however being the analysis based only on gravid females the sex was excluded. Also in this case, it was necessary to add the SVL log-transformed, air temperature as offset variable and the individual identity as random factor.

Microhabitat choice

The purpose of this analysis was to determine which variable influenced the microhabitat choice (summarized, as indicated above by means of PCA axes). Both models constructed for this

analysis were based on sampling carried out between 2012 and 2014 and the corresponding database included 608 captures. In the first model the response variable corresponded to the first PCA axis (PC1), while explanatory variables were sex, melanism and SVL (capture hour and month were excluded since solar radiation and NDVI index, represented by the PCA axes, were given as average values for the entire sampling season). As for models used in the thermoregulation analysis, SVL was log-transformed and the individual identity added as random factor. However, using here data collected from 2012 to 2014, also the year was added as random factor in order to control the effect of the sampling repetition along the three seasons. It was finally necessary to add a spatial correlation structure in order to deal with spatial correlation in residuals of the model. In this regard different correlation structures were tested according to Zuur *et al.* 2009 and the best model (Spherical correlation, function *corSpher*, package "nlme") was selected based on the Akaike's Information Criterion (AIC) (Zuur *et al.* 2009). In the second model, the response variable corresponded to the second PCA axis (PC2). Other elements and relative corrections corresponded exactly to those of the first model except for the correlation structure, where AIC test indicated indeed that the best spatial correlation structure was the Rational quadratic correlation (function *corRatio*, package "nlme"). Since the model for microhabitat choice was tested twice (first with PC1 and subsequently with PC2) a Bonferroni correction has been performed, thus reducing the significant threshold at 0.025 (Walker *et al.* 2009).

Results

Principal component analysis (PCA)

PCA for thermoregulation analysis

The principal component analysis for 2014 database was performed on four environmental predictors: altitude, slope, NDVI and solar radiation. Both PCA components (axes) retained and used in statistical models are shown in the Appendix 3. First component presented a proportion of variance equal to 0.36 while for the second one the value was 0.31 (Appendix 3). Components together explained 67,80 % of total variance, and the PCA plot is shown always in the Appendix 3. A principal component analysis was also carried out for the gravid females database, however its results and parameters are not shown since neither of the two axes had a significant influence on the internal temperature.

PCA for microhabitat analysis

As for thermoregulation analysis, the PCA was performed on four environmental predictors: altitude, slope, NDVI and solar radiation. Also in this case both PCA components (axes) retained and used in statistical models are shown in Appendix 4. First component presented a proportion of variance equal to 0.37 while for the second one the value was 0.28. Components together explained 64,50 % of total variance (Appendix 4). High values for the first PCA axis (PC1) are indicative of microhabitats characterized by high altitudes and solar radiation as well as by scarce plant productivity (low NDVI) and by a slight slope (Fig. 2A and 3A). High values for the second PCA axis (PC2) are indicative of microhabitats characterized by steep slope and scarce plant productivity as well as, by high solar radiation and low altitudes (Fig. 2A and 3A).

Thermoregulation

Following results were obtained after performing the backward selection on both models. Variables and interactions which resulted to be significant are shown in Tables 1A and 1B.

Generalized Linear Mixed Model (GLMM) performed on all vipers captured in 2014 did not show a significant effect of the colour ($P > 0.05$), indicating that there is not relevant difference in terms of internal temperature between melanistic and blotched vipers. Conversely, results highlighted a significant interaction between the second PCA axis (PC2) and the month ($F_{1,105} = 6.60$, $P = 0.0116$). This indicates that PC2 axis has an influence on internal temperature depending on the month. While month as covariate had no significant effect ($F_{1,105} = 0.96$, $P = 0.33$), PC2 axis was significant ($F_{1,105} = 7.77$, $P = 0.0063$). This indicates that vipers living in zones principally characterized by high vegetal productivity and altitude have a lower internal temperature. A significant effect was also found for the hour ($F_{1,105} = 5.88$, $P = 0.017$), indicating that snakes are cooler in the morning and their temperature rises with time passes. The last significant effect corresponds to SVL ($F_{1,105} = 11.29$, $P = 0.0011$) which shows that vipers with larger body size have a higher internal temperature.

Generalized Linear Mixed Model (GLMM) performed on the gravid females database showed a significant effect of the melanism ($F_{1,41} = 5.35$, $P = 0.0258$) with melanistic gravid females having higher internal temperature compared to blotched ones (Fig. 1). All other variables were not significant, neither alone nor in interactions.

Microhabitat choice

Following results were obtained after performing the backward selection on both models. Variables and interactions, which resulted to be significant, are shown in the Tables 2A and 2B. It is important to remember that for these two models a Bonferroni correction has been performed and the significant threshold was reduced at 0.025.

Generalized Linear Mixed Model (GLMM) performed using the first PCA axis (PC1) highlighted a significant effect of melanism ($F_{1,390} = 9.32$, $P = 0.0024$) indicating that along the PC1 axis microhabitat choice was substantially different between melanistic and blotched vipers. In other words, melanistic vipers seem to have tendency to choose microhabitats characterized by steeper slope and higher NDVI index as well as by lower solar radiation and altitude, compared to blotched ones. Conversely, blotched vipers showed a tendency to choose microhabitats characterized by higher altitude and solar radiation as well as by slighter slope and lower NDVI index, compared to melanistic ones (Fig. 2A and 2B).

Generalized Linear Mixed Model (GLMM) performed using the second PCA axis (PC2) highlighted, on the contrary, a significant effect of sex ($F_{1,390} = 11.97$, $P = 0.0006$) indicating that, along the PC2 axis, microhabitat choice was substantially different between males and females. In other words, females seem to have a tendency to choose microhabitats characterized by steeper slope and higher solar radiation as well as by lower altitude and plant productivity (NDVI), compared to males. Conversely, males showed a tendency to choose microhabitats characterized by higher NDVI index and altitude as well as by slighter slope and lower solar radiation, compared to females (Fig. 3A and 3B).

Discussion

Thermoregulation

The skin colour influence was observed only within gravid females with melanistic vipers reaching higher internal temperature compared to blotched ones (Fig. 1 and Table 1B). This temperature difference is probably related to the biological importance of reproductive statute. Indeed, several studies emphasize the dependence of various physiological aspects such as the embryos development on the thermal environment, showing that gravid females were

characterized by higher internal temperatures compared to non-gravid ones and males (Charland 1995; Chiaraviglio 2006; Crane & Greene 2008). However, only Schuerch *et al.* (submitted) demonstrated the effect of melanism on the body temperature within gravid females under experimental conditions and, in our knowledge, none tested this on free ranging individuals. Thus, this result is of considerable interest because it indicates that free ranging melanistic females can potentially use their peculiar thermoregulatory characteristics in order to reach higher body temperatures with consequent benefits during gestation. Nevertheless, it would be also interesting to analyse basking duration and frequency in order to also assess the influence of gravid females behaviour on body temperature difference between the two morphs.

Contrary to results obtained with the gravid females database, any difference in terms of internal temperature was found between melanistic and blotched vipers for the analysis including all vipers captured in 2014. In turn, the influence of variables like hour, body size and microhabitats (Table 1A) is quite logical. For example, dependence of the internal temperature on body size has already been shown (Bittner, King & Kerfin 2002). Even the effect of time of capture is rather trivial, with first sunlight hours characterized by a lower ambient temperature and consequently by lower internal temperature in vipers. Finally, also the microhabitat has an impact on the internal temperature and its influence varies according to the month (Table 1A). In fact, vegetal biomass variation along the season leads to a change of environmental conditions in vipers' microhabitats, which can significantly affect thermal parameters of these animals (in this regard, it is important to remember that the microhabitat summarized by the second PCA axis mainly contain the information related to NDVI index, as indicated in the Appendix 3). However, as indicated above and in line with results obtained in several studies, the variation of skin colour did not significantly affect the internal temperature of the two morphs (Table 1A) (Crisp *et al.* 1979; Forsman 1995; Tanaka 2005, 2007; Clusella-Trullas *et al.* 2009; Geen & Johnston 2014). This information could lead us to conclude that melanism has in

general no influence on the vipers' thermoregulation, or else that a selection pressure on the skin colour only exists for gravid females. However, it is very important to note that the advantage in terms of heat acquisition and maintenance related to darker individuals, is not limited to a body temperature increase (with consequent biological benefits), but can manifest itself in various other forms. In fact, the lack of difference in the internal temperature, could reside in a different behaviour between the two morphs, with melanistic vipers that may use their efficient thermoregulation in order to expose themselves for shorter time or less frequently, thus reducing predation risk. Or else, it is possible to imagine that darker morphs use their thermal advantage in order to colonize microhabitats characterized by harsher environmental conditions like low solar radiation, low air temperature or a soil particularly covered by vegetal structures. On the other hand, if we consider that melanistic individuals are less cryptic (Andrén & Nilson 1981), it is possible to imagine that these types of microhabitats provide them a greater protection from predators.

Microhabitat choice

The first analysis highlighted a difference in the microhabitats choice between melanistic and blotched vipers (Table 2A). More precisely melanistic morphs prefer microhabitats characterized by lower altitude and sun exposure and by higher vegetation cover, compared to blotched ones (Fig. 2). Such result resumes in part one of the hypotheses previously presented. Indeed, a better thermoregulation may explain the fact that darker individuals can inhabit microhabitats in which heat exchanges with the environment are less efficient (for instance, zones less exposed to the sun and marked by a higher vegetation cover). On the other hand, these types of microhabitats would allow melanistic vipers to better compensate their reduced camouflage ability. However, this result is not sufficient to demonstrate the theory according to which the choice of microhabitat would explain the absence of body temperature difference

between the two morphs. In fact, it is important to remember that the database used for thermoregulation analysis was substantially different (see statistical analysis paragraph in materials and methods section) and this, does not allow to evaluate the weight that microhabitat choice have on the body temperature of the two morphs. In other words, we can only suppose that thermal benefits of melanistic vipers are masked by the choice of thermally less favourable microhabitats. Furthermore, we should consider that also basking duration and frequency could influence the vipers' body temperature. On this regard it is plausible to hypothesize that a significant difference in thermal parameters has not been found because melanistic individuals reduces predation risk by exposing themselves for shorter time and less frequently.

The second analysis, on the contrary, indicated that males have a tendency to choose different microhabitats compared to females (Table 2B). More precisely they prefer zones overall characterized by higher vegetation cover and less exposed to the sun. The opposite tendency of females to inhabit sunnier areas characterized by a scarce vegetation cover is quite logical. Indeed, considering that almost half of females captured during these three years were gravid, it is possible to associate the microhabitat choice to the greater need for heat related to the reproductive statute. On the contrary males, not having gestation constraints, prefer inhabit spots less exposed and characterized by an abundant presence of vegetal structures, in which the predation risk is potentially reduced.

Conclusions and Perspectives

To date, various studies have been performed on the thermoregulation in reptiles and often they focused on species characterized by the presence of melanism. More precisely, thermal advantages linked to a darker pigmentation have been established in various circumstances under experimental conditions. For example, Tanaka (2005, 2007) shows that melanistic individuals belonging to the *Elaphe quadrivirgata* species had a greater rate of body

temperature increase compared to striped ones. Forsman (1995) highlighted superior thermoregulatory abilities in darker morphs of *Vipera berus* with melanistic individuals that enjoyed a greater heating rate and a slightly higher equilibrium temperature. Even melanistic individuals in the *Podarcis dugesii* species showed higher heating rate and equilibrium temperature compared to the green ones. Finally Schuerch *et al.* (submitted) demonstrated that melanistic gravid females of asp viper (*Vipera aspis*) enjoyed thermal advantages when experimentally exposed to cooler temperatures. However, as previously mentioned, benefits of these greater thermoregulatory abilities were rarely inferred in free ranging individuals, probably because of the lack of difference in body temperature between melanistic and non-melanistic morphs in natural environments (Crisp *et al.* 1979; Forsman 1995; Tanaka 2005, 2007; Clusella-Trullas *et al.* 2009; Geen & Johnston 2014). Results of this study conducted on free ranging asp viper, enabled us to demonstrate that melanistic gravid females were characterized by a higher internal temperature compared to blotched ones. Afterwards, the analysis performed on the microhabitat choice allowed us to explain how melanistic individuals may alternatively use their thermoregulation advantage to reduce the predation risk. Finally, concerning the absence of difference in body temperature between the two morphs, we can hypothesize that this may be due to a combined effect of the microhabitats choice and the behaviour in terms of basking duration and frequency.

Therefore, with the purpose to complete information related to the microhabitat choice, it would be very interesting to investigate also behavioural aspects within this polymorphic population. In other words, in order to have a more complete overview regarding the temperature control of these two morphs, it would be important to consider parameters such as basking duration and frequency throughout the day, but also along the season. The implementation of this type of analysis could reveal some of the secrets that still surround the thermoregulation topic in this particular asp viper population.

423 **Acknowledgements**

424 I am very grateful to Sylvain Dubey for his help throughout the project, especially during the
425 report drafting. In particular I also thank Johan Schürch and Naïké Trim for the help during data
426 collection on the field and I always thank Johan Schürch for the help during statistical analysis. I
427 would like to thank Jean-Nicolas Pradervand for giving me access to some environmental
428 predictors and Philippe Christe for having made available the material for internal temperature
429 measures. A special thanks is addressed to Sylvain Dubey research group for supporting costs of
430 the "education in handling venomous snakes" course.

431 I finally would like to thank Joaquim Golay, Alexandre Baillifard, Marie Strehler, Lisa Sannitz,
432 Maude Mayer, Benjamin Wolf, Athimed El Taher and Christophe Sahli for having contributed
433 in different ways during data collection.

434 **References**

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- 567

568 **Figures**

569 **Figure 1**

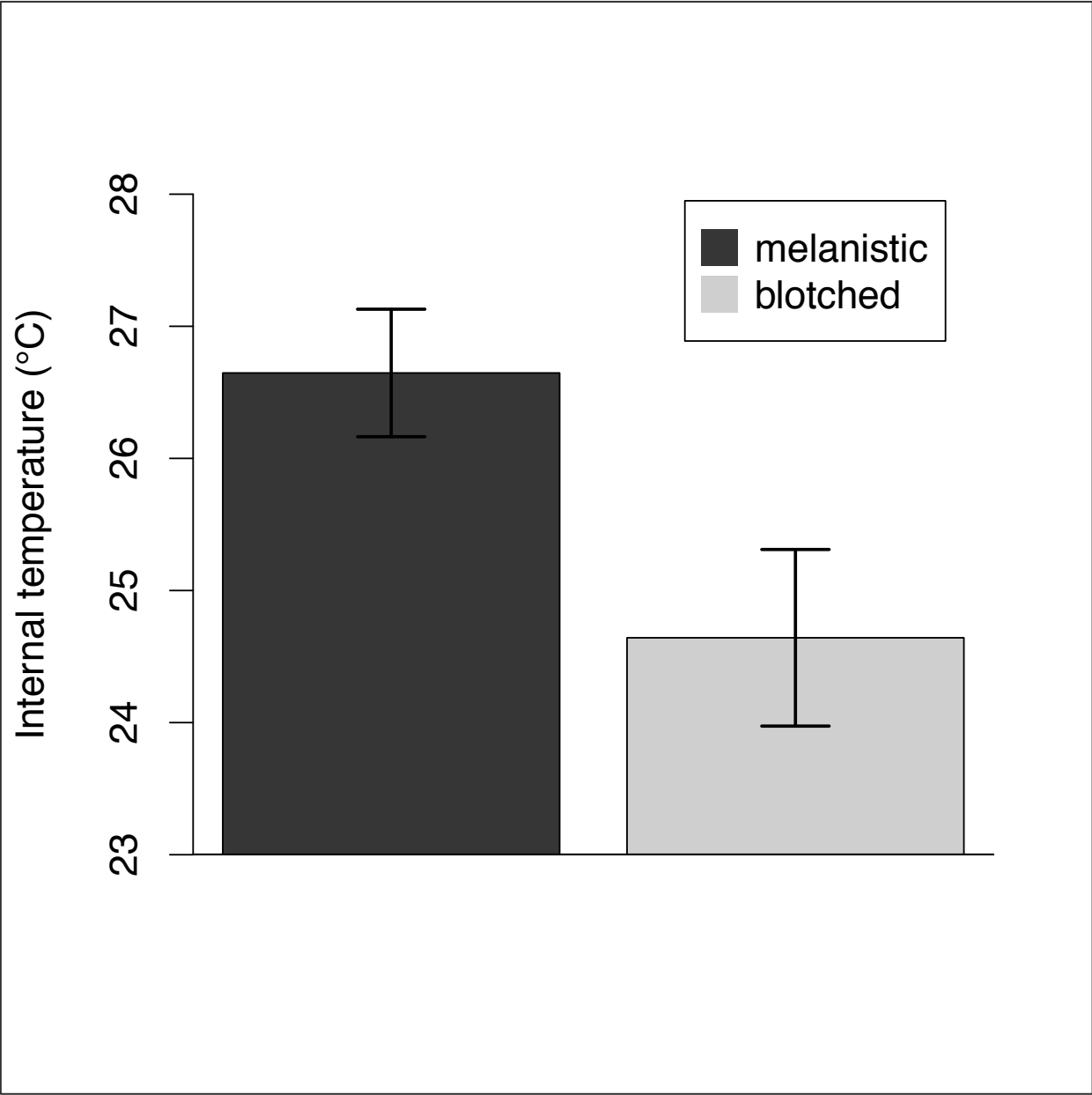


Figure 2

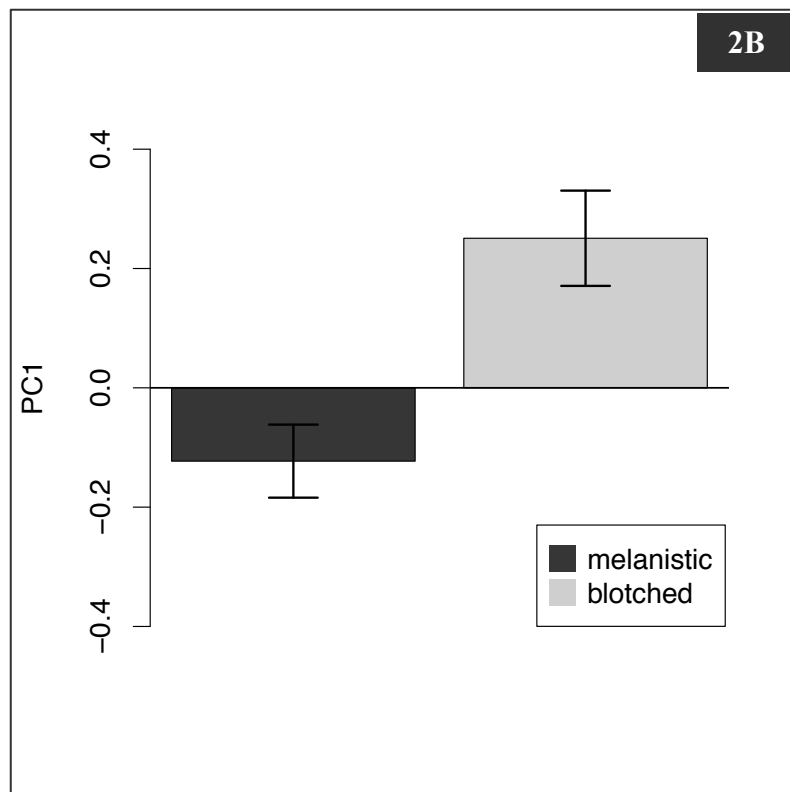
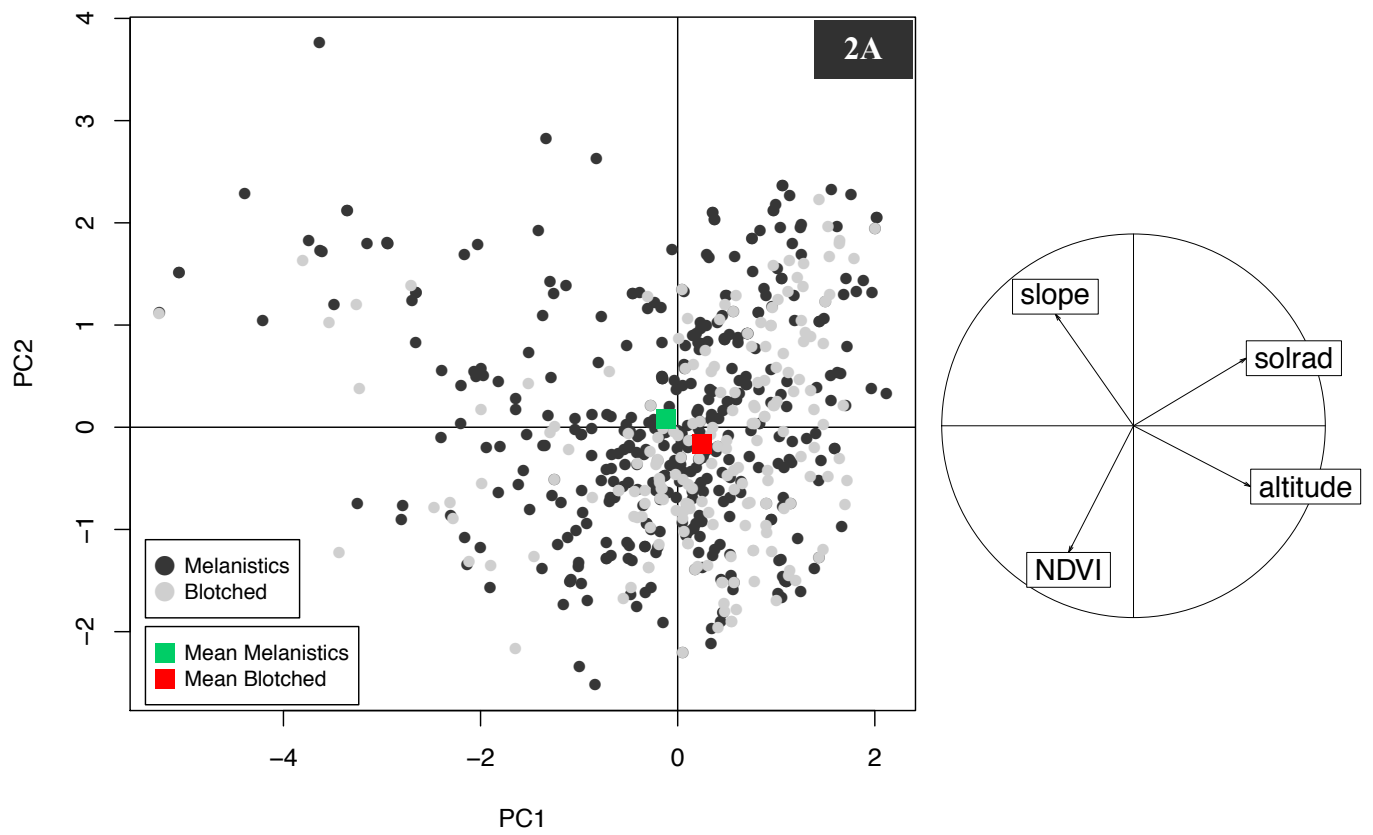


Figure 3

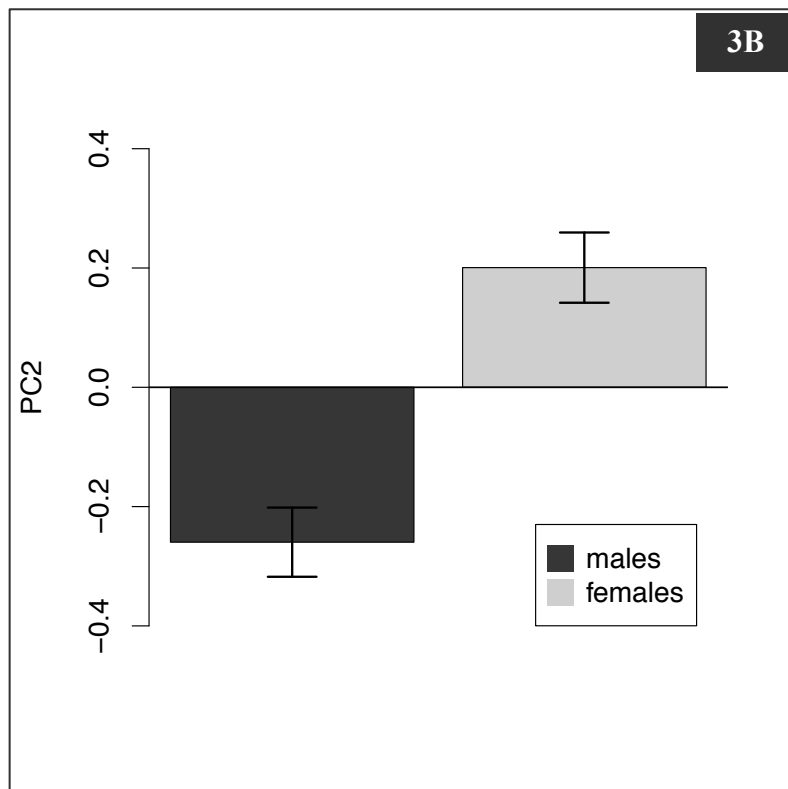
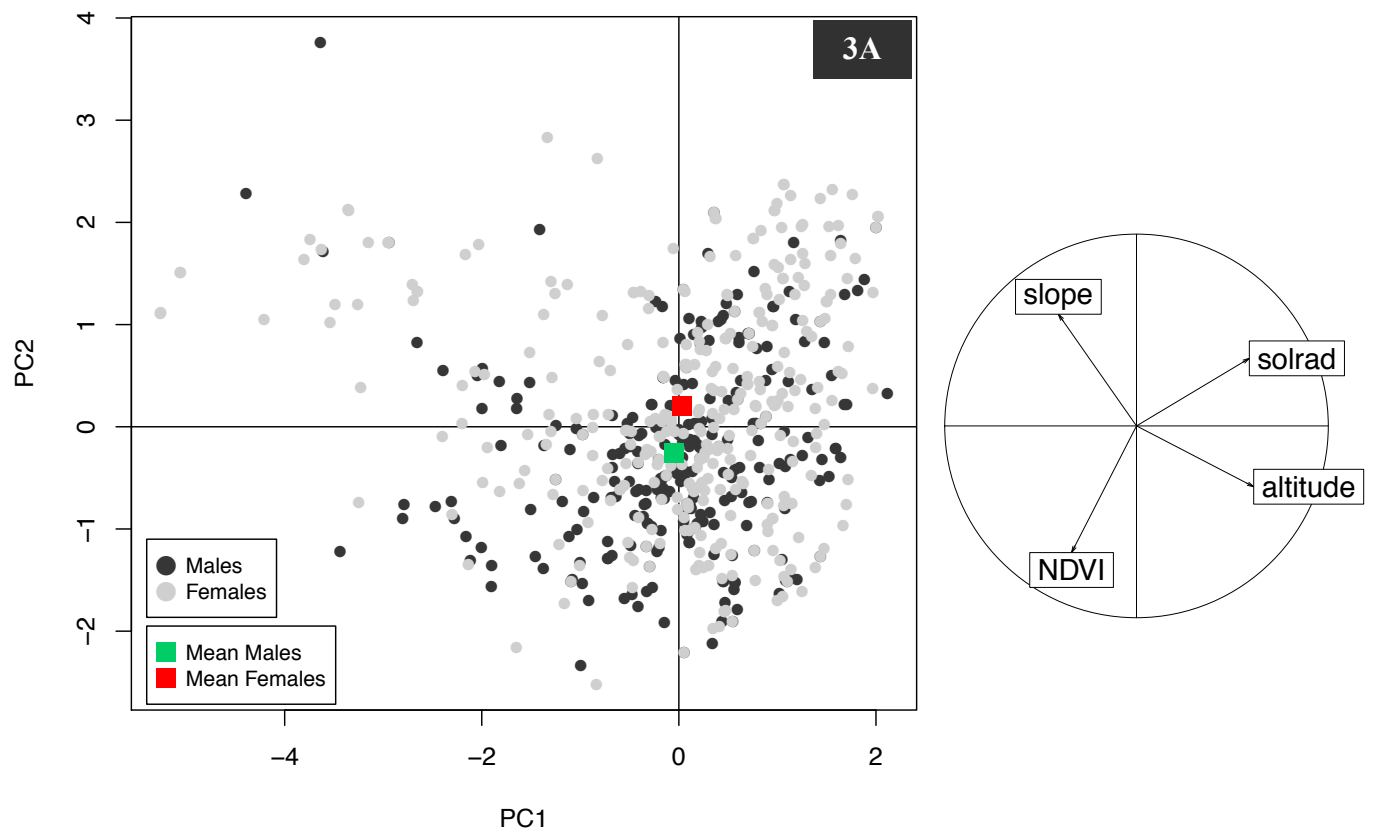


Figure legends

Figure 1 Average internal temperature for melanistic and blotched gravid females measured during 2014. Dark gray bar represents melanistic vipers while light gray one represents blotched vipers. Standard errors have been added on both bars. A significant difference in the average internal temperature can be observed between melanistic (26.65°C) and blotched (24.64°C) vipers.

Figure 2: Figure 2A shows the principal component analysis performed on environmental predictors such as solar radiation, altitude, NDVI index and slope. The analysis included all vipers captured between 2012 and 2014 and these latter were grouped here according to the skin colour. Dark gray points correspond to melanistic vipers while light grey points correspond to blotched ones. Green square represents the average of microhabitat values for melanistic vipers. Red square represents, by its side, the average microhabitat values for blotched vipers. With the help of correlation circle (on the right) it is possible to notice that, along PC1 axis, blotched vipers have greater tendency in choosing microhabitats characterized by higher altitude and solar radiation as well as, by slighter slope and lower vegetal productivity (NDVI), compared to melanistic ones. Figure 2B shows the average values of the first PCA axis (PC1) for melanistic (-0.12) and blotched (0.25) vipers. With the dark gray bar are represented melanistic vipers, while with light gray one are represented blotched ones. Standard errors have been added on both bars. Coherently with Figure 2A it is possible to notice that, based on the first axis, blotched vipers choose microhabitats significantly different compared to melanistic ones.

Figure 3: Figure 3A shows the same principal component analysis illustrated in the Figure 2A, nevertheless vipers are grouped here according to the sex. Dark gray points correspond to males while light grey points correspond to females. Green square represents the average of

595 microhabitat values for males while red square represents the average microhabitat values for
596 females. With the help of correlation circle (on the right) it is possible to notice that, along PC2
597 axis, males have tendency in choosing microhabitats characterized by higher vegetal
598 productivity (NDVI) and slighter slope as well as by higher altitude and lower solar radiation,
599 compared to females. Figure 3B shows the average values of the second PCA axis (PC2) for
600 males (-0.26) and females (0.20). With the dark gray bar are represented males, while with light
601 gray one are represented females. Standard errors have been added on both bars. Coehrently
602 with Figure 3A it is possible to notice that, based on the second axis, males choose
603 microhabitats significantly different compared to females.

604 **Tables**

605 **Table 1A** Results of Generalized Linear Mixed Model performed on vipers collected in 2014. Significant effects on the internal temperature (*P*-
606 value < 0.05) were found for hour, SVL and PC2 as well as for interaction between month and PC2. Individual identity was included in the model
607 as random factor.

	numDF	denDF	Value	Std. Error	t-value	F-value	P-value
Response variable: Internal temperature							
(Intercept)	1	207	-9.68	9.54	-1.01	1.03	0.3116
Month	1	105	0.14	0.15	0.98	0.96	0.3286
Hour	1	105	6.62	2.73	2.43	5.88	0.017
SVL	1	105	5.14	1.53	3.36	11.29	0.0011
PC2	1	105	-1.21	0.44	-2.79	7.77	0.0063
Month x PC2	1	105	0.33	0.13	2.57	6.60	0.0116

609

610 **Table 1B** Results of Generalized Linear Mixed Model performed on gravid females collected in 2014. Significant effect on the internal temperature
611 (*P*-value < 0.05) was found for melanism. Individual identity was included in the model as random factor.

	numDF	denDF	Value	Std. Error	t-value	F-value	P-value
Response variable: Internal temperature							
(Intercept)	1	53	24.66	0.71	34.73	1206.11	<.0001
Melanism [melanistic]	1	41	2.00	0.87	2.31	5.35	0.0258

612

613 **Table 2A** Results of Generalized Linear Mixed Model performed on vipers collected between 2012 and 2014. Significant effect on the first PCA
614 axis (PC1) was found for melanism. Individual identity and year were included in the model as random factors and, after a Bonferroni correction,
615 the significant threshold was lowered at 0.025.

	numDF	denDF	Value	Std. Error	t-value	F-value	P-value
Response variable: 1° PCA axis (PC1)							
(Intercept)	1	390	0.20	0.10	2.10	4.41	0.0364
Melanism [melanistic]	1	390	-0.38	0.12	-3.05	9.32	0.0024

616

617

618 **Table 2B** Results of Generalized Linear Mixed Model performed on vipers collected between 2012 and 2014. Significant effect on the second PCA
619 axis (PC2) was found for sex. Individual identity and year were included in the model as random factors and, after a Bonferroni correction, the
620 significant threshold was lowered at 0.025.

	numDF	denDF	Value	Std. Error	t-value	F-value	P-value
Response variable: 2° PCA axis (PC2)							
(Intercept)	1	390	0.06	0.07	0.94	0.88	0.3476
Sex [male]	1	390	-0.33	0.10	-3.46	11.97	0.0006

621

622 **Appendices**

623 **Appendix 1:** Pictures of a melanistic asp viper on the top and a blotched one on the bottom.

624 Both individuals were photographed in the studied area.



644 **Appendix 2:** Vegetation gradient which characterize the asp viper habitat in the studied area.

645 The following pictures show different microhabitat structures representative of our sampling

646 sites: Pictures 1A and 1B highlight an open area characterized by an almost total absence of

647 woody and shrubby vegetation. In particular picture 1A shows the classics heaps of stones

648 which allow for good sun exposure while providing refuges from predators. Picture 1B shows,

649 on the contrary, a wall lined by iron networks which have characteristics similar to those of the

650 heaps of stones. Picture 2 shows a site marked by the presence of a shrubby vegetation which

651 represent an intermediate microhabitat between the open area and the forest. Finally picture 3

652 shows the forest, marked by a strong presence of woody vegetation and characterized by a more

653 humid and fresh soil, especially during the summer period.



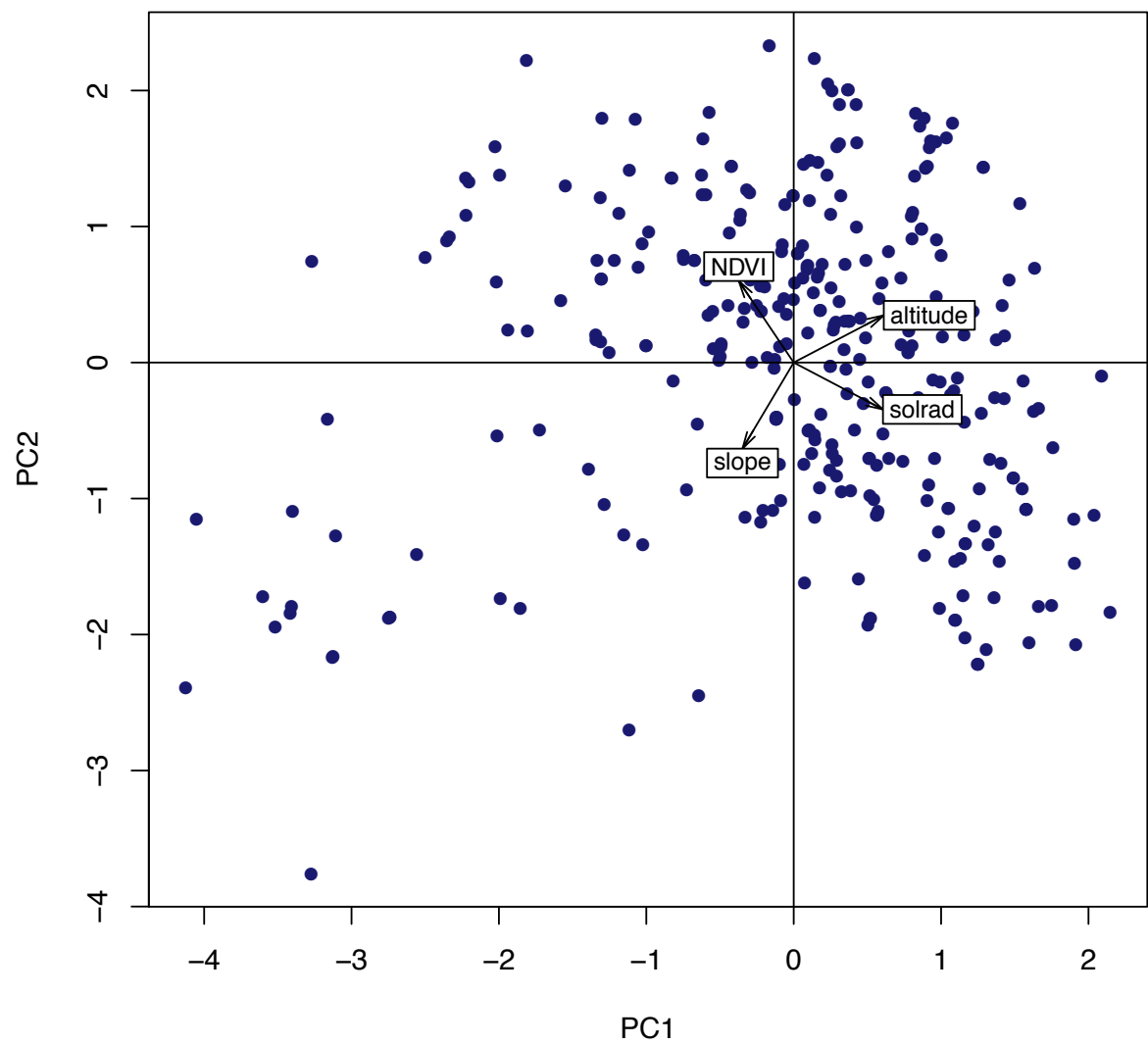
Appendix 3: Principal component analysis (PCA) performed on database including all vipers captured during 2014 and used in the thermoregulation model. Table 1A shows the importance of components listed by PCA, while table 1B highlights the four PCs and their rotation (which are coefficients of the linear combinations of our four variables). Only components highlighted in italic (*PC1* and *PC2*) have been retained in the analysis. Figure 1 finally shows the PCA plot.

Table 1A.

	<i>PC1</i>	<i>PC2</i>	<i>PC3</i>	<i>PC4</i>
Standard deviation	1.2073551	1.1214405	0.8449025	0.7555161
Proportion of variance	0.3644266	0.3144072	0.1784651	0.1427011
Cumulative proportion	0.3644266	0.6788338	0.8572989	1

Table 1B.

	<i>PC1</i>	<i>PC2</i>	<i>PC3</i>	<i>PC4</i>
Altitude	0.611	0.347	0.325	-0.633
Slope	-0.348	-0.633	0.571	-0.390
Solar radiation	0.606	-0.345	0.395	0.599
NDVI 2x2 m	-0.373	0.600	0.642	0.299



Appendix 4: Principal component analysis (PCA) performed on database including all vipers captured between 2012 and 2104 and used in microhabitat models. Table 1A shows the importance of components listed by PCA while table 1B highlights the four PCs and their rotation (which are coefficients of the linear combinations of our four variables). Only components highlighted in italic (*PC1* and *PC2*) have been retained in the analysis.

Table 1A.

	<i>PC1</i>	<i>PC2</i>	PC3	PC4
Standard deviation	1.2133685	1.0526814	0.9092685	0.7699543
Proportion of variance	0.3680658	0.2770345	0.2066923	0.1482074
Cumulative proportion	0.3680658	0.6451003	0.8517926	1

Table 1B.

	<i>PC1</i>	<i>PC2</i>	PC3	PC4
Altitude	0.611	-0.317	0.34	0.641
Slope	-0.407	0.582	0.608	0.354
Solar radiation	0.588	0.352	0.408	-0.603
NDVI 2x2 m	-0.339	-0.66	0.59	-0.317