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Leukocyte formula of the Walser's Viper (*Vipera walser)*

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Abstract. *Vipera walser* is a recently assessed species of North-Western Italian Alps, that has been regarded as an isolated population of *V. berus* until 2016, when it has been identified as a separate taxonomical unit according to molecular markers. Due to its restricted and fragmented range and the potential threat of climate change in mountain systems, it complies with the IUCN criteria to be classified as EN. In order to investigate, in part, the health status of this taxon, we have performed blood smears to describe whether a haematological parameter such as leukocytes is consistent with those of more widespread viperids of the Italian peninsula. Overall, we sampled 20 Walser's Vipers across the species range and characterised leukocyte formula. We found that lymphocytes were the

most common (~ 70% of total leukocytes). Eosinophils and heterophils were less abundant, while neutrophils and monocytes are the least represented. Our data is in accordance with that of other European viperids.

Keywords. *Vipera walser*, leukocyte differential count.

Vipera walser GHIELMI, MENEGON, MARSDEN, LADDAGA & URSENBACHER 2016 is a relict viper endemic to Alpine areas of North-Eastern Piedmont (Ghielmi et al., 2016). This viper lives exclusively in high altitude valleys up to about 2500 metres, in ecologically particular contexts, characterised by some of the highest rainfall in the entire Alpine region and an average annual temperature below 10 °C (Mercalli et al., 2008; Osservatorio Di Oropa - Meteo, 2022).

V. walser has an extremely limited geographical range, with a distribution area (Extent of occurrence - EOO) estimated at <1000 km^2 (Ghielmi et al., 2016). Therefore, it should be classified as "Endangered" (EN) according to the criteria of the IUCN Red List (2014) B1a/B2a, but the species conservation status has not been assessed yet. Given that the range of this species is strongly fragmented and that the area actually occupied (Area of occupancy - AOO) is less than 500 km^2, *V. walser* turns out to be one of the most threatened vipers in the world (Ghielmi et al., 2016). However, several studies are currently underway to clarify its taxonomic status, as recently its validity as a species has been questioned (Speybroeck et al., 2020; Doniol-Valcroze et al., 2021; Vanzo et al. 2024).

The population is already fragmented in two main subpopulations and, presumably, the complex topography of ridges and valleys might further increase the isolation among populations, as it was found in *V. berus* (Ursenbacher et al., 2009). Furthermore, such fragmentation implies an additional intrinsic threat factor, i.e., limited genetic variability compared to that of more widespread European vipers such as the adder and the asp viper (Ursenbacher et al., 2006; Ursenbacher, Conelli, et al., 2006; Ferchaud et al., 2011; Ghielmi et al., 2016). *V. walser* is considered a relict species that occurs in a very restricted range, so it can be regarded as an evolutionary dead end (Allendorf et al., 2012). *V. walser* is potentially threatened by decreasing habitat suitability due to both climate change (Ghielmi et al., 2016), and the abandonment of areas involved in agropastoral activities leading to natural reforestation (Carlson et al., 2014; Garbarino et al., 2014).

The presence of potentially pathological or stressful condition can significantly impact local and restricted populations, especially in endangered species (Schumacher, 2006; Buttke et al., 2015; Thomas et al., 2019). The leukocyte formula can be an important tool to assess the presence of inflammation and infection and can be used as an index of general stress and immune status of the animal (Blaxhall, 1972). In particular, in reptiles, heterophilia (increase in heterophils) and lymphocytopenia (decrease in lymphocytes) are the outcome of stress conditions; therefore, the relative proportion of heterophils over lymphocytes (i.e., H/L ratio) is often used as a composite measure of stress response (Davis et al., 2008; Stacy et al., 2011). Consequently, being able to provide baseline values of haematological parameters from wild populations is essential to evaluate possible threats and in species conservation (Stacy et al., 2011; Sacchi et al., 2020).

In this scenario, we have assessed for the first time the leukocyte formula of *V. walser*, in order to provide benchmarks that may be useful for assessing the health status of individuals of this species. Sampling took place via field surveys performed between May and October 2021: 20 adult individuals (13♀ and 7♂) of *V. walser* were captured across the entire distribution range of the species (as in Ghielmi et al., 2016). Fresh blood was sampled through tail clipping using surgical scissors (Duguy, 1970; Brown & Shine, 2018, 2022). This way to draw blood was not specifically designed for leukocyte analyses, but was a by-product of the methodology used for high quality DNA collection, which is the topic of another research project on the target species. Afterwards, the wounds were thoroughly disinfected with iodine tincture and eventually the individuals were released in their capture site. From each blood draw, a single-layer cell film was produced by depositing a small drop of blood at one end of the glass slide and placing a second glass slide close to the drop, slanted by 30-40 degrees, allowing the drop to adhere to the entire margin of the slide for capillarity (Nardini &

Girolamo, 2017). The latter glass was slid gently and quickly along the former to create a blood smear that was air-dried. Subsequently, smears were coloured using the May-Grünwald/Giemsa stain and stabilised through Entellan® (Vu et al., 2021). Two-five blood smears were prepared for each snake, and the best one was visually scanned by performing zig-zag scans across the slide. Leukocytes were classified as heterophiles, eosinophils, basophiles, neutrophils, lymphocytes, and monocytes (Fig. 1). These procedures were carried out using 40x magnification on an Optika B-383PLi microscope, distinguishing and counting on average 154 ± 8.9 leukocytes per sample.

Lymphocytes were the most common leukocytes (over 70% of total leukocytes). Eosinophils and heterophils were the second and third most abundant components. Neutrophils and monocytes are the least represented (Table 1). To test for differences in relative abundance of cell types between sexes, a non-parametric Mann-Whitney test was performed. No statistically significant difference was detected between sexes for all cell types ($W < 59$, $P > 0.29$).

Our investigation on *V. walser* is a first attempt to provide a benchmark of the leukocyte formula of wild populations in this species. Our data is consistent with available literature for other snakes from Europe (Duguy, 1970; Lisičić et al., 2013; Baycan et al., 2022) and South America (Troiano et al., 1997; Troiano et al., 1999; Grego et al., 2006, Carvalho et al., 2016), including Viperidae, and three major snake families (Colubridae, Pythonidae, and Boidae; Table 2). Notably, Lymphocytes are generally the most abundant white blood cell type and, consistently, heterophils and monocytes are generally the second- and third-most abundant ones, respectively. However, it is necessary to point out that across literature authors tend to identify and quantify different cell types according to necessity and interest; for instance, azurophils are sometimes identified as immature monocytes, according to cytochemical similarities (Lisičić et al., 2013), and used in their place (Ozzetti et al., 2015; Carvalho et al., 2016). In this matter, authors are not in accordance with one another and therefore interpreting and comparing leukograms can be sometimes complicated due to the terminology applied for cell type classification.

The implementation of heterophil and lymphocyte counts in past research has been correlated to stress so that higher H/L ratios are generally associated to higher stress levels (Davis et al. 2008). According to the published data we retrieved, a major variability in this measure was found as it can vary from low ratios (~ 0.11 in Carvalho et al., 2016 and our work) to very high values (~2.3 in Quadrini et al., 2018). Therefore, lacking marked clinical effects that correlate with higher values, we suggest using cautiously ratios of such kind to provide information about the health status of wild or captive populations of snakes. Consequently, we highlight the importance of the implementation of shared protocol and methodologies to undertake broad scale haematological studies of snake populations and to assess their relation to health and stress conditions.

In conclusion, with this work we provide, for the first time, information on some haematological parameters of the Walser's Viper, an endemic and endangered species of the Italian Alps, that might be of interest for future conservation measures. However, this work does not fully address this matter as it requires further investigations on health condition measures such as Body Condition Indices as well as comparative studies that take into account how sister species cope with the same threats in similar environmental conditions.

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TABLES

Table 1. Table of the leukocyte formula of the 13 females and 7 males of *V. walser* sampled for this study. For each leukocyte cell type, mean ± SD and range are shown.

% of	**Females**		**Males**		**Total**	
cell type	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Heterophils	6.9 ± 3.1	2.0 – 12.9	10.2 ± 6.6	2.9 – 19.3	8.0 ± 4.8	2.0 – 19.3
Eosinophils	10.4 ± 3.5	1.8 – 6.9	14.0 ± 8.5	3.9 – 27.9	11.7 ± 5.8	3.9 – 27.9
Basophils	4.3 ± 3.4	0.0 – 14-0	6.1 ± 5.7	1.3 – 18.0	4.9 ± 4.3	0.0 – 18.0
Monocytes	1.9 ± 2.6	0.0 – 8.5	0.8 ± 1.0	0.0 – 2.6	1.5 ± 2.2	0.0 – 8.5
Lymphocytes	74.8 ± 6.0	65.3 – 88.2	67.9 ± 14.1	46.6 – 85.4	72.4 ± 9.9	46.6 – 88.2
Neutrophils	1.7 ± 2.5	0.0 – 9.4	1.0 ± 1.5	0.0 – 3.6	1.4 ± 2.2	0.0 – 9.4

Table 2. Comparative table of the White Blood Cells cell type percentages among data available in literature and our study. WBC cell types are shown as follows: Lymphocytes - L, Heterophils - H, Eosinophils - E, Basophils - B, Monocytes - M, Neutrophils - N. For each cell type, data are reported as percentage mean ± SD when available, otherwise percentage range is provided. Data reported for Monocytes in italics refers to works where they were classified as azurophils.

Family	**species**	**cell type %**						**reference**
		L	**H**	**E**	**B**	**M**	**N**	
Viperidae	*Vipera walser*	72.4 ± 9.9	8.0 ± 4.8	11.7 ± 5.8	4.9 ± 4.3	1.5 ± 2.2	1.4 ± 2.2	this work
Viperidae	*Vipera ammodytes*	52.3 ± 8.7	12.6 ± 3.2	22.6 ± 4	5.3 ± 4.9	7	/	Baycan et al., 2022
Viperidae	*Vipera ammodytes*	19.61 - 65.17 (♂) 35.52 - 67.14 (♀)	4.52 - 48.02 (♂) 7.46 - 50.24 (♀)	4.98 - 32.35 (♂) 1.48 - 21.7 (♀)	0 - 4.83 (♂) 0 - 4.48 (♀)	*6.9 - 50.79 (♂)* *11.44 - 42.21 (♀)*	/	Lisičić et al. 2013
Viperidae	*Bothrops ammodytoides*	52.2 ± 6.9	12.2 ± 1.3	16.3 ± 1.8	1 ± 0.3	8.2 ± 0.9	/	Troiano et al., 1999
Colubridae	*Oxyrhopus guibei*	39.1 ± 11.4	15.1 ± 10.8	/	8 ± 5.7	*37.8 ± 10.8*	/	Ozzetti et al., 2015
Colubridae	*Xenodon neuwiedii*	36.9 ± 10.5	42.9 ± 10.3	/	7.9 ± 5.3	*42.9 ± 10.3*	/	Ozzetti et al. 2015
Boidae	*Corallus hortulanus*	25 ± 8.18	37 ± 14.87	/	0.8 ± 1.21	1.4 ± 1.8	/	Quadrini et al. 2018
Pythonidae	*Python bivittatus*	18.22 ± 12.56	42 ± 12.52	1 ± 1.94	0.22 ± 0.44	0.33 ± 0.71	/	Quadrini et al. 2018
Boidae Viperidae	*Boa constrictor, Bothrops jararaca, Crotalus durissus*	58.6 - 78.2	6.6 - 17.1	/	/	*15 - 24.8*	/	Carvalho et al. 2016

CAPTIONS TO FIGURE

Figure 1. Different leukocyte cell types detected in a sampled blood smear along the visual transects. Respectively, in each panel are shown: a) large heterophile (dot-dashed circle), a lymphocyte (dashed circle) and a blood platelet (solid circle); b) monocyte; c) basophile; d) heterophile; e) eosinophile.