Ecological Observations on Hybrid Populations of European Plethodontid Salamanders, Genus *Speleomantes*

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Abstract: *Speleomantes* are the only plethodontid salamanders present in Europe. Multiple studies have been performed to investigate the trophic niche of the eight *Speleomantes* species, but none of these studies included hybrid populations. For the first time, we studied the trophic niche of five *Speleomantes* hybrid populations. Each population was surveyed twice in 2020, and stomach flushing was performed on each captured salamander; stomach flushing is a harmless technique that allows stomach contents to be inspected. We also assessed the potential divergence in size and body condition between natural and introduced hybrids, and their parental species. Previously collected data on *Speleomantes* were included to increase the robustness of these analyses. In only 33 out of 134 sampled hybrid *Speleomantes* we recognized 81 items belonging to 11 prey categories. The frequency of empty stomachs was higher in females and individuals from natural hybrid populations, whereas the largest number of prey was consumed by males. We compared the total length and body condition of 685 adult salamanders belonging to three types of hybrids and three parental (sub)species. Three group of salamanders (one hybrid and two parental species) showed significantly larger size, whereas no difference in body condition was observed. This study provided novel ecological information on *Speleomantes* hybrid populations. We also provided insights into the potential divergence between hybrids and parental species in terms of size and body condition. We discuss our findings, and formulate several hypotheses that should be tested in the future.

Keywords: *Speleomantes*; *Hydromantes*; trophic niche; body condition; cave biology; biospeleology; parental species; diet; size; capture-mark-recapture

1. Introduction

European cave salamanders of the genus *Speleomantes* are the only plethodontids living in the Europe, and are almost all endemic to Italy [1]. Seven of the eight *Speleomantes* species (*S. ambrosii*, *S. italicus*, *S. flavus*, *S. supramontis*, *S. imperialis*, *S. sarrabusensis*, *S. genei*) live exclusively in Italy, while the range of one species (*S. strinatii*) extends to part of French Provence [1]. Each species is distributed in a well-defined area, and no range overlap exists; *Speleomantes* distribution is likely shaped by geomorphology [2,3]. For *S.*

ambrosii, the river Magra marks the separation of the two allopatric subspecies: the western *S. ambrosii ambrosii* and the eastern *S. a. bianchii* [1,4]. Although some phenotypic variability can be observed among *Speleomantes* [5–7], a valid method to phenotypically distinguish among species/subspecies is still lacking, thus their identification is based mostly on geography [1,3]. The southern distribution of the latter reaches the norther limit of *S. italicus*, creating a narrow contact zone in which viable hybrid populations are found [1,8] (see Figure 1 of Ref. [8]) These natural hybrid populations exhibit some genetic divergence, which is mainly influenced by the relative abundance of individuals of each parental species: in the northern part of the range there are populations of *S. a. bianchii* introgressed with *S. italicus*, whereas in the southern area, the opposite occurs [8]. However, hybrids do not show a clear divergence of phenotypic characters from their parental species; therefore, hybrids can also only be recognized on the basis of their geographic distribution [8,9]. In addition to this natural hybrid zone, hybrids between *S. ambrosii* and *S. italicus* are also related to a human-mediated translocation. In 1983, for scientific purposes individuals of both *S. italicus* and *S. a. ambrosii* were introduced into a natural cave in southern Tuscany, outside the natural range of *Speleomantes* [1,10]. Thirty years after its introduction, the population was genetically characterized and none of the individuals had a pure genotype. For 77% of individuals, the majority of alleles (>75%) matched alleles specific to *S. a. ambrosii*; for 6% of individuals, the majority of alleles matched those of *S. italicus*; and for 16% of individuals, alleles of *S. a. ambrosii* and of *S. italicus* were recombined [11]. However, the lack of ecological information on this population prevents us from evaluating the potential difference between these non-natural hybrids and the hybrids living in the natural hybrid zone, and to assess the trophic relationships between these populations and the local fauna [8,12]. Here we provide the first assessment of the size, body condition, and diet of both natural and introduced hybrid populations of *Speleomantes*.
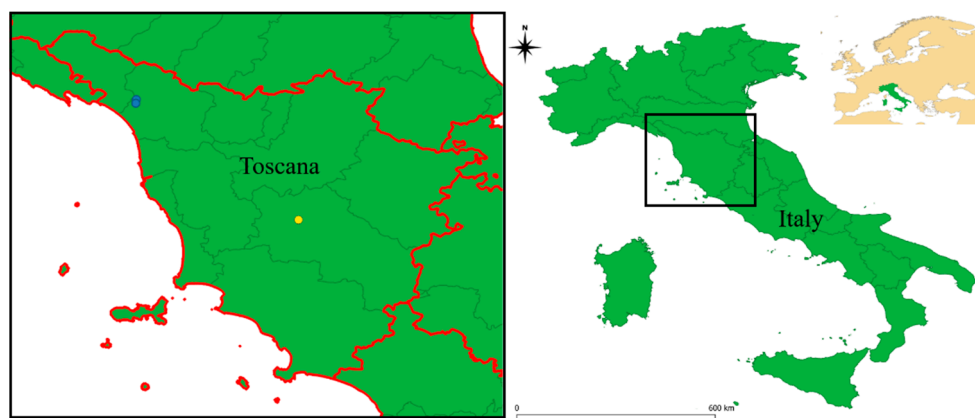

Figure 1. Map indicating the sampled populations. Blue labels indicate natural hybrid populations, whereas yellow indicates those introduced outside the natural distribution of *Speleomantes*. The coordinates of the surveyed sites are not provided to increase species protection [13].

2. Materials and Methods

In 2020 we surveyed five hybrid populations of *Speleomantes* that inhabit natural caves (Figure 1); each population was surveyed twice, once before (29 June–14 July) and after (3–10 September) the aestivation period [1].

Three populations are located within the natural hybrid zone between *S. italicus* and *S. ambrosii bianchii* occurring in north-western Tuscany (Province of Lucca); two populations included introgressed *S. italicus* with >10% of *S. ambrosii* alleles, whereas the other is a *S. ambrosii* population introgressed with >10% of *S. italicus* alleles [8]. The natural hybrid populations were selected following the genetic characterization of Ruggi, Cimmaruta, Forti and Nascetti [9]. The two other hybrid populations are found in southern Tuscany (Province of Siena), where cave salamanders are not native and have been introduced [1].

One of these two populations was discovered during this study. In the surroundings of the cave where *Speleomantes* were introduced [10], some authors (CC, EL, SM) have found and explored another cave about 53 m in a straight line from the previous one; because several *Speleomantes* were observed during the exploration, the individuals from this second cave were considered to be an additional population [14]. Surveys and animal handling were performed by taking measures to avoid the spread of pathogens (i.e., using disposable gloves and disinfecting boots and equipment after each survey). During each survey, all captured salamanders were placed in a perforated plastic box (60 × 40 × 20 cm). After salamanders were captured, we recorded the following data in sequence: individual sex (males were recognized by the presence of the distinctive mental gland, females were salamanders with SVL ≥ 40 mm without the mental gland, and remaining salamanders were considered juveniles) [1]; weight (using a digital scale, 0.01 g); a photo was taken from the dorsal view of individuals positioned along a reference card [6]; the harmless stomach flushing technique was used to evaluate *Speleomantes* foraging activity [15]. All salamanders were released at their collection points. The photos were analyzed with ImageJ software to measure the total length (TL) of the salamanders and estimate the snout-vent length (SVL) [16]. Stomach contents were preserved in a 75% ethanol solution and subsequently observed under an optical microscope, where prey items were recognized and counted following [17]. When we were unable to recognize any item at the order level, we considered the content as "unidentifiable"; when no remains were found, the stomach was considered "empty". The dorsal pattern of salamanders was used for individual recognition [18].

We used binomial Generalized Linear Mixed Models (GLMMs) to assess the potential effects that the considered variables may have on the stomach condition. Four individuals were captured twice, so we only used data from their first capture. We used stomach condition (empty/full) as the dependent variable, and the independent variables were salamander sex (male, female, juvenile) and hybrid identity (natural = *S. a. bianchii* × *S. italicus*; introduced = *S. a. ambrosii* × *S. italicus*); because the frequency of empty stomach changes over time [19], the survey month was added, together with the identity of the cave, as a random variable. Similarly, we used a GLMM to assess the potential correlation between the number of recognized prey and the independent variables mentioned above; cave and individual identity were still used as random variables.

We also performed a comprehensive analysis by combining these data with previously published datasets [5,8,20] to evaluate differences in size and body condition between hybrids and parental species. In these previous studies, the threshold to discriminate between adults and juvenile was set on the base of the smallest TL measured among males, which was 69 mm; in this study the smallest male had a SVL of 44 mm and TL of 69 mm. For each site, only the survey with the highest number of measured salamanders was considered, with the exception of hybrid data, in which pattern recognition [18] allowed the inclusion of individuals captured for the first time during the second survey. We used a Linear Mixed Model (LMM) to evaluate the potential differences in adult size (TL) between different groups of salamanders (*S. a. ambrosii*, *S. a. bianchii*, *S. italicus*, *S. a. ambrosii* × *S. italicus*, *S. a. bianchii* × *S. italicus*). A Shapiro–Wilk test showed a non-normal distribution of data related to *Speleomantes* size (log-transformed TL, W = 0.98, $p < 0.001$); however, LMM is appropriate for the analysis of non-normal distributed data [21]. The log-transformed TL was used as a dependent variable, and the group of salamanders as the independent variable. Considering the natural divergence in maximum size occurring between males and females [1], the sex of salamanders was used as a random factor. We used the Residual Index (RI) as a proxy of the body condition of the salamanders; this index provides information on the difference between the observed and the expected body mass [22,23]. To calculate the RI, we first log-transformed weight and TL, and then extracted residuals from the regression analysis for each species/hybrid group, in order to avoid bias due to different size [22,23]. *Speleomantes* body condition peaks during the foraging periods (i.e., when precipitation is higher and temperature relatively cold), and is

poorest during inactivity periods (i.e., when climatic conditions are too hot and dry), when the salamanders mostly consume energy that was previously stored [1,24,25]. Therefore, in this analysis we only used data collected before the aestivation period (June–July). We used an LMM to assess the potential correlation between the RI (dependent variable) and two independent variables: the sex of the salamanders and the species group. Considering that data were collected over different periods, we included the month and year of the survey, in addition to the identity of the cave, as random factors. The significance of GLMM and LMM variables was tested with a likelihood ratio test.

3. Results

In the hybrid populations, we obtained 138 salamander detections corresponding to 134 individuals; four individuals (one male and three females) were observed twice. The size of the recaptured individuals (both SVL and TL) did not change between the two surveys: the difference between the first and second measurement was <2 mm; this difference is comparable to measurement error [16]. Most of the individuals sampled (90) had an empty stomach. The frequency of empty stomachs was significantly different between sexes (χ^2 = 7.31, df = 2, p = 0.026) and type of hybrid (β = 4.41, SE = 1.14, χ^2 = 14.91, df = 1, p < 0.001); the frequency of empty stomachs was higher in females and individuals from natural hybrid populations. Eleven individuals had stomach contents in a state of advanced digestion and, therefore, the contents were considered not identifiable. We were able to recognize a total of 81 prey items from 33 individuals; the recognized prey belonged to 11 different categories: Sarcoptiformes (1), Mesostigmata (1), Araneae (4), Pseudoscorpiones (4), Polydesmida (3), Isopoda (8), Hemiptera (1), Hymenoptera (1), Coleoptera (3), Coleoptera_larva (1), and Diptera (55). Diptera were the most consumed prey: they were observed in 32 individuals, representing 67% of recognized prey. The number of prey consumed was significantly affected by the sex of salamanders (χ^2 = 10.74, df = 2, p = 0.005); the largest number of prey was consumed by males.

When we combined the data of hybrid populations with those from previous surveys, we obtained data on the size and body condition of 678 salamanders (additional details in Figure 2). The maximum and average ($\pm$SD) size (TL) measured for the salamander groups considered were: *S. a. ambrosii*, females max. 125 and average 91 ($\pm$14) mm, and males max. 105 and average 92 ($\pm$6) mm; *S. a. bianchii*, females max. 114 and average 89 ($\pm$12) mm, and males max. 124 and average 105 ($\pm$12) mm; *S. italicus*, females max. 120 and average 94 ($\pm$13) mm, and males max. 118 and average 101 ($\pm$8) mm; *S. a. bianchii* × *S. italicus*, females max. 119 and average 90 ($\pm$15) mm, and males max. 119 and average 95 ($\pm$8) mm; *S. a. ambrosii* × *S. italicus*, females max. 134 and average 98 ($\pm$20) mm, and males max. 108 and average 103 ($\pm$2) mm. The size of the adult salamanders significantly differed between the groups ($F_{4,506}$ = 4.61, p = 0.001); *S. italicus*, *S. a. bianchii*, and *S. a. ambrosii* × *S. italicus* hybrids had the largest size (Figure 2). No significant differences in body condition were observed between sexes ($F_{2,641}$ = 0.69, p = 0.5) or species groups ($F_{4,25}$ = 0.16, p = 0.956).
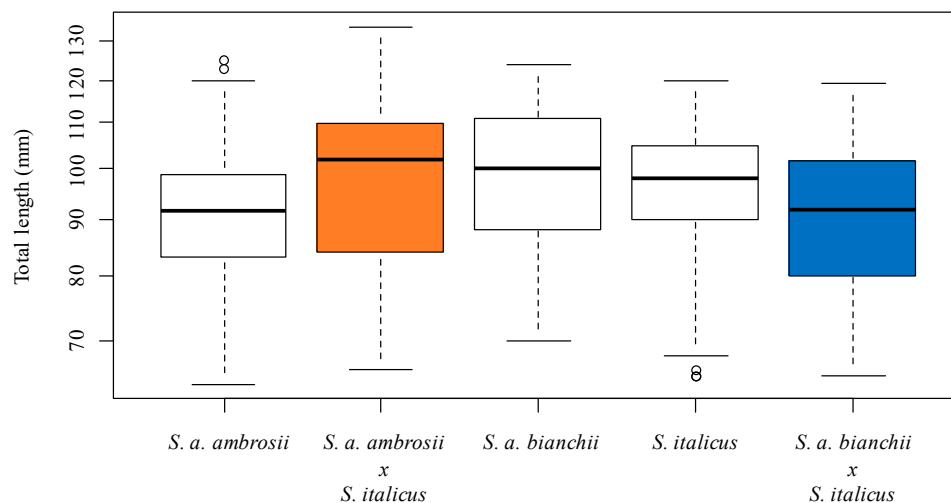
Size of adult *Speleomantes*

Figure 2. Boxplots showing the average size (TL) of adult *Speleomantes* considered in this study: *S. a. ambrosii* (163), *S. a. ambrosii* × *S. italicus* hybrids (28), *S. a. bianchii* (46), *S. italicus* (150), *S. italicus* × *S. a. bianchii* hybrids (125). The red box indicates introduced hybrids, blue natural hybrids, while in white the parental species.

4. Discussion

Studies on hybrid populations of *Speleomantes* have been extremely limited [8,11], leaving their morphological and ecological traits largely unknown. Despite our limited sample size, especially in regards to diet, we were able to obtain interesting information to provide the basis for future research. For example, we provide the first documentation relating to the natural colonization of a new environment performed by the introduced *Speleomantes*. Forty years after their release [10] the salamanders managed to occupy at least a nearby subterranean environment with a stable population. It is therefore of great importance to regularly monitor these hybrid populations to see if they will continue to extend their distribution.

The trophic niche of *Speleomantes* is subject to temporal variations [19,26] and is therefore strongly influenced by the fluctuating availability of potential prey [27]. With this study we were able to gather only limited information on the diet of *Speleomantes* hybrids. Most of the prey consumed were Diptera, which can be highly abundant near the entrance of subterranean environments, especially during the hot season [28,29]. This is in agreement with previous studies on the *Speleomantes* diet [16,30,31]. We also observed generally higher male foraging activity, whereas natural hybrids appear to forage less. Natural hybrids are distributed in a small area of the Apuan Alps (north-western Tuscany), a territory characterized by different lithology and vegetation compared to that of other studied salamanders [1,32,33]. Diverging environmental characteristics may offer different prey availability or simply alter the peak of foraging activity in these *Speleomantes* populations [24,27]. Therefore, further and prolonged investigations on multiple hybrid populations are needed to better delineate their trophic niche [17], evaluate potential divergences [27,30], and evaluate whether they increase foraging opportunities by expanding their microclimatic niche.

A further limitation of this study is the lack of genetic information on hybrid *Speleomantes* populations. Hybrid populations can undergo complex evolutionary phenomena, for example, with selective advantages for some components of their genome. To date, the available information on the genetic characteristics of hybrid populations is limited to allozyme data, which cannot capture the complexity of genomic processes [8]. Collecting genome-wide information on salamanders is challenging because their very large genome size makes the application of different genomic tools, such as RAD sequencing, problem-

atic. However, recent developments can allow more affordable analysis of genome-wide variation, even in species with large genomes, and in the coming years this could improve our understanding of evolutionary processes affecting these populations [31,32].

In this study we observed larger size in introduced hybrids (*S. a. ambrosii* × *S. italicus*), *S. italicus*, and the *S. a. bianchii* subspecies. We do not have data on the potential genetic effect on the size of hybrid populations. However, ecological conditions are more likely to play an important role in driving the evolution of this adaptive trait. It has been shown that *Speleomantes* are in thermal equilibrium with their surrounding environment, and that larger individuals require more time to thermoconform, and are thus potentially able to exploit a less suitable microclimate for a longer period [8,20,33], allowing them to extend their activity period and increase foraging [24]. The larger size observed in introduced hybrids may be the result of a warmer and drier local climate, whereas the smaller size of natural hybrids may have been determined by the local colder and moister climatic conditions. This remains a hypothesis that deserves further investigation. Furthermore, introduced hybrids showed a lower frequency of empty stomachs compared to other population; thus, it is possible that higher food availability allows for better feeding and growth. However, this hypothesis is not supported by the lack of differences in the body condition index. Considering the larger size of the introduced hybrids (and the associated better tolerance of harsher conditions), we recommend regular monitoring of these populations to better control their range expansion, especially in the context of global warming [34,35].

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Data Availability Statement: Data analysed in this study can be found at this link https://figshare.com/s/387b39c7de65dfc32298.

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