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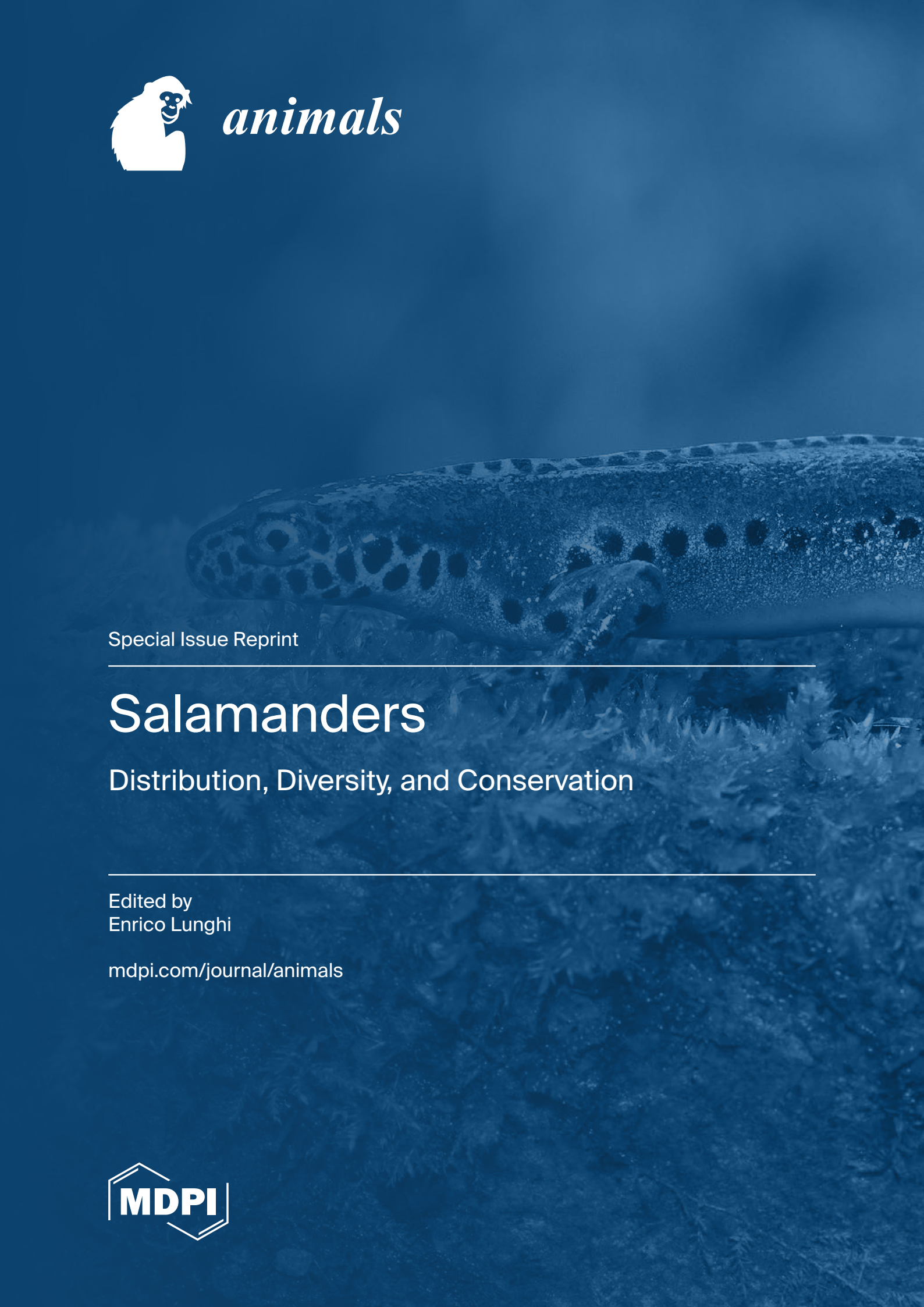

Special Issue Reprint

Salamanders

Distribution, Diversity, and Conservation

Edited by
Enrico Lunghi

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Salamanders: Distribution, Diversity, and Conservation

Salamanders: Distribution, Diversity, and Conservation

Editor

Enrico Lunghi

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Editor
Enrico Lunghi
Department of Life, Health and
Environmental Sciences (MeSVA)
University of L'Aquila
L'Aquila
Italy

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

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Article

Evolutionary Insights into the Relationship of Frogs, Salamanders, and Caecilians and Their Adaptive Traits, with an Emphasis on Salamander Regeneration and Longevity

Bin Lu

Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China; lvbin@cib.ac.cn

Simple Summary: Amphibians have unique traits, such as regeneration and longevity in salamanders, frequent vocalization in frogs, and degenerative vision in caecilians. The genetic basis of these traits is not well understood. This study aimed to investigate the genetic changes underlying these unique traits, especially salamanders' regeneration and longevity, by comparing the genes of amphibians to other vertebrates. I found that salamander genomes have undergone accelerated adaptive evolution, especially for development-related genes. Several salamanders' genes are under positive selection and/or share mutations with other long-lived and regenerative vertebrates, suggesting that these genes are important for these unique traits. This study could help us to better understand the mechanisms of regeneration and aging, which could lead to the development of new ways to improve human health and well-being.

Abstract: The extant amphibians have developed uncanny abilities to adapt to their environment. I compared the genes of amphibians to those of other vertebrates to investigate the genetic changes underlying their unique traits, especially salamanders' regeneration and longevity. Using the well-supported Batrachia tree, I found that salamander genomes have undergone accelerated adaptive evolution, especially for development-related genes. The group-based comparison showed that several genes are under positive selection, rapid evolution, and unexpected parallel evolution with traits shared by distantly related species, such as the tail-regenerative lizard and the longer-lived naked mole rat. The genes, such as *EEF1E1*, *PAFAH1B1*, and *OGFR*, may be involved in salamander regeneration, as they are involved in the apoptotic process, blastema formation, and cell proliferation, respectively. The genes *PCNA* and *SIRT1* may be involved in extending lifespan, as they are involved in DNA repair and histone modification, respectively. Some genes, such as *PCNA* and *OGFR*, have dual roles in regeneration and aging, which suggests that these two processes are interconnected. My experiment validated the time course differential expression pattern of *SERPINI1* and *OGFR*, two genes that have evolved in parallel in salamanders and lizards during the regeneration process of salamander limbs. In addition, I found several candidate genes responsible for frogs' frequent vocalization and caecilians' degenerative vision. This study provides much-needed insights into the processes of regeneration and aging, and the discovery of the critical genes paves the way for further functional analysis, which could open up new avenues for exploiting the genetic potential of humans and improving human well-being.

Keywords: extant amphibians; evolution; regeneration; aging

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1. Introduction

Modern amphibians (Lissamphibia) possess some of the most intriguing features among vertebrates. Salamanders, including both aquatic larval axolotl and metamorphosed newts (*Cynops*; *Notopthalmus*; and *Pleurodeles*, the Iberian newt), have the remarkable ability to regenerate entire limbs, a tail, and even parts of the brain, eye, and heart [1,2]. However, there are some key differences in the regeneration modes of newts and axolotls. Newts

regenerate lens and muscle tissues through dedifferentiation, while axolotls regenerate muscle tissues through stem cell activation [3–6]. It remains to be determined whether these differences reflect a higher degree of cell plasticity in newts. In addition, newts can regenerate more body parts than axolotls. Axolotls can only regenerate the eye lens during the first two weeks after hatching [5], while newts can regenerate the eye lens throughout their lifespan, and their ability to regenerate the lens does not decline with age or the number of lens removal/regeneration cycles [6]. Research using salamanders as a model system has gained tremendous insights into the developmental and physiological process of regeneration [7–9]. We now know that the extracellular matrix (ECM) plays a critical role in directing cell growth and migration, and nerves and immune cells are essential for regeneration [8,10–12]. When macrophages were removed, salamanders lost their ability to regenerate and instead formed scar tissue [8]. Other vertebrates, including human, lose their regenerative potential with age, largely because of failure to maintain tissue homeostasis [13]. Furthermore, salamanders have one of the longest life spans for their body size [14], which naturally raises the possibility of genetic interactions underlying regeneration and aging [15,16]. Other traits, such as the diverse body plans of modern amphibians, the vocalization of frogs, and many traits associated with the fossorial lifestyle of caecilians, are just as amazing (Figure 1A). However, the genetic mechanisms behind these traits remain largely elusive. In addition, persisting controversies surrounding the origin of Lissamphibians and the relationships among the three main groups have hindered comparative analysis [17].

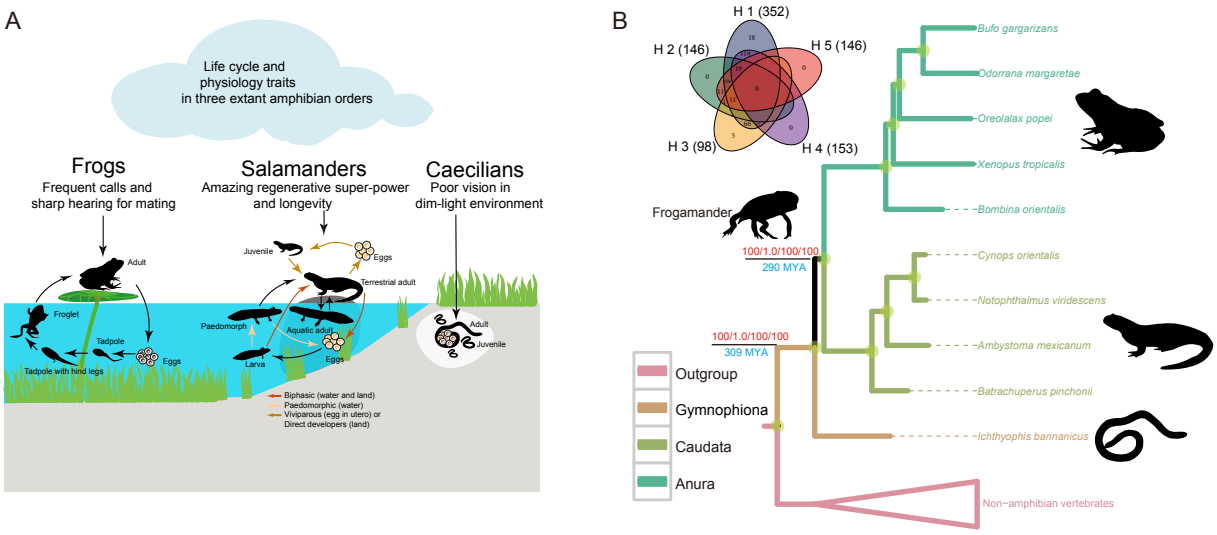

Figure 1. Physiology traits and phylogenomic analysis of extant amphibians. (**A**) The most prominent characteristics of frogs, salamanders, and caecilians. (**B**) Phylogenomic tree. The red numbers on the important nodes represent support values from the maximum likelihood (ML), Bayesian (PB), maximum pseudo-likelihood estimates (MPE), and maximum parsimony "Genes as characters" (GAC) methods. The blue numbers below the branch represent the origin date of the extant amphibians (309 MYA) and the living frogs and salamanders (290 MYA). Upper left is a Venn diagram of gene clusters supporting the different phylogenetic hypotheses, as shown in Figure S1. The numbers in brackets represent the quantity of genes that cannot reject a specific hypothesis.

The recent boom in genomic work provides an excellent opportunity to explore the genetic architecture of major evolutionary changes. Amphibians have only one published salamander genome [18], which is largely limited by their extremely large genome sizes. Alternative approaches, such as transcriptome sequencing, are commonly used [19,20]. The enormous amount of available genomic resources enables large-scale and in-depth comparative analyses. Furthermore, several distantly related vertebrates share some phenotypic traits with amphibians; for instance, lizards can regenerate tails, and naked mole

rats and Brandt's bats also have long lifespans [21]. This provides opportunities to examine potentially shared genetic mechanisms of the same trait. Although it has been argued that parallel/convergent evolution at the molecular level is often rare because there could be several genomic routes leading to the same phenotype, shared genetic changes would provide strong evidence for adaptive evolution [22].

Using bioinformatics and a comparative analysis of genomic data, I explored the potential genetic mechanisms of major traits in modern amphibians, in particular, the regenerative healing and anti-senescence capabilities of salamanders. My specific objectives were (1) to address the long-debated question of how the three orders of modern amphibians (Anura, Gymnophiona, Caudata) are related to each other as well as to other vertebrate groups; and (2) based on the resulting tree, I explored the genetic architecture of several traits of the three groups of modern amphibians, with a focus on the regeneration capacity and longevity of salamanders, as well as the vocalization and hearing of frogs and the degenerative visual function of caecilians.

2. Materials and Methods

2.1. Sample Collection, Transcriptome Sequencing, and De Novo Assembly

Transcriptome sequences for five amphibian species were acquired, including Yunnan caecilians (*Ichthyophis bannanicus*; collected in Jinghong, Yunnan Province, China, in 2018), Baoxing tooth toads (*Oreolalax popei*; collected from Baoxing County, Sichuan Province, China, in 2018), oriental fire-bellied toads (*Bombina orientalis*; collected from Qingdao, Shandong Province, China, in 2018), stream salamanders (*Batrachuperus pinchonii*; collected from Baoxing County, Sichuan Province, China, in 2018), and Chinese fire-bellied newts (*Cynops orientalis*; collected from Wuxue, Hubei Province, China, in 2018). All specimens were identified using a morphological method [23]. For evolutionary analyses, I collected samples from two adult individuals of each species, one male and one female. Individuals were euthanized and dissected immediately after death. RNA was separately extracted from the brain, liver, heart, skeletal muscle, and gonad tissues of these individuals using the Trizol protocols (Invitrogen, Carlsbad, CA, USA) and mixed in approximately equal quantities. For the limb regeneration experiment, adult newts were anesthetized and placed in a sterile dish. The right forelimb zeugopod was amputated using sterile scissors. The newts were then returned to their home tanks and monitored for recovery. The proximal healing tissue was harvested at 0 h, 1 day, 5 days, 10 days, and 20 days post-amputation, and RNA was separately extracted using the Trizol protocols (Invitrogen, Carlsbad, CA, USA). Three newts were used for each time window of limb regeneration. The concentration and integrity of total RNA was examined using agarose gel electrophoresis, a NanoPhotometer spectrophotometer (IMPLEN, Westlake Village, CA, USA), and an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). cDNA libraries were constructed and subsequently sequenced on an Illumina HiSeq2000 platform, which was carried out by Novogene Inc. (Beijing, China). Approximately 4 G of raw data of paired-end reads was obtained for each transcriptome.

Quality filtration and de novo assembly were performed. The raw reads were first cleaned by filtering out the adapter sequences using Trimmomatic v0.36 [24]. Reads with more than 10% unknown base calls and low-quality reads (<Q20) were also discarded. Preliminary assemblies were produced using Trinity v2.9.0 [25] according to the published protocols [26]. To ensure the quality of the assembly, I also performed a multiple K-mer and multiple coverage cutoff values assembly using ABySS v2.0 [27,28]. Combinations of five K-mer lengths (21, 31, 41, 51, and 61) and six coverage cutoff values (2, 3, 6, 10, 15, and 20) were used, and 30 raw assemblies were produced. Sequence overlaps and redundancies were then eliminated to produce the final assembly using the programs CD-HIT-EST v4.8.1 [29] and CAP3 v10.2011 [30].

2.2. Phylogenetic Analyses and Date Estimation

2.2.1. Construction of the Raw Phylogenomic Datasets

Genomic data or transcriptome data of five additional amphibian species were obtained from previous studies, including the green odorous frog (*Odorrana margaretae*) [31], Asiatic toads (*Bufo gargarizans*) [32], axolotl (*Ambystoma mexicanum*) [33], eastern newt (*Notophthalmus viridescens*) [34], and western clawed frog (*Xenopus tropicalis*) [35]. A total of ten species were used to represent the three extant orders. Humans and the green anole were used as outgroups.

Putative orthologous genes were identified using the program HaMSTR v1.6.0 (Hidden Markov Model-based Search for Orthologs using Reciprocity) [36] based on its core ortholog database. Amino acid alignments were generated using Clustal Omega v1.2.4 [37] with the default parameters. Codon alignments were generated based on the amino acid alignments. A set of randomly sampled genes was selected, and their alignments were manually checked to ensure the performance of the program. Two raw supermatrix datasets were constructed, one by concatenating the coding regions of the nucleotide sequences (CDS) and the other by concatenating their corresponding amino acid sequences.

2.2.2. Data Filtration

To remove or reduce potentially detrimental effects of several confounding factors in phylogenomic reconstruction, I filtered the data using four approaches. First, it is well known that uneven distributions of missing data can affect phylogenetic inferences [38,39]. I used the Alistat v1.7 to quantify the sparseness of the concatenated alignments. Second, the completeness score value ranging from 0 to 1 was estimated. Selecting informative subsets of supermatrices increases the chances of finding the correct trees [40]. I also used mare v0.1.2 [40] to assess the information content of genes in the supermatrices by measuring potential phylogenetic signals and data coverage. Genes with no information content were eliminated. Third, I investigated the influence of base compositional heterogeneity (CH) on the phylogenetic reconstruction using BaCoCa v1.1 [41]. The RCFV (Relative Composition Frequency Variability) values across all taxa were calculated for the complete datasets [42]. The higher the RCFV value, the higher the degree of compositional heterogeneity. Chi-square tests were conducted, and a significance level was set at 0.01, where a p-value below 0.01 indicates that the composition significantly deviates from homogeneity, according to the author's suggestion. Heterogeneous genes were excluded from the supermatrices. Fourth, the common phylogenetic assumption assumes that evolutionary processes can be modeled and DNA sequences "evolved under globally stationary, reversible and homogeneous (SRH) conditions" [43–45]. In fact, CH across the sequences is common, and the evolutionary process is more complex than the model assumption. Non-SRH conditions will introduce systematic errors during the tree reconstruction process and even generate false phylogenetic conclusions [46,47]. I used the program SymTest to assess whether the concatenated sequences are consistent with evolution under SRH conditions. In addition, p-value heatmaps were generated to visualize which sequence pairs could be assumed to have evolved under SRH conditions and which ones violated this assumption.

After eliminating "noisy" genes and sites, select optimal data subsets were extracted from the raw supermatrices of both the CDS and amino acid sequence and then used in the phylogenomic analyses.

2.2.3. Phylogenomic Tree Construction

I analyzed the two filtered supermatrices using both the maximum likelihood (ML) and Bayesian inference (BI) methods. ML trees were inferred using RAxML v8.0.19 [48] with the site-homogeneous JTT-F+G and GTR+G models, and the BI tree was inferred using PhyloBayes v3.3 [49] with the site-heterogeneous CAT-GTR model. The best-fit partitioning schemes and substitution models for the supermatrices were determined by PartitionFinder v2.1.1 [50]. The Bayesian Information Criterion (BIC) was chosen to compare partitioning schemes and models of molecular evolution.

I also applied ML approaches on each filtered gene to obtain all the individual gene trees. The tree sets were used to evaluate alternative phylogenetic hypotheses and to infer species trees.

I evaluated five phylogenetic hypotheses concerning the relationships among the three orders of modern amphibians. The first three hypotheses assume a monophyletic origin for Lissamphibians. (1) The Batrachia hypothesis proposes a frog–salamander sister relationship [51–54]. (2) The Procera hypothesis proposes a salamander–caecilian sister relationship [55–57]. (3) I also examined the frog–caecilian sister relationship, although no previous study has suggested the existence of this relationship. The last two hypotheses assume a paraphyletic origin for Lissamphibians, which were suggested by paleontological data. (4) Caecilians are more closely related to amniotes than to salamanders and frogs [58], while salamanders and frogs form a monophyletic group [59–61]. (5) Caecilians and salamanders are sisters, and together, they cluster with amniotes without frogs [62,63]. For each gene, RAxML was used to compute the per-site log-likelihood values of the five constrained topologies with the GTR+G substitution model. AU tests [64] were then performed with CONSE v 0.20 [65] to calculate *p*-values. A small *p*-value (0.05) for a topology indicates that the topology is significantly worse than the best one and should be rejected. For each hypothesis, I recorded the genes that could not be rejected by the AU tests. The *p*-values of each individual gene for each topology were converted into heatmaps using Phylcon v1.0 [66].

I also used two supertree approaches to construct species trees from individual gene trees. It is well known that gene trees are not always consistent with species trees due to incomplete lineage sorting and other biological reasons [47]. Here, I applied a pseudo-likelihood approach under the multi-species coalescent model [47,67] to overcome potential limitations and to obtain maximum pseudo-likelihood estimates (MPEs) of species trees from a collection of individual gene trees. I also used the parsimony-based "genes as characters" (GAC) approach [68] to infer species trees. This method treats each gene as a single-ordered multi-state character, and individual gene trees are described by step matrices. All the characters were weighted equally because no reasonable a priori information exists. I assumed that these genes evolved largely independently to satisfy the independent character assumption of parsimony analysis. Finally, parsimony analyses were performed in PAUP. A heuristic search was employed with TBR branch swapping and 100 random addition replicates. Bootstrap values were estimated with 1000 replicates.

2.2.4. Divergence Time Estimates

Data were acquired for ten additional vertebrates, including zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), Amazon molly (*Poecilia formosa*), coelacanth (*Latimeria chalumnae*), Shedao pit-viper (*Gloydius shedaoensis*) [69], alligator (*Alligator mississippiensis*), chicken (*Gallus gallus*), opossum (*Monodelphis domestica*), elephant (*Loxodonta africana*), and mouse (*Mus musculus*). Most data were downloaded from Ensembl [70]. The dataset included 22 vertebrate species representing all major lineages. This dataset provided multiple calibration points, and it also formed the basis for all downstream analyses.

Multiple calibration points provide more realistic divergence time estimates overall [71]; thus, I used four calibration points, including both soft minimum and maximum time constraints: (1) bird–mammal split (min 312.3 MYA, max 330.4 MYA); (2) human–toad split (min 330.4 MYA, max 350.1 MYA); (3) crocodile–lizard split (min 259.7 MYA, max 299.8 MYA); and (4) the origin of crown Osteichthyes (min 416.0 MYA, max 421.75 MYA). A relaxed molecular clock Bayesian method implemented in MCMCTREE v4.9 [72,73] in PAML v4.9 was used to estimate the divergence time among the three modern amphibian orders. I only used the 2nd codon of the CDS and amino acid datasets to estimate the divergence date to avoid potential problems associated with saturation. The results from molecular clock estimates were compared to the ages of Batrachian fossils from the early Permian.

2.3. Test for Selections on Lissamphibia

2.3.1. Lineage-Specific dN/dS and dS Estimates

The evolutionary rate ratio of divergence at nonsynonymous and synonymous sites, dN/dS, is a widely used indicator to measure the magnitude of natural selection acting on protein-coding genes [74]. A lower dN/dS ratio indicates a strong purifying selection against protein changes, and an elevated dN/dS ratio suggests a weak purifying selection or a strong positive selection in favor of protein alterations.

The dN/dS calculation was based on the 22-species dataset, which includes all major lineages of vertebrates and allows us to compare Lissamphibia to other vertebrates. I also constructed a reduced dataset with only the ten amphibians. This dataset has fewer taxa but more and longer orthologs, which allows us to perform an in-depth analysis of genes involved in adaptive and parallel evolution.

The dN/dS ratio for each lineage was estimated using a maximum likelihood approach [75], implemented in CODEML of the PAML 4 software [76]. The free-ratio branch model, which allows the dN/dS ratio to vary for different branches, was run on each ortholog and the concatenated supermatrices. Abnormal values (dN/dS > 5) were excluded from the analysis. A mean dN/dS value for each major group was calculated by averaging the ratio of all terminal branches within the group. I also calculated the number of synonymous substitutions (dS) to represent the rate of neutral evolution. Furthermore, I used PHAST v1.5 [77] to estimate the substitution rates for 4-fold degenerate sites in the concatenated supermatrices.

2.3.2. Rapidly Evolving GO Categories

The dN/dS ratio of the Gene Ontology (GO) category can partially reflect the evolutionary rate of a functional module. I identified rapidly evolving GO categories (REGOs) in the five major groups of vertebrates. During the transition from water to land, vertebrates experienced many major changes in their anatomic structure; therefore, I investigated the evolutionary pattern of developmental functions in extant amphibians and compared the proportion of REGOs involved in development between the major groups. I also compared development-related genes within each major group. Salamanders have an extended life span and remarkable regeneration ability during any stage of their life cycle; therefore, I further compared the adaptive evolutionary rate of GOs in modern salamanders with that in their ancestors. If development- and/or aging-related GOs accelerated adaptive evolution in living salamanders relative to their ancestors, this may suggest that salamanders' superpower continually evolved and improved. Conversely, it may imply that these great changes occurred in the salamanders' ancestors, and extant salamanders just inherited this ability. The Wilcoxon rank sum test was used to compare the dN/dS ratios of a particular gene or GO category to that of the other genes or categories as background.

2.3.3. Identification of Genes under Positive Selection

The branch-site model implemented in the program CODEML v4.9 was used to detect positively selected genes (PSGs) along a specific lineage. The model assumes that foreground branches are under positive selection and background branches evolve in a neutral fashion. I compared the selection model (alternative model, dN/dS > 1) and the neutral model (null model, dN/dS = 1) using a likelihood ratio test (LRT). A chi-square test was conducted for each gene to assess statistical significance. Multiple testing was corrected by applying the false discovery rate (FDR) method. For a gene, if the selection model has a significantly higher likelihood than the neutral model (FDR-adjusted p-value < 0.05), this indicates that these genes on the foreground branch might have experienced positive selection.

2.3.4. Identification of Fast-Evolving Genes

To identify the fast-evolving genes (FEGs) in Lissamphibia, I ran a one-ratio branch model and a multi-ratio branch model with CODEML in PAML to estimate the global and local dN/dS ratios, respectively. The one-ratio model assumes that all branches have

been evolving at the same rate (null hypothesis), and the multi-ratio model allows the foreground branch to evolve at a different rate (alternative hypothesis). Salamanders, frogs, and caecilians were set as the foreground. The LRT was employed to compare the one-ratio and the multi-ratio branch models. The *p*-values of the chi-square test were adjusted using FDR correction for multiple testing. If a gene had an FDR-adjusted *p*-value of <0.05 and a higher dN/dS in the foreground branch than in the background branch, it was considered an FEG in the foreground branch. Functional enrichment analyses for FEGs were carried out using DAVID bioinformatics resources [78].

2.4. Test for Parallel Evolution

I tested patterns of parallel evolution between modern amphibians and three distantly related vertebrates, which share the characteristics of interest with amphibians and have relevant data. The green anole (*Anolis carolinensis*) is capable of regenerating its tail; the Brandt's bat (*Myotis brandtii*) has longevity, super hearing for echolocation, and reduced visual capacity [79,80]; and the naked mole rat (*Heterocephalus glaber*) has longevity, cancer resistance, pain insensitivity, degenerated hearing, and poor visual perception [81–85]. In addition, zebrafish, chicken, and humans were also included for comparison. Ancestral sequence reconstruction was carried out using both ANCESTOR v1.0 [86] and CODEML.

To reduce the influence of uncertainty and individuality, I only focused on amino acid changes shared by all members in a group (e.g., all salamanders examined). I first identified amino acid positions where changes only occurred in two lineages: an amphibian group and a distantly related vertebrate species that share the same phenotypic trait of interest. If they share the same amino acid residue and are derived from the same ancestral amino acid residue, these changes were defined as "parallel". If the changes resulted in different amino acid states in the extant species, the changes were classified as "common". Common changes may be a possible indicator of adaptation accomplished via multiple different amino acids at the same position (reference). CONVERG v2.0 [87] was used to compute the probabilities that the observed parallel substitutions are attributable to random chance.

Genes with parallel changes or common changes were then compared to PSGs and FEGs, and overlapping genes were subjected to further analysis. Orthologs from other bats, including three echolocating bats—the little brown bat (*Myotis lucifugus*), David's myotis (*Myotis davidii*), and vampire bat (*Desmodus rotundus*)—and one non-echolocating bat, the black flying fox (*Pteropus alecto*), were downloaded for comparison. The alignment quality of these candidate genes and the degrees of conservation of the region with amino acid changes were manually checked. Furthermore, candidate genes associated with human/mouse diseases were determined through exploring disease databases, such as OMIM (http://omim.org (accessed on 1 November 2022)) and GeneCards (www.genecards.org (accessed on 1 November 2022)) for human and MGI (http://www.informatics.jax.org (accessed on 1 November 2022)) for mouse, which focus on the relationship between disease phenotype and genotype.

2.5. Prediction of Functional Impact of Variants

I used SIFT v6.2.1 [88], PROVEAN v1.1.5 [89], and PolyPhen-2 v2.2.2 [90] to predict the possible effect of unique amino acid substitutions and positively selected and parallel mutations on protein structure and function.

I retrieved information on protein domains and important sites and the 3D structure of proteins from the InterPro database [91] and RCSB PDB [92], respectively. Amino acid conservation scores across the sequences of candidate genes were calculated using the Rate4Site algorithm [93,94] on the ConSurf server [95]. The scores were converted by multiplying by -1 so that higher scores indicate higher conservation. Local average conservation was calculated by fitting a cubic smoothing spline to the per-site conservation scores using the smooth.spline method in R. Unique amino acid changes were mapped onto conservation domain plots and the protein 3D structure.

2.6. Quantitative Real-Time PCR

The proximal healing tissue was harvested at 0 h, 1 day, 5 days, 10 days, and 20 days post-amputation. RNA was extracted using the Trizol protocols (Invitrogen) and then reverse transcribed using a PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa) according to the manufacturer's instructions. The primers for OGFR were forward: 5′-CAGCCCAATGGTGTTCCTGAT-3′; and reverse: 5′-GCGGACAAACCTTTCTTTCA-3′. The primers for SERPINI1 were forward: 5′-ATTTAAGGGATCTATCTGAGGCC-3′; and reverse: 5′-CACCCAGCCATTGATGTGTT-3′. The primers for ACTIN were forward: 5′-AGATCTGGCACCACACCTTC-3′; and reverse: 5′-CAGTGGTACGACCAGAAGCA-3′. The qPCR reactions were performed on a BioRad CFX96 Real-Time PCR Detection System using the EvaGreen 2X qPCR MasterMix (Abm). The thermal cycling parameters were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 52 °C, and 30 s at 72 °C. Three replicates were performed at each time point, and failed reactions were excluded for the analysis. The relative expression level of unigenes was normalized to ACTIN as the comparative Ct and calculated using the delta delta Ct ($2^{-\Delta\Delta}$Ct) method.

3. Results and Discussion

3.1. Robust Support for Frog–Salamander Sister Relationship

I gathered data from ten amphibian species to infer the origin of and relationships between three extant amphibian orders (Figure 1B). After filtering the one-to-one ortholog dataset, I ultimately utilized 369 coding sequences (CDSs) (second codon positions and first + second codon positions) and 772 protein sequences for the phylogenomic analyses. The datasets had relatively full coverage (completeness score: 0.7–1.0; Figure S2A), high information content (information content: 0.6–1.0; Figure S2B), and a low degree of CH (RCFV value < 0.025; Figure S3) and were least affected in terms of violation of the assumption of evolution under global SRH conditions (Figure S4).

Both the partitioned maximum likelihood (ML) and Bayesian inference (BI) analyses of the concatenated data strongly supported the monophyly of Lissamphibia and the frog–salamander sister relationship (the Batrachia hypothesis; Figure 1B). In addition, two independent supertree analyses, including the maximum pseudo-likelihood estimates (MP-EST) from a collection of individual gene trees [96] and the genes as characters approach (GAC) [68], both found the same topology as the concatenated data tree, providing strong support for the Batrachia hypothesis (Figure 1B).

I also examined the conflicts among individual genes and detected the degrees of support of various genes for the alternative hypotheses. The AU test indicated that the Batrachia hypothesis (H1) was supported by the vast majority of the data, with 352 of 369 CDSs and 708 of 772 proteins supporting the hypothesis (Figures 1B and S5), while the Procera hypothesis (H2), which posits that salamanders and caecilians are sister taxa, was supported by only 146 of 369 CDSs and 274 of 772 proteins. Surprisingly, the paraphyletic origin hypothesis (H4), which posits a close relationship between caecilians and amniotes and a sister relationship between salamanders and frogs, was supported by a large number of genes (153 of 369 CDSs and 374 of 772 proteins). The frog–caecilian sister relationship (H3) received the lowest support, with only 98 of 369 CDSs and 194 of 772 proteins supporting it.

Genome-scale data provide opportunities for resolving difficult phylogenetic relationships [97,98]. My data and analysis provide strong support for the monophyly of Lissamphibia and the salamander–frog sister relationship, consistent with the findings of recent studies [99–101].

3.2. Fossil-Compatible Divergence Time of Lissamphibia

A total of 22 vertebrate species (Figure 2) were included in this analysis. I used four calibration points from vertebrates (Figure S6) to estimate the divergence time of Lissamphibia. The results indicate that modern amphibians arose in the late Carboniferous, about 309 MYA (Figures 1B and S6). This is consistent with the fossil record; amphibian-like early temnospondyls (e.g., *Amphibiamus*) appeared in the Carboniferous [102]. The origin of

Batrachia was estimated to take place in the early Permian (~290 MYA; Figures 1B and S6). This is extremely close to the estimated age of the "frogamander", *Gerobatrachus hottoni* (~290 MYA), from Texas during the early Permian [59]. *Gerobatrachus hottoni*, who possessed a large frog-like head and a salamander-like tail, was considered the closest relative of Batrachia [59]. Furthermore, the origins of living frogs and salamanders were dated back to the late Triassic (~200 MYA) and middle Jurassic (~170 MYA), respectively (Figure S6), which was close to or matched the fossil study well (~185 MYA for frog origin and ~170 MYA for salamander origin) [103].

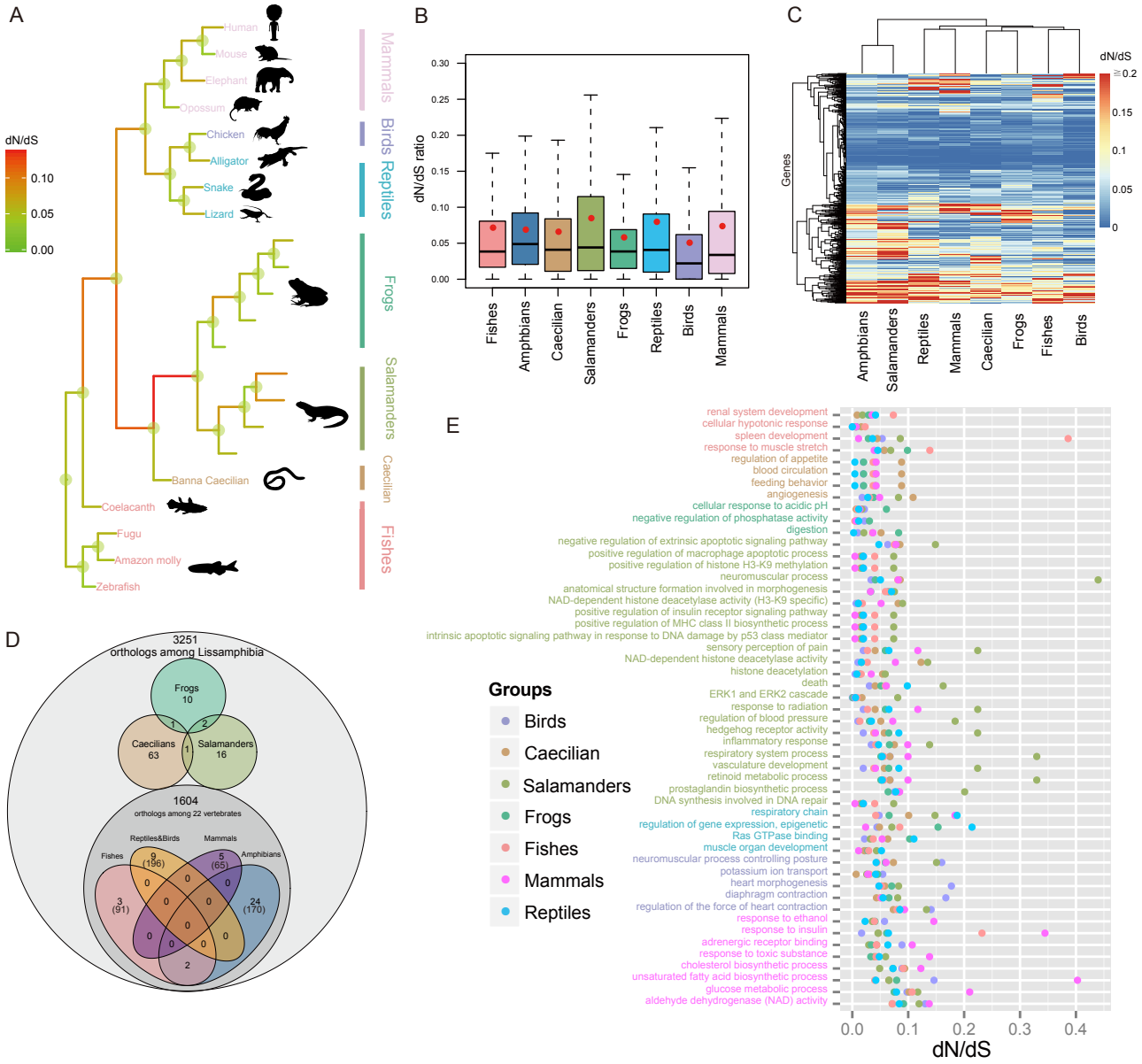

Figure 2. Accelerated rate of adaptive evolution in salamanders. (**A**) dN/dS ratio tree of 22 vertebrates shows that salamanders have a higher dN/dS ratio than any other vertebrate group. (**B**) The boxplot of dN/dS ratios for all genes in each major vertebrate group, which shows that salamanders have a much higher mean dN/dS ratio than any other group ($p \leq 0.025$ for all pairwise tests). (**C**) The clustered heatmap of dN/dS ratios of major vertebrate groups also shows that salamanders are distantly related to other vertebrate groups in terms of dN/dS ratio. (**D**) Venn diagram showing number of PSGs and FEGs (in brackets) in Lissamphibia and other vertebrates. (**E**) The rapidly evolving GO categories (REGOs) in Lissamphibia and other vertebrates show that salamanders have a significantly higher number of REGOs than any other vertebrate group.

My age estimates were younger than most previous molecular studies [59] but are mostly consistent with the fossil evidence. For example, the origin of Lissamphibia was estimated to be as young as the early Permian at 294 million years ago (MYA) [54] or as old as the late Devonian period at 369 MYA [104]. Most previous studies used only one or a few genes, which may contribute to the variation and discrepancy.

3.3. Rapid Evolution of Salamander Genes

To gain insight into the evolution of Lissamphibia, I compared aspects of their coding gene architecture to those of the other major vertebrate lineages (Figure 2A), including the evolutionary rate and genes or gene clusters that are potentially under positive selection.

The dN/dS ratio has been widely used as a measurement of the rate of adaptive evolution. The colored dN/dS tree shows that salamanders exhibited a higher ratio of dN/dS relative to all the other vertebrate groups (Figure 2A). Furthermore, the distribution of dN/dS ratios among the salamander lineages was significantly larger than that of the other vertebrate groups (Wilcoxon rank sum test (WRST), $p < 0.03$ for all pairwise tests; Figure 2B). Additionally, a cluster analysis for the vertebrate dN/dS ratios found that salamanders were located on a lone branch at the base of the clustering tree and were very different from the other vertebrates (Figure 2C). Evidently, globally elevated evolutionary rates for protein-coding genes likely occurred in salamanders' genomes.

This accelerated evolution can either be due to selection (reduced purifying selection and/or increased positive selection in favor of protein alterations) or a high mutation rate, which can be measured as synonymous mutations [105].

To test whether salamanders have high mutation rates, I compared the mutation rates at synonymous sites (dS) among the major lineages of vertebrates, which has often been used to represent the neutral mutation rate. Interestingly, salamanders exhibited an extremely low dS compared to other vertebrates (Figure S7; WRST $p < 2.9 \times 10^{-40}$ for all pairwise tests). On average, the dS of salamanders was approximately one-third of the dS of caecilians and frogs. I further estimated rates for the four-fold degenerate sites (4D), which evolve the closest to the neutral rate [106]. As expected, the color pattern of the 4D mutation rate tree is very similar to that reconstructed using synonymous sites (Figure S8).

Clearly, salamanders do not have elevated mutation rates, and the high dN/dS ratios are due to selection forces. I further explored aspects of selection forces below. Previous work showed that salamander mitochondrial genomes also display high dN/dS ratios, although their comparisons were restricted to salamanders and frogs [107].

I also noticed that both the common ancestors of Lissamphibia and Batrachia demonstrated higher dN/dS ratios than the extant amphibians (Figure 2A). These are likely a reflection of the rapid genomic changes required during their early adaptation to terrestrial life.

3.4. Detection of Selection

I identified a series of fast-evolving genes (FEGs; Table S1) and positively selected genes (PSGs; Table S2) in salamanders, frogs, and caecilians (Figure 2D). The number of candidate genes was conservative because my analyses were conducted on groups of species rather than individual species. This approach helps to reduce the impact of uncertainty and individual variations. Further analysis was conducted on some FEGs and PSGs of interest.

To investigate the lineage-specific evolution of function modules in the genome, I identified rapidly evolving GO categories (REGOs) unique to each major group of vertebrates. For Lissamphibia, many REGOs were associated with developmental processes, including the development of the circulatory (blood circulation, regulation of blood pressure), respiratory, sensory, and immune systems and morphogenesis (Figure 2E). Developmental process-related REGOs accounted for a higher proportion in Lissamphibia (33%) than in other vertebrate groups (13–19%), including birds who have highly specialized anatomic structures (23%) (Figure 3A).

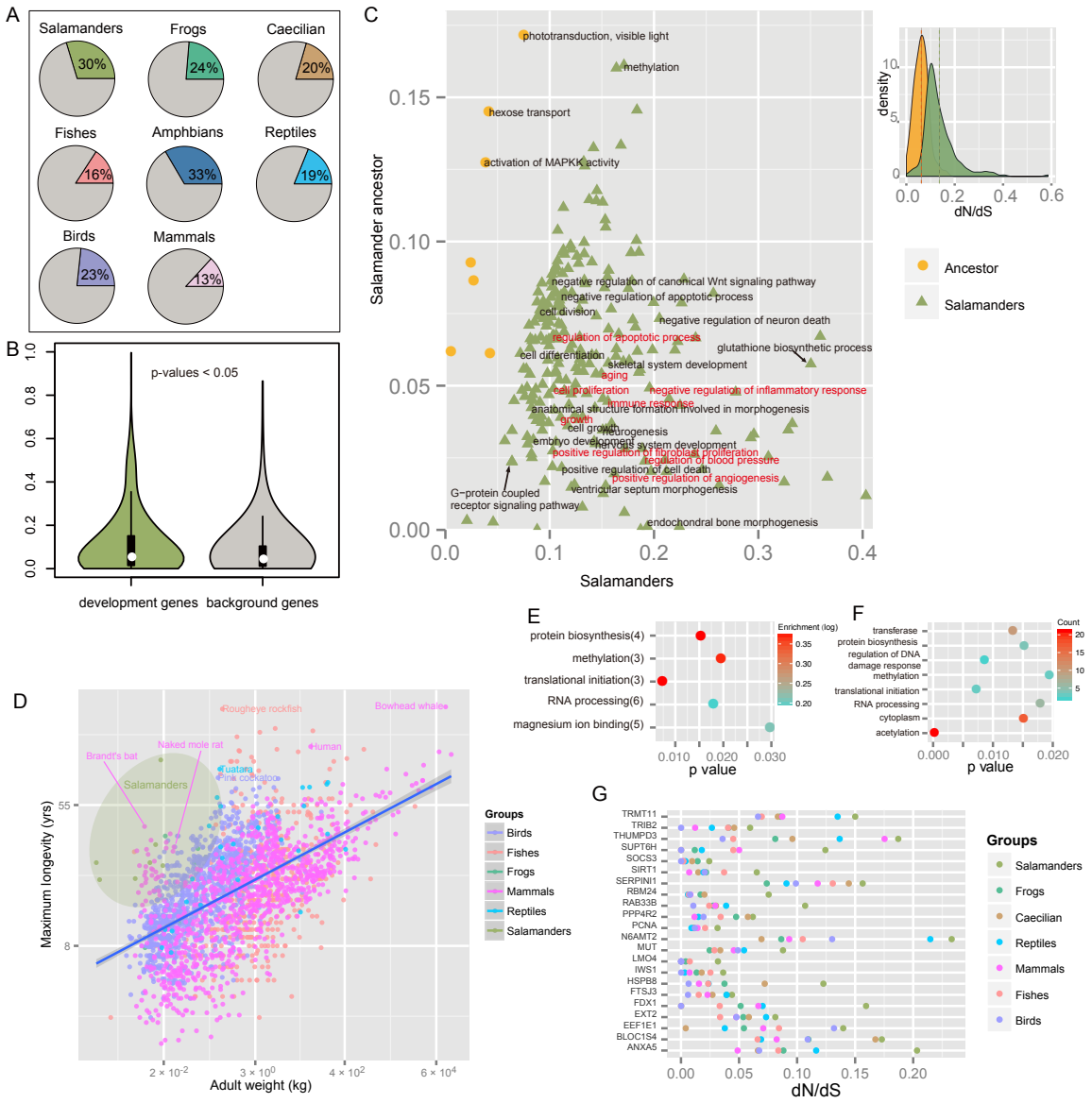

Figure 3. Genetic potential underlying salamanders' regenerative capability and longevity. (**A**) Proportion of rapidly evolving GO categories (REGOs) involved in development is highest in salamanders. (**B**) Salamanders' developmental genes evolved significantly faster than other genes. (**C**) Extant salamanders' development-related and aging-related GO categories evolved significantly faster than those of their ancestors. (**D**) Salamanders fall into the category of exception to the rule of "Larger animals live longer", which is shown by the yellow-green color. (**E**) Top five enriched GO terms from salamanders' fast-evolving genes (FEGs). (**F**) Top eight GO terms for salamanders' FEGs. (**G**) Selected salamanders' FEGs may be responsible for regenerative capability and longevity.

3.5. Regeneration- and Development-Related Genes in Salamanders

Salamanders have a superior ability to regenerate limbs compared to other vertebrates. I specifically examined genes and GO categories that are linked to development and regeneration in salamanders. I also compared salamanders to another vertebrate, the green anole, which also has a regeneration ability.

The regulation of apoptosis activity plays a key role in local cell dedifferentiation [108,109], which is the first and perhaps the most crucial stage of regeneration. Not surprisingly, I detected several rapidly evolving GO categories associated with the regulation of apoptotic pathways that are unique to salamanders (Figure 2E). Notably, one REGO of regulation of apoptotic cell death was triggered by the tumor suppressor p53 (Figure 2E), which has been proven to be

critical for regeneration [110]. In addition, a fast-evolving gene, *EEF1E1*, which is involved in the negative regulation of cell population proliferation and positive regulation of apoptotic processes, shows signals of parallel evolution between salamanders and green anoles (Figure 4A; L108F; $p < 1 \times 10^{-6}$). Furthermore, some REGOs were involved in macrophage regulation and the MHC biosynthetic process (Figure 2E). This is consistent with the essential role of macrophages in successful healing and regeneration [8]. Many FEGs in salamanders, such as *ANXA5*, *SIRT1*, *RAB33B*, *HSPB8*, etc. (Figure 3G), may be involved in the coagulation process, inflammatory processes, and autophagy during the initial stage of regeneration.

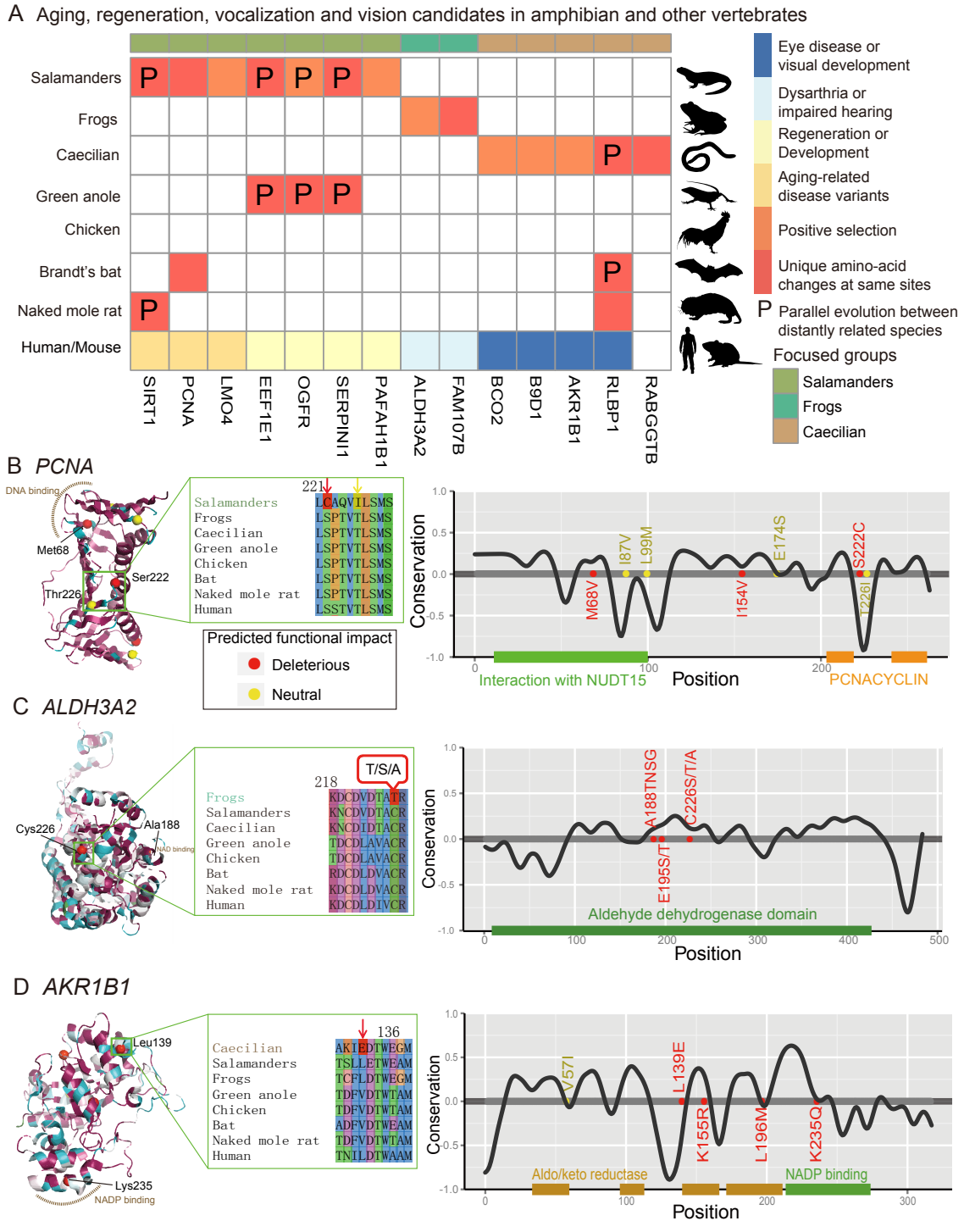

Figure 4. Aging, regeneration, vocalization, and vision candidates in extant amphibians and other vertebrates. (**A**) Candidates under positive selection were identified, and those with shared common

or unique position mutations in focused lineages and who have undergone parallel evolution are marked. Associated diseases are also labeled with different colors. (**B**–**D**) Salamander, frog, and caecilian candidates, respectively. **Left**: Crystal structure of candidates. Purple-red represents conserved regions, and cyan represents variable regions. The important domains and lineage-specific mutations are highlighted in the structure. Residue color represents the strength of the functional impact. Insert: alignment of an example residue with functional impact. **Right**: The conservation scores, domain, and lineage-specific mutation positions. The black curve shows the cubic smoothing spline of the amino acid conservation scores; higher scores indicate higher conservation.

Unlike mammals, salamanders have a scar-free healing capacity, mainly due to their fibroblasts, which form the early blastema rather than scars and control the regeneration process [111]. I detected positive selection acting on the salamanders' *PAFAH1B1* gene, which plays a crucial role in the directed migration of fibroblasts during wound healing and stem cell division [112]. Furthermore, two amino acid changes (L37F and I200V) were specific to salamanders. L37F is located in the LisH domain, which may be required to activate dynein, and I200V is in the WD40 repeat; both mutations were predicted to have strong functional impacts. In addition, the ERK pathway, whose activity is another key difference in cellular reprogramming between salamanders and mammals [9], and the retinoid metabolic process, which may play an important role in the early phase of regeneration [113], were also found to have undergone the most rapid evolution in salamanders (Figure 2E).

The second stage of regeneration is similar to the development process in that it involves proliferation, redifferentiation, and growth. I detected several development-related REGOs that were unique to salamanders, including anatomical structure formation, neuromuscular process, hedgehog receptor activity, and vasculature development, among a few others. (Figure 2E). I detected a strong signal of natural selection on a cell proliferation-related gene, LMO4. One site in its LIM-type zinc finger (Znf) domain (region 23–83), which acts as an interface for protein–protein interactions, was under positive selection (Ala24; Bayes empirical Bayes posterior probabilities (BEBPP) = 0.996). I also detected seven FEGs involved in differentiation and development, including *TRIB2*, *RBM24*, *EXT2*, *SOCS3*, *BLOC1S4*, *MUT*, and *SERPINI1* (Figure 3G and Table S1). Furthermore, the gene *SERPINI1*, which is associated with central nervous system development, was detected to have undergone parallel evolution between salamanders and green anoles (Figure 4A; $p < 1 \times 10^{-6}$). A parallel change (F118Y) was predicted to be harmful. The prostaglandin biosynthetic process, a pathway that was recently reported to be closely associated with regenerative capacity in mice (*15-PGDH*) [114] and lizards (*PTGIS* and *PTGS1*) [115], was detected to have evolved most rapidly in salamanders (Figure 2E; *PTGES2* and *PTGS2*). The mutation position of *PTGES2* was shared among salamanders (E183A), echolocating bats (E183D), and naked mole rats (E183D). Unlike *PTGS1*, which is constitutively expressed, *PTGS2* is upregulated during inflammation and contributes to cell proliferation, angiogenesis, apoptosis inhibition, and immune response suppression [116].

Furthermore, I found that the REGOs related to development in salamanders were the most common group in vertebrates (Figure 3A). Did the development-related genes drive the accelerated evolution of salamanders and contribute to regeneration? The answer seems to be yes, as the evolutionary rate of development- and regeneration-related genes were significantly higher than that of the other background genes (Figure 3B; $p = 0.039$). In contrast, a similar pattern was not observed in any other vertebrate group (Figure S9).

How did salamanders obtain such an amazing regenerative ability? To answer this question, I compared the extant salamanders with their most recent common ancestors (MRCAs). Interestingly, many functional modules pertaining to the regeneration process, including the apoptotic process, immune responses, proliferation, angiogenesis, growth, morphogenesis, and aging, have undergone faster adaptive evolution in the extant salamanders relative to their MRCAs (Figure 3C). This is to say, salamanders were constantly evolving their regenerative capacity. Continuous self-improvement may have helped salamanders improve their genetic potential to heal wounds after injury and also delay aging.

For example, the opioid growth factor receptor (*OGFR*) is an important regulator of cell proliferation, tissue growth, cancer, cellular renewal, wound repair, and angiogenesis [117]. In salamanders, *OGFR* likely evolved under positive selection (Figure 4A), and Lys198 was identified as a positively selected site (BEBPP = 0.995). It is intriguing that Val190 and Pro231 of *OGFR* are parallel and common changes in salamanders (V190L, P231K) and lizards that can regenerate their tails (V190L, P231R) (Figure 4A), and all mutations were predicted to probably have a damaging effect on protein function. *OGFR* can interact with *TERF2IP*, a regulator of telomere length that is tightly bound to aging, raising a potential correlation between regeneration ability and aging.

To investigate the potential effect of *OGFR* and *SERPINI1* on regeneration, I used real-time quantitative PCR (qPCR) to measure their expression levels in the healing limb blastemata of Chinese fire-bellied newts (*Cynops orientalis*) at 0 h, 1 day, 5 days, 10 days, and 20 days post-amputation. The expression of both genes varied significantly at almost every time point compared to that at 0 h (Figure 5). Both genes exhibited similar trends along the time course, with gradually decreasing expression over time, reaching a minimum around the fifth day, and then gradually increasing expression. The expression level of one gene, *SERPINI1*, on the 20th day was almost restored to that at 0 h. Previous studies found that activation of *OGFR* prevents cell proliferation [118], and knockdown of *SERPINI1* reduces the outgrowth of neurons [119]. Thus, the observed fluctuations in expression levels over time may point to a dynamic regulation of cell proliferation and axonal growth during limb regeneration.

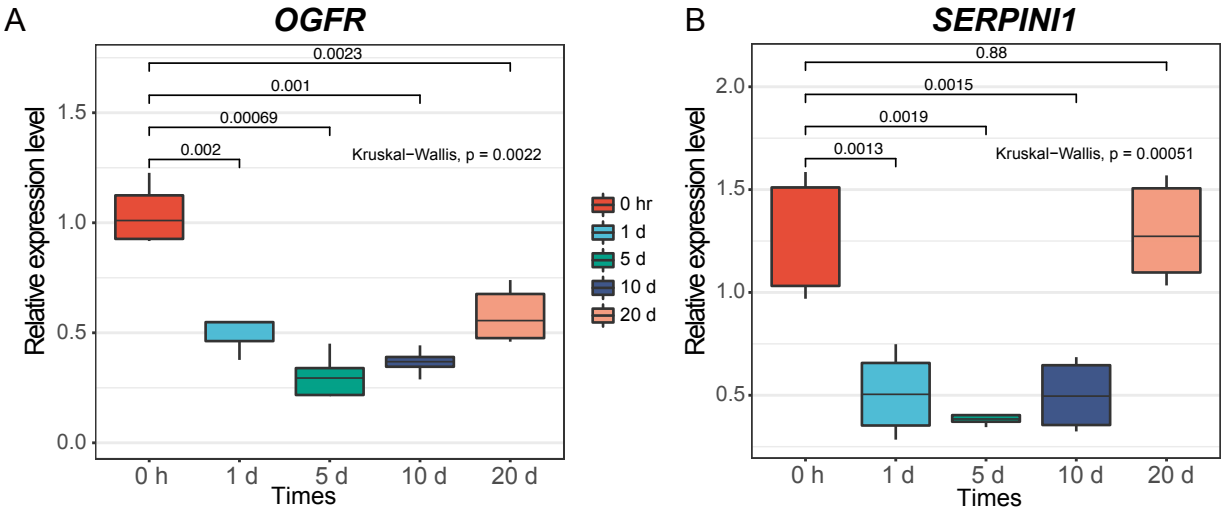

Figure 5. Expression patterns measured by qPCR during the time course of limb regeneration. (**A**) *OGFR*. (**B**) *SERPINI1*. Two biological replicates, each with three technical replicates, were measured at each time point.

This study identified several critical genes that are linked to regeneration. Many of these genes were expected and/or consistent with the findings of previous studies [111,120]. Interestingly, three genes (*EEF1E1*, *OGFR*, and *SERPINI1*) displayed significant signals of parallel evolution in salamanders and lizards, both of which possess regenerative capacities, implying a functional convergence for their regenerative mechanisms. Specific changes in these candidates in salamanders may explain, in part, why humans cannot regrow perfectly like salamanders. It is well known that some genes are only activated in specific tissues and/or conditions, such as in healing tissues [121]. Therefore, some genes may have been missed in my screening, which is a limitation of this study. Although I validated two regeneration-related candidates (*OGFR* and *SERPINI1*) by qPCR in healing blastemata and confirmed that their expression levels change significantly during limb regeneration, this was a limited solution.

3.6. Longevity-Related Genes in Salamanders

Lifespan commonly correlates with body mass for most animals. This rule is named "Larger animals live longer", although there are several exceptions in mammals, fish, reptiles, and birds (Figure 3D). It is noteworthy that this is not the case for most salamanders (Figure 3D). Olm (*Proteus anguinus*) is possibly the longest-living salamander, which can live over 100 years, even though its body mass is only about 20 g. In contrast, the longest-living mammal (bowhead whale) can live over 200 years but with a 100,000,000 g weight. The average lifespan of salamanders is about 18 years, calculated based on 70 species of salamanders, which is significantly higher than that of frogs (about 12 years; WRST $p = 2.9 \times 10^{-5}$) in the case where there is no significant difference in body mass between the sister groups (WRST $p = 0.219$).

How do salamanders extend their life span? The "rate of living" (ROL) theory of aging (the faster the metabolism, the shorter the lifespan) may partly answer this question (Figure S10; Spearman correlation = -0.65, $p = 7.5 \times 10^{-45}$), although there are numerous outliers. Salamanders possess the lowest metabolic rates among tetrapods [122], which can greatly help them reduce the accumulation of damage from reactive oxygen species (ROS) in the body and slow aging. Furthermore, the lowest metabolic rates may also lead to the extremely low mutation rates in salamanders due to the link between the frequency of oxidative damage and the likelihood of DNA change [123]. In other words, longevity seems to be negatively correlated with the mutation rate, which is similar to longer-lived rockfish (although the authors mainly focused on mitochondrial mutations) [124].

It is now clear that the genetic mechanisms underlying aging can be conserved across distantly related species [125]. Increasing evidence indicates that epigenetic factors have critical roles in aging [126]. I examined genes and GO categories that are related to epigenetics and compared them between salamanders and other long-living vertebrates.

A large number of REGOs in salamanders are involved in histone modification, particularly methylation and acetylation, and were significantly enriched (Figure 3E,F). The FEGs included *SIRT1*, *IWS1*, *SUPT6H*, *FTSJ3*, *THUMPD3*, and *TRMT11* (Figure 3G).

I further examined several candidate genes in more detail. *SIRT1*, also known as NAD-dependent deacetylase sirtuin-1, plays a critical role in metabolic regulation. It is an important genetic modulator for aging and longevity in humans, mice, worms, flies, and yeast [127]. *SIRT1* evolved much more rapidly in salamanders (dN/dS = 0.065) than in any other animals (dN/dS $\leq$ 0. 037). I uncovered two interesting amino acid changes occurring at crucial regions of *SIRT1* in salamanders. One mutation (K375R) located in the catalytic core small domain (365–417) of the sirtuin family domain (deacetylase sirtuin-type), a key component responsible for NAD+ binding and the histone deacetylation reaction (associated with aging), was predicted to be possibly harmful to protein function. In addition, K375R is a parallel change that occurred in both salamanders and the long-lived and cancer-resistant naked mole rat. It is possible that K375R may be directly related to longevity in salamanders. Another salamander-specific mutation (I227L) is in a nuclear localization signal (223–230) and a region that is the site of interaction with *CCAR2* (*DBC1*), a partner that can inhibit *SIRT1* deacetylase activity. I227L may decrease the interaction between *SIRT1* and *CCAR2* and delay aging. In addition, *SIRT1* is involved in the insulin-like signaling (ILS) pathway, which evolved rapidly in salamanders and is a metabolic signaling pathway involved in controlling life span in many species [125]. *SOCS3*, another FEG, is also involved in ILS regulation (Figure 3G). Notably, *IWS1* and *SUPT6H*, both evolving the fastest in salamanders, interact with each other and form a complex to control histone modifications [128]. In addition, *IWS1* has at least eight salamander-specific mutations (K581R, E605D, V612A, S646G, K653R, T700S, R716K, and V763L).

The DNA damage response and repair, which counter the constant assaults by endogenous and environmental agents on DNA, are critical for maintaining genetic stability and are considered to be the key to aging and longevity. I detected that DNA repair-related GOs evolved most rapidly in salamanders (Figure 2E; *PCNA*, *SIRT1*, and *PPP4R2*). Proliferating cell nuclear antigen (*PCNA*), which is essential for DNA replication and

damage repair [129], was associated with aging in humans, rats, and long-lived bowhead whales [130,131]. It is highly conserved even between plants and animals, indicating a strong selection pressure for structure conservation in order to interact with its partners. Correspondingly, numerous mutations (Figure 6A) in *PCNA* resulted in impaired DNA replication and damage repair [132]. Notably, salamander *PCNA* evolved at a rate more than two times faster than any other vertebrates (Figure 3G; and Table S1), which results in up to seven unique amino acid mutations present in all salamander species, and of these, three were predicted to be deleterious (Figures 4B and 6A).

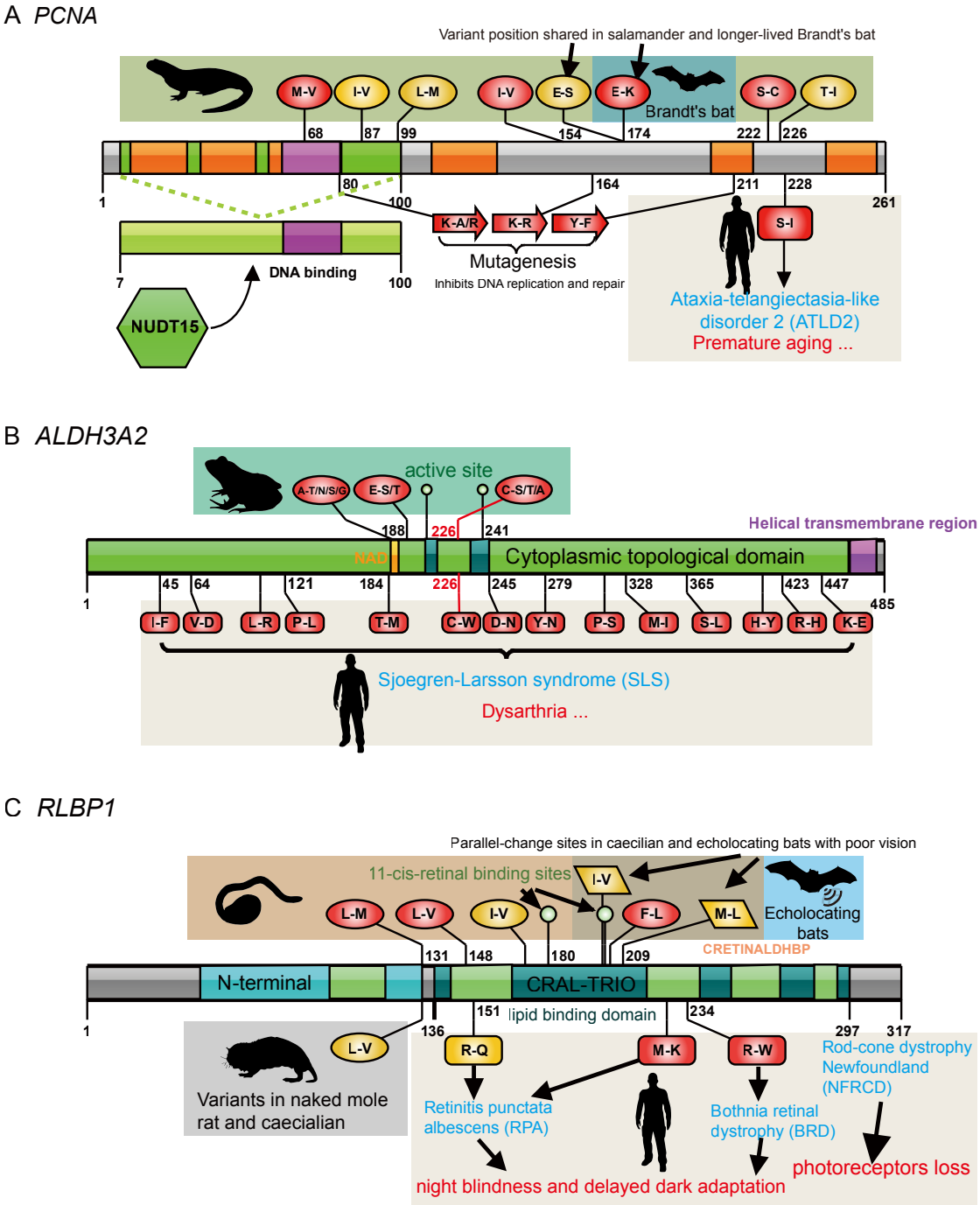

Figure 6. Specific and shared variants of candidate genes in extant amphibians and distantly related vertebrates with similar traits. (**A**) PCNA in salamanders. (**B**) ALDH3A2 in frogs. (**C**) RLBP1 in caecilians. Color of residue represents the strength of the functional impact. Red: harmful; yellow: neutral.

It is worth noting that three amino acid substitutions (M68V, I87V, and L99M) are located in the region that interacts with *NUDT15* (Figure 6A), a partner that is important in protecting *PCNA* from degradation [133]. One important mutation (M68V) is in the conserved DNA-binding region (61–79) and was considered to be deleterious (Figures 4B and 6A). These three changes were expected to strengthen the physiological interaction between *PCNA* and *NUDT15* and enhance the stability of *PCNA* in salamanders. It has been shown that the S228I mutation in humans significantly decreases *PCNA* interactions with *FEN1*, *LIG1*, and *ERCC5* and can give rise to an age-related syndrome (ataxia telangiectasia-like disorder 2 (ATLD2)) in which the clinical features include premature aging, a short stature, development delay, neurodegeneration, and hearing loss due to impaired DNA repair (Figure 6A) [134]. Here, I uncovered two salamander-specific amino acid mutations (S222C and T226I) near position 228, and S222C was predicted to have a damaging effect on *PCNA* structure (Figure 4B). Another interesting position in *PCNA* is 174, which is a well-conserved position in vertebrates because common changes were found in salamanders (E174S) and the longer-lived Brandt's bat (E174K) (Figure 6A). In contrast, this is not the case for other vertebrates, even for normal-lived bats (including the little brown bat, vampire bat, David's myotis, and black flying fox). Considering the key role of *PCNA* in the DNA damage response, these amino acid changes may be directly related to minimizing the negative effects of ROS, which aid in slowing the aging of salamanders. Moreover, it is well known that telomeres shorten with age, which is in part because of the end replication problem. *PCNA* may contribute to the maintenance of proper telomere length via semi-conservative replication. Moreover, although salamanders' aging-related module evolved significantly faster than that of their MRCAs (Figure 3C), the DNA repair process did not, raising the possibility that salamanders' MRCAs already possessed a stronger ability to repair DNA damage.

It is intriguing that the sensory perception of pain evolved much faster (greater than two times) in salamanders than in any other vertebrate (Figure 2E). A recent study indicated that loss of pain perception can directly delay aging via knocking out *TRPV1* pain receptors [135]. I studied *PTGS2*, a regenerative candidate that was mentioned previously, which is also responsible for the sensory perception of pain. This study's findings once again suggest that longevity and regeneration are likely to be closely related. Another similar case is *LMO4*. *PCNA*, *EEF1E1*, *OGFR*, and *SIRT1* also play dual roles in life expectancy and regenerative capacity. With this in mind, it is not difficult to imagine how salamanders can perfectly regenerate complex structures, even when aging.

3.7. Vocalization- and Hearing-Related Genes in Frogs

Vocalization is the primary form of communication of most frogs during their breeding season. I detected several positively selected genes that are potentially linked to vocalization and hearing.

The gene fatty aldehyde dehydrogenase (*ALDH3A2*) bore the signatures of positive selection. *ALDH3A2* is a critical gene associated with Sjögren–Larsson syndrome (SLS), which is characterized by dysarthria and a few other symptoms in humans. The three sites of *ALDH3A2* (Ala188, Glu195, and Cys226) that were under natural selection in frogs awakened my interest. These sites are located in the highly conserved aldehyde dehydrogenase domain (Figure 4C), and Ala188 is in a small core region (185–190) of the NAD-binding domain (Figure 6B). Importantly, amino acid changes at position 226, which varied in frogs, can cause SLS in humans (Figure 6B). Furthermore, many other variants in *ALDH3A2* can also lead to SLS [136], implying the importance of conserved sites to maintain the function of *ALDH3A2*.

FAM107B is a candidate associated with the sensory perception of sound. For frog *FAM107B*, I detected a unique amino acid change (A46S), which probably has a harmful effect on protein structure. I also observed three unique amino acid mutations in *FAM107B* in naked mole rats (H34R, L37F, and Q41L), who have lost much of their ability to localize sounds due to their subterranean lifestyle. In addition, *FAM107B* knockout mice exhibit

impaired hearing [137]. Furthermore, TIMM10, which is involved in the sensory perception of sound, evolved the fastest in frogs (Table S1).

3.8. Vision-Related Genes in Caecilians

Similar to naked mole rats, most caecilians live a subterranean lifestyle and have degenerated visual functions. I examined the molecular basis for the poor vision of caecilians and identified the top nine candidates. These included six genes that are potentially involved in visual perception/stimulus and/or the retinoic acid metabolic process, including three PSGs (*BCO2*, *JUNB*, and *CALR*), two FEGs (*RABGGTB* and *CLN5*), and one parallel-evolved gene (*RLBP1*).

The retinaldehyde-binding protein 1 (*RLBP1*) gene revealed an interesting pattern of parallel evolution. *RLBP1* is a crucial visual protein expressed in the retinal pigment epithelium and is involved in the retinal "visual cycle" [138]. Mutations in *RLBP1* cause rod–cone dysfunction and severe vision loss, and they are associated with numerous eye diseases such as night blindness, delayed dark adaptation, and loss of color vision (Figure 6C). I observed six unique missense mutations in *RLBP1*, of which five are located in the CRAL-TRIO domain, a hydrophobic binding pocket for 11-cis-retinal binding (Figure 6C). All known mutations in the CRAL-TRIO domain have been proven to cause a series of severe visual diseases due to impaired 11-cis-retinal binding and release triggered by *RLBP1* structural transitions [139]. Furthermore, I detected one common mutation in *RLBP1* between caecilians (L131M) and naked mole rats (L131V; Figure 6C) and two parallel changes between caecilians and echolocating bats (Brandt's bat, little brown bat, and David's myotis; I201V and M209L, $p = 0.000894$), which all live in dim-light environments and display a reduced visual capacity. Interestingly, a non-echolocating bat, the black flying fox, which has excellent eyesight, did not have changes at Ile201 and Met209. Considering the essential role of *RLBP1* in the conversion of photobleached opsin molecules into photosensitive visual pigments, these shared changes may be responsible for the complete or partial color blindness of these species and imply a convergent evolution due to their dark habitats.

A PSG, *AKR1B1*, was detected with the positively selected sites Val57 (BEBPP = 0.975) and Leu139 (BEBPP = 0.982). It is an important gene associated with retinal disease and cataracts in humans. It is noteworthy that all the caecilian-specific deleterious mutations occurred in important regions, especially the NADP-binding motif, which is located in a large, deep, elliptical pocket in the C-terminal end of *AKR1B1* (Figure 4D). A parallel site mutation (T136A, $p = 0.548152$) was only detected in caecilians and echolocating bats but not in non-echolocating bats. Another two PSGs (*B9D1* and *KLF4*) participate in camera-type eye development, whereas positive selection sites (Gln21 for *B9D1*, BEBPP = 0.998; Ser421 for *KLF4*, BEBPP = 0.994) and unique deleterious amino acid changes (T73S for *B9D1* and S421N for *KLF4*) may be associated with the small-sized eyes of caecilians.

4. Conclusions

To conclude, this study provides new insights into the origin of Lissamphibia and the genetic basis of adaptive traits of extant amphibians, particularly the regeneration ability and longevity of salamanders. The discovery of these critical genes will set the stage for further functional analyses. With the recent developments in gene editing technology, the importance and function of these candidate genes can be tested, which will provide much-needed clues to understanding the processes of regeneration and aging. All of these will open new avenues to understanding their genetic systems and to exploiting the genetic potential of humans and improving human well-being.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13223449/s1, Figure S1. Phylogenetic hypothesis about origin and relationship of three orders of extant amphibians. (A) Monophyletic origin: Batrachia hypothesis (H1); Procera hypothesis (H2); frog–caecilian sister relationship (H3). (B) Paraphyletic origin: caecilian–amniote and salamander–frog relationships (H4); (salamanders (caecilians, amniotes)) (H5); Figure S2. Completeness score and information content. (A) Completeness score of species pairwise comparison. A high score represents a high degree of shared site coverage (green), while a low score represents a low degree of shared site coverage (red). (B) Information content of potential phylogenetic signal for each gene. Dark blue represents a high information content, light blue represents a low information content, and red (0) represents no information content; Figure S3. Density plot showing degree of compositional heterogeneity for each gene. (A) Amino acid sequences; (B) CDS sequences. The lower the RCFV value, the lower the degree of compositional heterogeneity in that gene; Figure S4. Global stationary, reversible, and homogeneous (SRH) conditions of species pairwise comparison for different datasets (A-I). The higher the *p*-value, the more consistent its evolution with evolution under SRH conditions; Figure S5. Supporting conditions for the five phylogenetic hypotheses concerning the relationship among the three orders of extant amphibians. (A) AU test based on amino acid sequences. Each vertical line represents a gene, and different colors represent different *p*-values from the AU tests. A small *p*-value (dark green) indicates that a gene rejects a topology. (B) AU test based on CDS sequences. (C) Venn diagram based on amino acid sequences. Numbers in brackets represent the quantity of genes that cannot reject a specific hypothesis. (D) Venn diagram based on CDS sequences; Figure S6. Estimation of origin and divergence date of extant amphibians. (A) Clock of protein. (B) Clock of 2nd codon of CDS. Numbers on nodes are age estimations, and blue bars represent 95% confidence intervals. Time unit is ten billion years. Fossil records are in red point on node; Figure S7. Estimation of mutation rates of the synonymous sites (dS). (A) Boxplot of dS values for each major vertebrate group. Red point is the mean value of the dN/dS ratio. Salamanders' dS ratios were significantly smaller than any other vertebrates ($p < 2.9 \times 10^{-40}$ for all pairwise tests). (B) Clustered heatmap of dN/dS ratio of major vertebrate groups. Red color represents a high dN/dS ratio; blue color represents a low dN/dS ratio. (C) dS value tree of 22 vertebrates. Red color represents high dS values, and green represents low dS values; Figure S8. Four-fold degenerate site tree of 22 vertebrates. Color represents mutation rate of 4-fold degenerate sites. Red color represents high rates and green represents low rates; Figure S9. dN/dS ratio comparison of development-related genes and the other genes in three orders of extant amphibians and four other major vertebrates. Wilcoxon rank sum test *p*-values are presented. Only salamanders' dN/dS ratio of development-related genes was significantly greater than the other genes ($p = 0.039$); Figure S10. Scatter diagram of relationship between metabolic rate and max lifespan; Table S1. Fast-evolving genes for salamanders, frogs, and caecilians; Table S2. Positively selected genes for salamanders, frogs, and caecilians.

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Abbreviations

4D	Four-fold degenerate sites
BEBPP	Bayes empirical Bayes posterior probabilities
BI	Bayesian inference
BIC	Bayesian Information Criterion
CDS	Coding sequence
CH	Compositional heterogeneity
ECM	Extracellular matrix
FDR	False discovery rate
FEGs	Fast-evolving genes
GAC	Genes as characters
GO	Gene Ontology
HaMSTR	Hidden Markov Model-based Search for Orthologs using Reciprocity
LRT	Likelihood ratio test
ML	Maximum likelihood
MPEs	Maximum pseudo-likelihood estimates
PSGs	Positively selected genes
RCFV	Relative Composition Frequency Variability
REGOs	Rapidly evolving GO categories
ROL	Rate of living
SRH	Stationary, reversible, and homogeneous conditions
WRST	Wilcoxon rank sum test

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Disentangling Exploitative and Interference Competition on Forest Dwelling Salamanders

Giacomo Rosa, Sebastiano Salvidio and Andrea Costa *

Department of Earth, Environment and Life Sciences (DISTAV), University of Genova, Corso Europa 26, 16126 Genova, Italy
* Correspondence: andrea-costa-@hotmail.it

Simple Summary: Exploitative competition and interference competition differ in the way access to resources is modulated by a competitor. Exploitative competition implies resource depletion and usually produces spatial segregation, while interference competition is independent from resource availability and can result in temporal niche partitioning. Here, we inferred the presence of these two patterns of competition on a two-salamander system in Northern Italy. We found evidence supporting interference competition and temporal niche partitioning.

Abstract: Exploitative competition and interference competition differ in the way access to resources is modulated by a competitor. Exploitative competition implies resource depletion and usually produces spatial segregation, while interference competition is independent from resource availability and can result in temporal niche partitioning. Our aim is to infer the presence of spatial or temporal niche partitioning on a two-species system of terrestrial salamanders in Northern Italy: *Speleomantes strinatii* and *Salamandrina perspicillata*. We conducted 3 repeated surveys on 26 plots in spring 2018, on a sampling site where both species are present. We modelled count data with N-mixture models accounting for directional interactions on both abundance and detection process. In this way we were able to disentangle the effect of competitive interaction on the spatial scale, i.e., local abundance, and from the temporal scale, i.e., surface activity. We found strong evidence supporting the presence of temporal niche partitioning, consistent with interference competition. At the same time, no evidence of spatial segregation has been observed.

Keywords: exploitative competition; interference competition; multi-species N-mixture models; salamanders; temporal niche partitioning

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1. Introduction

Identifying which ecological processes influence the occurrence and local abundance of a species is of fundamental interest among ecologists and conservationists. Local abundance for a given species is expected to reach its maximum when environmental features correspond to the optimum of its ecological requirements [1,2]. However, the local rise in abundance or density of a species as resources or suitability of a given area increase is counterbalanced and limited by biotic interactions with other organisms that share the same ecological niche, usually through density-dependent competitive interactions [3,4].

Even though the study of competition laid its root early in the field of ecology [5], nowadays, the topic is still alive and debated, and the consequences of competitive interactions are still attracting interest among the scientific community. This happens at the theoretical [6–8] and practical level [9,10]. Competitive interactions can occur at the inter- or intra-specific level and can involve various niche axes and mechanisms. For instance, organisms compete directly for mates (e.g., [11,12]) and physical space, such as habitat or shelter use [13], and indirectly for food resources [14] or other niches, such as call space [15]. Regardless of the mechanism or origin of competitive interactions, they usually occur when

one species prevents the access of its competitors to a shared resource. This exclusion from use of a resource usually takes place in two different ways: exploitative competition or interference competition [13,16,17]. These two types of competition differ substantially in the way they affect the access of one species to a given resource. In the case of exploitative competition, some organisms make better use of the same limiting resource, directly reducing the availability of the shared resource for other individuals [18]. By contrast, interference competition occurs when one organism actively prevents another organism from accessing a resource, independently from its availability, for instance by means of territorial or aggressive behaviors [13,16].

Both types of competition are assumed to have significant effects on population dynamics, local abundance, community assembly, and species coexistence [5,19,20]. Indeed, exploitative competition can either lead to the exclusion of a species from a given habitat [18], resulting in a density reduction of one competitor, or can trigger resource partitioning among different niche axes [21–23]. Interference competition can trigger competitive exclusion [3], causing behavioral changes, for instance by shifting species activity patterns [13,17]. One example of interference competition is the shift to crepuscular activity of a species that avoids the presence of a more nocturnal competitor [17].

Likewise, different kinds of competitive interactions can result in different layouts of niche and resource partitioning, minimizing niche overlap and competitive exclusion [13]. For instance, when two species or organisms segregate over different microhabitats, we observe spatial niche partitioning; conversely, when organisms exploit the same set of resources but in different time frames, we observe a partitioning along the temporal niche [2,24]. In this respect, exploitative and interference competition are also hypothesized to have different outcomes for what concern spatial or temporal segregation/partitioning. Exploitative competition has been found to have an effect on the local abundance or spatial displacement of one or both competitors (e.g., [21]). This is because of a density-dependent effect on limited resources, in which the under-performing competitor must rely on the leftover resource by the best-performing competitor [25,26]. Conversely, interference competition usually produces niche shift patterns that are consistent with temporal niche partitioning, i.e., when species access the same resource in different timeframes in order to reduce their interactions with the competitor (e.g., [17,27,28]).

In the present study, our aim was to test the occurrence of exploitative or interference competition in a two-species system of forest dwelling salamanders, the Strinati's cave salamander (*Speleomantes strinatii*) and the northern spectacled salamander (*Salamandrina perspicillata*). These two salamanders are found syntopically in NW Italy, where they rely on the same set of trophic resources, particularly soil-dwelling invertebrates [29,30]. We used binomial N-mixture models that account for directional biotic interaction on both the local abundance and the timing of surface activity to alternatively test the occurrence of spatial niche partitioning or temporal niche partitioning, after modelling abundance and activity on environmental covariates. We expected that (i) in the case of exploitative competition, we should observe a negative interaction of one species on the local abundance of the other, this being a negative density-dependent effect; or (ii) in the case of interference competition we should observe a negative interaction of one species on the surface activity of the other, this outcome representing niche partitioning on the temporal axis.

2. Materials and Methods

2.1. Study Framework

To test for the presence of exploitative or interference competition, we randomly selected 26 permanent plots under a meta-population design [31,32], in one site in Northern Italy where both salamanders are present. We counted salamanders in all plots during three repeated surveys over a short time period. We then used count data to model abundance and activity interaction occurring between the species, using a two-species N-mixture model [31], while incorporating environmental covariates [33–38].

2.2. Study Species

The first focal species is the Strinati's cave salamander *Speleomantes strinatii*, a plethodontid salamander with a maximum total length of about 128 mm. The second focal species is the northern spectacled salamander *Salamandrina perspicillata*, that belongs to family Salamandridae; this salamander is smaller, not exceeding 100 mm in total length. Strinati's cave salamanders possess direct development and are fully terrestrial, while northern spectacled salamanders have a biphasic life cycle, lay eggs in the water, and become fully terrestrial after metamorphosis, with only reproductive females entering the water for egg deposition [39,40]. Adults of both species are lungless (*S. strinatii*) or have vestigial lungs (*S. perspicillata*), and gas exchange occurs mainly through their skin. Both species are usually found in the talus and the leaf litter of mixed broadleaved woodlands, and their surface activity is mainly regulated by the amount of rain or soil moisture [29,37,41]. In forest environments, the diet of *S. strinatii* and *S. perspicillata* has been extensively studied, by means of stomach flushing: both species feed on a large number of invertebrate taxa and share many trophic resources [29,30]. However, at the population level *S. strinatii* has been described as a generalist predator [29], while *S. perspicillata* is a specialized predator on springtails and mites [30].

2.3. Study Site

The study area is located in North-Western Italy, on the Apennine Mountain range of the Piemonte region, where the two studied species occurs sympatrically and are often found syntopically. The sampling area is located at an altitude of 600 m a.s.l. in the municipality of Mongiardino Ligure (44°38′24″ N; 9°03′00″ E), Alessandria province. The site is crossed by a first order Apennine stream and is characterized by a Supra–Mediterranean mixed deciduous forest, dominated by European Oak (*Quercus pubescens* Willd., 1805; [42]). We selected, individually marked, and GPS-positioned 26 square plots in the proximity of the stream banks, each one measuring 30 m^2 (5.5 m side). The minimum distance among plots was about 20 m.

2.4. Salamanders' Sampling

In spring 2018 (16 April–25 May), the same observer visited all sites three times during daytime (8–11 a.m.) and in favorable weather conditions for salamander's activity (e.g., during or immediately after light rain). During each survey the observer searched for salamanders within each plot for four minutes [37,43], checking the leaf litter, inspecting rock crevices with a flashlight, and lifting temporary shelters such as superficial rocks and dead wood in search of salamanders. Since both species are known to be active during or immediately after rain and considering that inactive individuals retreat underground and are usually unavailable for sampling, we considered both individuals encountered on the forest floor and those found under temporary shelters as active on the surface [37,38,41]. We assigned each individual to one of the two study species and their abundance in each plot recorded.

2.5. Environmental Covariates

Within each marked plot at a depth of 20 cm, we obtained five measurements of soil relative humidity using a digital moisture meter (Extech MO750). All measurements were obtained on the same day (20 April 2018), 4 days following a 50 mm precipitation event. The average of these five measurements was considered as a plot-specific proxy, describing the soil moisture retention potential (MOIST). From a digital elevation model (DEM; 20 m mesh size) of the study area, we calculated two covariates: the duration of direct insolation (INSOL), expressed in hours, and the topographic position index (TPI). This latter index expresses the topographic position of each cell within the landscape, assuming positive values for cells located on ridges or hilltops and negative values for cells located in depressions [44]. Moreover, for each sampling session, we recorded the day of the year (DAY; i.e., the continuous count of the number of days beginning each year from

1 January). We obtained the temperature of the survey (TEMP) and the accumulated rain in the 72 h prior to sampling (RAIN) from local weather stations. We conducted terrain analyses with software SAGA 7 [45].

2.6. Data Analysis

We analyzed our repeated count data of *S. strinatii* and *S. perspicillata* using a co-abundance formulation of the static binomial N-mixture model of Royle [31], accounting for directional biotic interactions [32], both on the abundance of salamanders and on their activity pattern. Binomial N-mixture models estimate the latent abundance state (N) at site i (Ni), assuming $Ni \sim Poisson(\lambda)$, where λ is the expected abundance over all sites, by using repeated counts C at site i during survey j (Cij) to estimate individual detection probability p, assuming $Cij \mid Ni \sim Binomial(Ni,p)$. Both parameters can be modelled as a function of environmental covariates trough a *log* or *logit* link, respectively. In order to model the possible effect of exploitative competition on local abundance, we stacked two N-mixture models, using the latent abundance of the larger species (i.e., *S. strinatii*) as a covariate in the abundance model of *S. perspicillata* [34–36]. Therefore, we added a co-abundance interaction effect term (γ) to the model [33] to estimate the overall effect of the abundance of Strinati's cave salamanders on the abundance of the northern spectacled salamander [32]. Likewise, to model the possible presence of interference competition resulting from reduced activity of the smaller species when the larger one is active, we considered the detection probability p of both species to be a rough proxy of species' surface activity. This approach is widely shared and often successfully used in ecological studies assessing species interactions [37,38,46]. Therefore, we added another interaction term (ε) on the detection process, using the detection probability *pij* of *S. strinatii* as a covariate on the detection of *S. perpicillata*. Prior to building our model, we standardized covariates and checked them for collinearity, considering a cut-off for inclusion of Pearson r < 0.7 [47,48]. We modelled the detection process of two species as follows:

$$[1; \textit{S. strinatii}]\ logit(pij_{Ss}) = \alpha 0 + \alpha 1 * DAYij + \alpha 2 * TEMPij + \alpha 3 * RAINij + \delta ij$$

$$[2; \textit{S. perspicillata}]\ logit(pij_{Sp}) = \alpha 0 + \alpha 1 * DAYij + \alpha 2 * TEMPij + \alpha 3 * RAINij + \varepsilon * pij_{Ss} + \delta ij$$

where the subscripts *Ss* and *Sp* stand for *S. strinatii* and *S. perspicillata*, respectively, $\alpha 0$ is the intercept, $\alpha 1$–$\alpha 3$ are covariate effects, ε is the directional interaction term on the activity between the two species, and δ is a normally distributed random effect that accounts for possible over dispersion in the detection process [49]. Similarly, we modelled the abundances of the two species as:

$$[3; \textit{S. strinatii}]\ log\ (NiSs) = \beta 0 + \beta 1 * MOISTi + \beta 2 * INSOLi + \beta 3 * TPIi + \eta i$$

$$[4; \textit{S. perspicillata}]\ log\ (NiSp) = \beta 0 + \beta 1 * MOISTi + \beta 2 * INSOLi + \beta 3 * TPIi + \gamma * NiSs + \eta i$$

where the subscripts *Ss* and *Sp* stand for *S. strinatii* and *S. perspicillata*, respectively, $\beta 0$ is the intercept, $\beta 1$–$\beta 3$ are covariate effects, *Ni* is the latent abundance of adults at site i, γ is the co-abundance effect of adults on juveniles, and η is a normally distributed site-level random effect [49]. We estimated model parameters using a Bayesian approach with Markov chain Monte–Carlo methods, using uninformative priors. We ran three chains, each one with an adaptive phase of 10,000 iterations, followed by 350,000 iterations, discarding the first 50,000 as a burn-in and thinning by 100. We considered that chains reached convergence when the Gelman–Rubin statistic (R-hat) was <1.1 [50]. We used the 90% highest density interval of the posterior distribution as a credible interval (CRI), and considered covariates on both abundance, detection, and biotic interaction to have a significant effect when CRI did not cross the zero. In order to assess model fit [51–53], we employed posterior predictive checks based on χ^2 statistics as a measure of the discrepancy between observed and simulated data and calculated a Bayesian p-value accordingly [49]. Analyses were

conducted calling program JAGS (V4.3.0; [54]) from the R environment with package "JagsUI" (V1.5.1; [55]).

3. Results

During our sampling, we counted 96 salamanders: 61 *S. strinatii* and 35 *S. perspicillata*. For both species, the co-abundance N-mixture model was in a good fit (posterior predictive checks: Bayesian *p*-value for *S. strinatii* = 0.46, and for *S. perspicillata* = 0.51). For all monitored parameters, convergence resulted successful (maximum R-hat = 1.059). The complete list of parameters' estimates, along with their respective 90% CRI, for both species, is reported in Table 1.

The two species showed different detection probabilities, with *S. strinatii*, the larger species, being more active on the forest floor (p = 0.43; CRI = 0.21–0.66) when compared to *S. perspicillata* (p = 0.32; CRI = 0.08–0.62). None of the survey covariates had a clear effect on p (CRI crossed zero, in all cases), although there was an 89.7% probability that TEMP had a negative effect on the detection of *S. perspicillata* (Pd, posterior probability of direction; Table 1). The two species also showed different per-site abundances, with *S. strinatii* (λ = 0.70; CRI = 0.25–1.15) being less abundant than *S. perspicillata* (λ = 2.10; CRI = 0.48–4.02).

Overall, the two species showed differential responses to the same set of environmental predictors, both for the ecological process and their detection/activity. Indeed, the abundance of *S. strinatii* was positively affected by MOIST (Figure 1) and negatively affected by INSOL (Figure 2), while abundance of *S. perspicillata* was only negatively affected by INSOL (Figure 2).

Finally, for what concern the two interaction terms, the one on the surface activity of *S. perspicillata* was negative and significant ($\varepsilon = -3.76$; CRI = −7.66–−0.09), highlighting that the surface activity of *S. strinatii* has a strong negative effect on the activity of *S. perspicillata* (Figure 3). At the same time, the interaction term on local abundance was also negative ($\gamma = -0.18$), but its CRI largely crossed the zero (CRI = −0.56–0.20; pd = 79.9%; Figure 3).

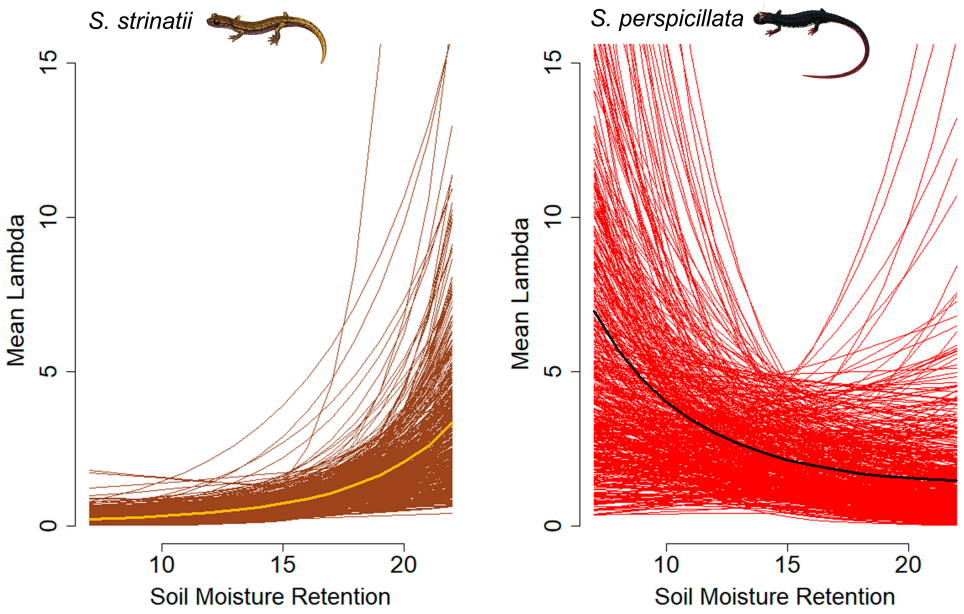

Figure 1. Plots showing the effect of the soil moisture retention potential (MOIST) on salamander abundance: *Speleomantes strinatii* on the **left** and *Salamandrina perspicillata* on the **right**. Bold lines represent the posterior mean, while the thin lines represent 500 random draws from the posterior distribution.

Table 1. Complete list of the parameters estimated for the detection process, abundance, and interactions for both *S. strinatii* and *S. perspicillata*. Mean = mean obtained from the posterior distribution; 90% CRI = 90% highest density region of the posterior distribution; pd = probability of direction.

Speleomantes strinatii				*Salamandrina perspicillata*			
Parameter	**Mean**	**90% CRI**	**pd**	**Parameter**	**Mean**	**90% CRI**	**pd**
Detection				**Detection**			
Mean p	0.43	0.21–0.66	-	Mean p	0.32	0.08–0.62	–
DAY	0.04	−3.9–−4.3	50.7	DAY	−0.43	−4.85–3.61	57.0
TEMP	1.32	−1.7–4.4	76.6	TEMP	−2.59	−5.88–0.97	89.7
RAIN	1.33	−1.56–4.32	77.0	RAIN	−1.81	−5.2–1.37	82.0
Abundance				**Abundance**			
Mean λ	0.70	0.25–1.15	-	Mean λ	2.10	0.48–4.02	-
MOIST	0.88	0.25–1.57	98.7	MOIST	−0.48	−1.30–0.29	85.0
INSOL	−0.76	−1.31–−0.20	99.2	INSOL	−1.41	−2.34–−0.37	99.5
TPI	−1.11	−0.48–0.24	70.0	TPI	−0.33	−0.99–0.36	79.6
				Interactions			
				ε	−3.76	−7.66–−0.09	94.8
				γ	−0.18	−0.56–0.20	79.9

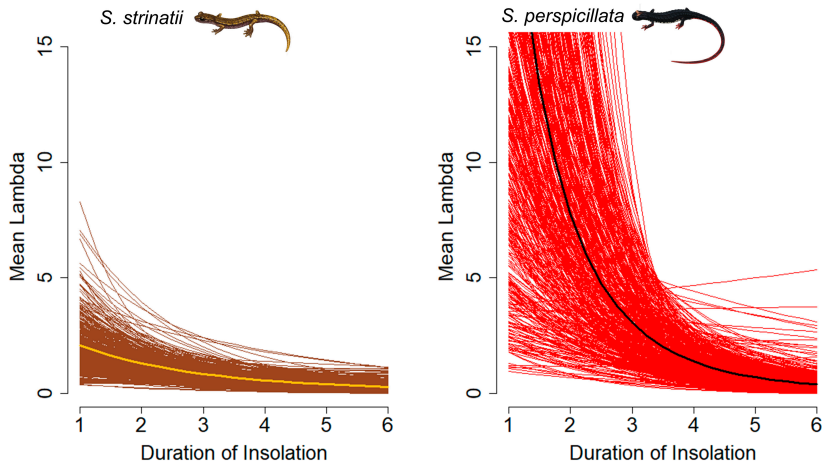

Figure 2. Plots showing the effect of duration of direct insolation (INSOL) on salamander abundance: *Speleomantes strinatii* on the **left** and *Salamandrina perspicillata* on the **right**. Bold lines represent the posterior mean, while the thin lines represent 500 random draws from the posterior distribution.

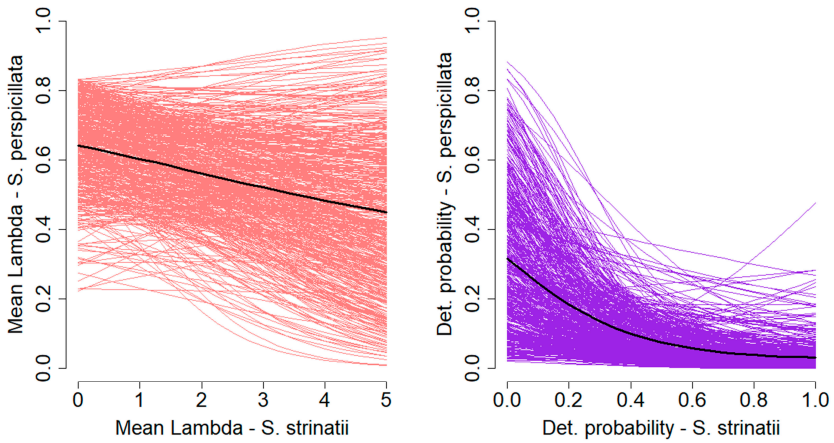

Figure 3. Plots showing the effect of surface activity (**left panel**; interaction terms ε) and abundance of *S. strinatii* (**right panel**; interaction term γ) on the surface activity and abundance of *S. perspicillata*. Bold lines represent the posterior mean, while the thin lines represent 500 random draws from the posterior distribution.

4. Discussion

Identifying the ecological processes that impact the presence and population size of a species holds significant importance for ecologists and conservationists, since the maximum local abundance of a species is observed when environmental characteristics align with its ecological requirements. On the other hand, the increase in local abundance or density of a species due to improved resources or suitability of a particular area is constrained by biotic interactions with other organisms that partially overlap the same ecological niche. These interactions typically occur as density-dependent competitive interactions, which can balance and limit local abundance. These competitive interactions can arise within or between species, encompassing a range of niche axes and mechanisms. For instance, organisms engage in direct competition for mates and physical space, such as habitats or shelters, as well as indirect competition for food resources or other niches, such as call space. Irrespective of the mechanism or origin of these competitive interactions, they typically emerge when one species hinders its competitors' access to a shared resource. This exclusion from resource utilization generally occurs through two distinct pathways: exploitative competition or interference competition. These two forms of competition differ significantly in their impact on a species' access to a particular resource. Exploitative competition arises when certain organisms make more efficient use of the same limited resource, thus directly reducing its availability for other individuals. Conversely, interference competition takes place when one organism actively obstructs another organism's access to a resource, regardless of its availability.

In this study, we found evidence that interference competition, rather than exploitative competition, is shaping the co-occurrence of *Speleomantes strinatii* (Strinati's cave salamander) and *S. perspicillata* (northern spectacled salamander), after accounting for differential habitat requirements. That is, after modelling ecological requirements of the two species by means N-mixture models using appropriate covariates on both the abundance and detection processes, we still detected some significant interaction between the two species, which is attributable to a biological interaction [32–36]. Specifically, after accounting for environmental covariates, we found that the activity of *S. strinatii* negatively affected the surface activity of *S. perspicillata*, indicating that the observed difference in surface activity between the two species can be explained by temporal niche partitioning. This suggests that the two species avoided direct interactions on the forest floor by being active at different times. Conversely, we did not find any effect of Strinati's cave salamander's abundance on northern spectacled salamander's abundance at the scale at which the study has been conducted. This indicates that resource depletion (i.e., exploitative competition) probably does not limit the local abundance of either species in the study area. The overall findings of this study align with previous research on interference competition, which has been observed to produce niche shift patterns consistent with temporal niche partitioning [17,27,28].

In contrast, our results did not provide support for the hypothesis of exploitative competition, which influences local abundance or spatial displacement of one or both competitors due to density-dependent effects on limited resources [21,25,26]. The lack of evidence for exploitative competition in this two-species system may imply that resource availability for these salamander species is not a major ecological limiting factor, or that they have developed mechanisms partitioning resources in a way that avoids direct competition [13]. Indeed, *S. perspicillata* and *S. strinatii* share some of the same trophic resources, but (i) display different trophic strategies [29,30], (ii) forage successfully in slightly different weather conditions [56], and (iii) when they both are active on the forest floor, *S. perspicillata* reduces the foraging intensity on the core prey items composing its diet, suggesting some kind of interference with *S. strinatii* [57].

Such niche differentiation likely relaxes competition for food, explaining why we did not detect exploitative effects. Temporal niche partitioning due to interference competition has been observed in several taxa, including amphibians [58], birds [59], and mammals [60]. For other forest salamanders, Jaeger and colleagues [58,61,62] found evidence of temporal niche partitioning at the intraspecific level in the eastern red-backed

salamander (*Plethodon cinereus*), which was driven by interference competition for food resources and territories during the breeding season. However, interference competition has also been found to produce negative effects on the fitness and survival of competitors. Hairston et al. [63] found that interference competition between *P. cinereus* and *P. richmondi* resulted in reduced growth rates and survival of *P. richmondi* due to aggressive interactions with *P. cinereus*. Similarly, Petranka and Smith [64] found that interference competition between *P. cinereus* and *P. glutinosus* resulted in decreased survival and growth rates of both species.

Salamandrina perspicillata in the study site, despite being the outperformed species (i.e., its overall activity is reduced by the surface activity of *S. strinatii*), is roughly three times more abundant than *S. strinatii*. This is further evidence for the lack of a density-related exploitative competition on this two-species system, otherwise *S. perspicillata* abundance should be reduced. This also suggests the presence of behavioral strategies to minimize the overlap in their realized temporal niches. This may have been facilitated by their ability to successfully exploit similar prey resources at different times, thus minimizing the intensity of their competitive trophic interactions [2,24].

5. Conclusions

Our results support the hypothesis that interference competition promotes temporal niche partitioning in terrestrial salamanders. By shifting their surface activity, *S. strinatii* and *S. perspicillata* can co-occur at high densities while minimizing competitive interactions. On the other hand, trophic niche differentiation, divergences in metabolism, prey selection or behavioral traits, together with a high prey abundance may help to prevent the emergence of competition for food resources and may promote species coexistence [65]. Nevertheless, further studies in different ecological situations and adopting different sampling frameworks specifically designed to model surface activity should be conducted to help clarify the role of temporal or spatial niche partitioning in shaping the coexistence of these salamander species.

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An Isolated and Deeply Divergent *Hynobius* Species from Fujian, China

Zhenqi Wang [1,†], Siti N. Othman [2,†], Zhixin Qiu [1], Yiqiu Lu [1], Vishal Kumar Prasad [2], Yuran Dong [1], Chang-Hu Lu [1] and Amaël Borzée [2,3,*]

1 The Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Nanjing Forestry University, Nanjing 210037, China
2 Laboratory of Animal Behaviour and Conservation, College of Biology and the Environment, Nanjing Forestry University, Nanjing 210037, China
3 Jiangsu Agricultural Biodiversity Cultivation and Utilization Research Center, Nanjing 210014, China
* Correspondence: amaelborzee@gmail.com
† These authors contributed equally to this work.

Simple Summary: What does not have a name is difficult to understand and protect. Upon the unexpected discovery of an *Hynobius* salamander in Fujian province, China, we worked on understanding its relationship with other species and ultimately describing it. Please welcome the Fujian Bamboo Salamander to science, a segregated species based on genetics and morphology. While it is related to other southern mainland Chinese species, it may have diverged earlier and share some similarities with morphology and behavior with the Anji salamander. The Fujian Bamboo Salamander is special as it produces vocalization when under threat. The species is, however, incredibly rare, fitting the definition of Critically Endangered in the IUCN Red List of Threatened Species.

Abstract: It is important to describe lineages before they go extinct, as we can only protect what we know. This is especially important in the case of microendemic species likely to be relict populations, such as *Hynobius* salamanders in southern China. Here, we unexpectedly sampled *Hynobius* individuals in Fujian province, China, and then worked on determining their taxonomic status. We describe *Hynobius bambusicolus* sp. nov. based on molecular and morphological data. The lineage is deeply divergent and clusters with the other southern Chinese *Hynobius* species based on the concatenated mtDNA gene fragments (>1500 bp), being the sister group to *H. amjiensis* based on the *COI* gene fragment, despite their geographic distance. In terms of morphology, the species can be identified through discrete characters enabling identification in the field by eye, an unusual convenience in *Hynobius* species. In addition, we noted some interesting life history traits in the species, such as vocalization and cannibalism. The species is likely to be incredibly rare, over a massively restricted distribution, fitting the definition of Critically Endangered following several lines of criteria and categories of the IUCN Red List of Threatened Species.

Keywords: Hynobidae; species description; bamboo forest

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1. Introduction

Anthropogenisation of landscapes, and other human activities, have brought the world to the sixth great mass extinction [1,2]. The threats to species are not equal, and large-bodied species [3], as well as species with narrow spatial ranges [4], are principally impacted. The toll on species is staggering [5], and between 900 and 130,000 species have become extinct since the 1500s (www.iucnredlist.org/statistics; accessed on 3 May 2023). Similarly, species that have not yet been described are going extinct before being documented [6] and without known impacts [7] (as seen, for instance, in spiders [8]). Biodiversity loss is close to a tipping point [9], and conservation actions are the last tool to maintain evolutionary patterns free of anthropomorphic selection [10]. However, for conservation

to be achieved, practitioners need to know what to protect, and thus species need to be described currently, conservation does not correspond to threat status [11], and we can only protect what we know.

With 41% of species listened as threatened by the IUCN Red List of Species (www.iucnredlist.org; accessed on 3 May 2023), amphibians are the most threatened animal class, with habitat loss being one of the principal drivers of species decline [12,13]. For the status of amphibians to improve, a clear taxonomy is first needed, and despite the large number of species described in China every year [14], many species are still in need of formal description or taxonomic revision. This need is especially true for southern China, a hotspot for biodiscovery [15] and conservation needs [16].

All *Hynobius* salamanders species were expected to have been described in China, although the taxonomic resolution of the genus is still an ongoing work, with some recent descriptions in other range countries of the genus, including the Republic of Korea [17] and Japan [18–20]. There are five described *Hynobius* species in mainland China, all part of the Southern Chinese group and all meant to be breeding in lentic water bodies, and four species on Taiwan Island, belonging to a segregated phylogenetic group and breeding in small cool mountain streams [21]. All these species are terrestrial, partially fossorial, and breed through larval development in water bodies. The species in southern China belong to the *Hynobius chinensis* group and include *H. chinensis* Günther, 1889 [22], *H. amjiensis* Gu, 1992 [23], *H. guabangshanensis* Shen, 2004 [24], *H. maoershanensis* Zhou, Jiang and Jiang, 2006 [25] and *H. yiwuensis* Cai, 1985 [26].

A *Hynobius* salamander was reported from Fujian province in 1978 and identified as *H. chinensis* [27]. However, following the multiple species descriptions since then, the distribution of *H. chinensis* is now known to be restricted to the Hubei province, and the morphological differences between the species have been clarified, including embryos [28]. The *Hynobius* salamander collected in 1978 from Fujian is therefore considered an undescribed species, and no additional individual has been found in the area since then, potentially resulting from local extirpations. As a result, upon encountering *Hynobius* salamanders in Fujian, we tested for phylogenetic clustering within the *H. chinensis* clade, then proceeded to determine their taxonomic status with phylogenetic tools, and accordingly described a new species with specific morphological characters.

2. Materials and Methods

2.1. Field Sampling

We found two individuals of the genus *Hynobius*, one in January and one in August 2022, from Quxi village, Liancheng County, Fujian, China (25.566° N, 116.938° E). We do not provide precise GPS coordinates here to protect the species from harvesting for the pet trade. The two individuals were found in a bamboo forest (*Phyllostachys* cf. *edulis*) about 1500 m above sea level. As ten days (24–26 January and 4–10 August) of fieldwork during adequate sampling seasons and times of day resulted in only two adult individuals, and the population size at this location, and likely for this species, is likely lower than 200 individuals, we followed the IUCN recommendation on ethical sampling [29]. We did not collect the individuals but instead orally swabbed them to obtain genetic materials and measured them (see Section 2.5 Morphometry) before releasing them at the point of capture to avoid a further threat to the species. In addition, we observed two waterbodies with eight pairs of egg sacs in January 2022, and we collected one egg sac deposited by the species for morphological measurement and to study the development of larvae in the species before releasing them at the point of capture. We used the entire body of an embryo from the egg sacks collected that died at an early developmental stage to extract DNA for a third individual (ID: 22HyF007). We consider this individual unlikely to be related to the adults swabbed due to the distance between the egg sacks and the adults in view of the dispersal abilities of the genus [30].

2.2. Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were strictly followed. All animal sample collection protocols complied with the current laws of the People's Republic of China. All observations and experiments conducted in this study are in agreement with the ethical recommendations of the College of Biology and the Environment at Nanjing Forestry University (IACUC approval number 2022014). We did not collect adult individuals as voucher specimens, in line with the IUCN recommendation on research involving species at risk of extinction [29]. Instead, we relied on one of the individuals raised from the egg mass.

2.3. DNA Preparation

We extracted the total genomic DNA for all three samples using the Qiagen DNeasy Blood & Tissue kit (QIAGEN Group, Hilden, Germany) according to the manufacturer's protocol. We then performed standard Polymerase Chain Reaction (PCR) amplifications for all 15 samples in 20 µL of total reaction per tube, containing 35 to 50 ng/µL of template DNA (Table 1). The final concentrations of the other PCR reagents were such as 0.125 µM for each forward and reversed primer, 1× Ex taq Buffer (Takara; Shiga, Japan), 0.2 mM of dNTPs Mix (Takara; Shiga, Japan), 1.875 mM of magnesium chloride ($MgCl_2$), 0.1 unit/µL of Ex taq (HR001A, Takara; Shiga, Japan), and double distilled water added to make up the final volume. The PCR thermal profiles for each primer fragment are described in Table 1.

Table 1. PCR information and protocols to amplify DNA fragments. Three mtDNA fragments were selected to study the phylogenetic position of the candidate *Hynobius* species. The primer pair for the Cyt*b* fragment was designed in-house, and the other sequences come from the literature. The length of each PCR amplicon is estimated in base pairs.

Fragment	Primer	5′-3′	Length	Source	PCR Condition
mtDNA—*COI*	LCO1490	GGTCAACAAATCATAAAGATATTG	680 bp	[31]	95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 40 s, and final elongation at 72 °C for 7 min
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	680 bp	[31]	
16S rRNA	L02510	CGCCTGTTTATCAAAAACAT	500 bp	[32]	95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 40 s, and final elongation at 72 °C for 7 min
	H03063	CTCCGGTTTGAACTCAGATC	500 bp	[33]	
mtDNA-Cyt*b* (in-house design)	CytbHy-F1	TGTAGACCTCCCAACCCCC	780 bp	This study	95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55–60 °C for 30 s and 72 °C for 60 s, and final elongation at 72 °C for 10 min
	CytbHy-R1	CGTAGGCGAATAAGAAATACCACT	780 bp	This study	

2.4. Molecular Analyses

We trimmed all DNA sequences of the three gene fragments, isolated from the three *Hynobius* individuals, and aligned them with their most homologous sequences retrieved from Genbank (see accession numbers and references in Supplementary Table S1). To test the phylogenetic relationship of East Asian Hynobid salamanders, we verified the monophyly of the candidate species and made sure it is not an introduced population of any of the other species; we reconstructed Bayesian Inference (BI) trees for four independent datasets: (i) 16S rRNA gene (*n* sequence = 97, length = 528 bp), (ii) protein-coding Cytochrome b (Cyt*b*) gene (*n* sequence = 117, length = 630 bp), (iii) protein-coding cytochrome c oxidase subunit I (*COI*) gene (*n* sequence = 84, length = 567 bp), and (iv) concatenated 16S rRNA, Cyt*b* and *COI* (*n* sequence = 29, length = 1451 bp; Supplementary Table S1). We searched for the best-fit sequence evolutionary model for each gene fragment using PartitionFinder v.2.1.1 (Canberra, Australia) [34]. We set the model selection to the Bayesian Information Criterion (BIC) and greedy search settings, and we standardized the estimation parameters of the partition model to unlinked branch length and fit to "Mr. Bayes" mode.

Here, the software recovered seven partitions based on a single non-coding and three coding codon's positions (best sequence substitution models in Table 2). Using the information of best substitution models for the gene fragments, we then reconstructed the BI trees based on the four datasets using Mr Bayes v.3.2.7 (Rochester, NY, USA) [35]. For

each dataset, we ran the analysis for 10 million iterations of MCMC, with four independent chains, and resampled at each 1000 generation of the run until the trees reached convergences. We defined convergence as split frequency values lower than 0.05 at the end of all analyses and values higher than 200 for the Estimated Sample Size (ESS) for each parameter in the diagnostic reports provided by the software.

Table 2. Sequence substitution model. Best sequence substitution model for each gene fragment of the candidate *Hynobius* species are related sequences used for the reconstruction of the Bayesian Inference trees using Mr. Bayes v.3.2.7 [35], predicted and configured in PartitionFinder v. 2.1.1 [34].

Mitochondrial Gene Fragment	Type	Partition's Strategy	Best Sequence Substitution Model
16S rRNA	Non-coding ribosomal	1–528 bp	HKY + I + G
Cyt*b*	Protein-coding	Exon by three codons' position (1–630 bp; 2–630 bp; 3–630 bp)	GTR + I + G
COI	Protein-coding	Exon by three codons' position (1–567 bp; 2–567 bp; 3–567 bp)	GTR + I + G

Next, we constructed a haplotype network to identify the inheritance pattern between the sequences of our candidate species and its homologous sequences. Due to the strong posterior probability for the candidate species in the *COI* gene tree, and recommendations from the literature [36], we used the same dataset to analyze its haplotype distribution, consisting of 84 Hynobiids individuals (n taxa = 18) inferred from the *COI* gene fragment from individuals distributed across continental East Asia. Furthermore, we selected the *COI* alignment dataset due to its adequacy in the number of taxa for robust analysis in comparison to the concatenated dataset. We assigned the sequences to their respective species group and generated the haplotypes using DNAsp v.6.12.03 (Barcelona, Spain) [37]. We then determined the network of haplotypes using a statistical analysis of parsimony with TCS [38], implemented in PopART v.1.7 (Dunedin, New Zealand) [39]. Lastly, we mapped and visualized the distribution of the species involved in the haplotype network using QGIS v.2.18.0 (Chicago, IL, USA) [40].

Finally, to estimate the evolutionary divergence over sequence pairs between species, we analyzed 29 sequences of concatenated 16S rRNA, Cyt*b*, and *COI* using MEGA v.11.0.13 (State College, PA, USA) [41]. We assigned the sequence data to 18 groups based on species identity, and we removed all ambiguous positions for each sequence pair using the pairwise deletion option. There were a total of 1451 positions in the final dataset, and we computed the evolutionary divergence using the Maximum Composite Likelihood model [42].

2.5. Morphometry

For the morphological analyses, we measured the characters listed by Shen et al. [24], Lai and Lue [21], Borzée and Min [17], and Chen et al. [43] as they provide the largest dataset available for continental East Asian *Hynobius* species. See Figure 1 in Chen et al. [43] for a graphic representation of the following variables. We used digital calipers (model 1108–150, Insize; Suzhou, China) to the nearest 0.1 mm three times per individual and averaged, following the recommendations of Borzée and Min [17]. The measurements were: total length from the snout to the tail end (TOL); length of body from tip of snout to anterior angle of vent (SVL); tail length from anterior angle of vent to tip of tail (TL); tail height as the height of the tail at its highest point (TH); tail width as the width of the tail at its widest point (TW); head length from tip of snout to gular fold (HL); head width immediately posterior to jaw articulation (HW); snout length from the anterior border of the eye to the tip of the snout (SL); interocular distance, measured between medial margins of eyelids (IOD); laterally measured diameters of eyes (DE); head height as the height of the head at its highest point (HH); internarial distance, measured between the medial margins of the nares (IND); body width at axilla (BW); trunk length from axilla to groin (AG); forelimb length from anterior insertion to tip of second toe (FOL); hindlimb length from anterior insertion to tip of third toe (HIL). We also counted the number of

costal grooves (COS) for all individuals, and the number of horny vomerine teeth (VT) for four individuals.

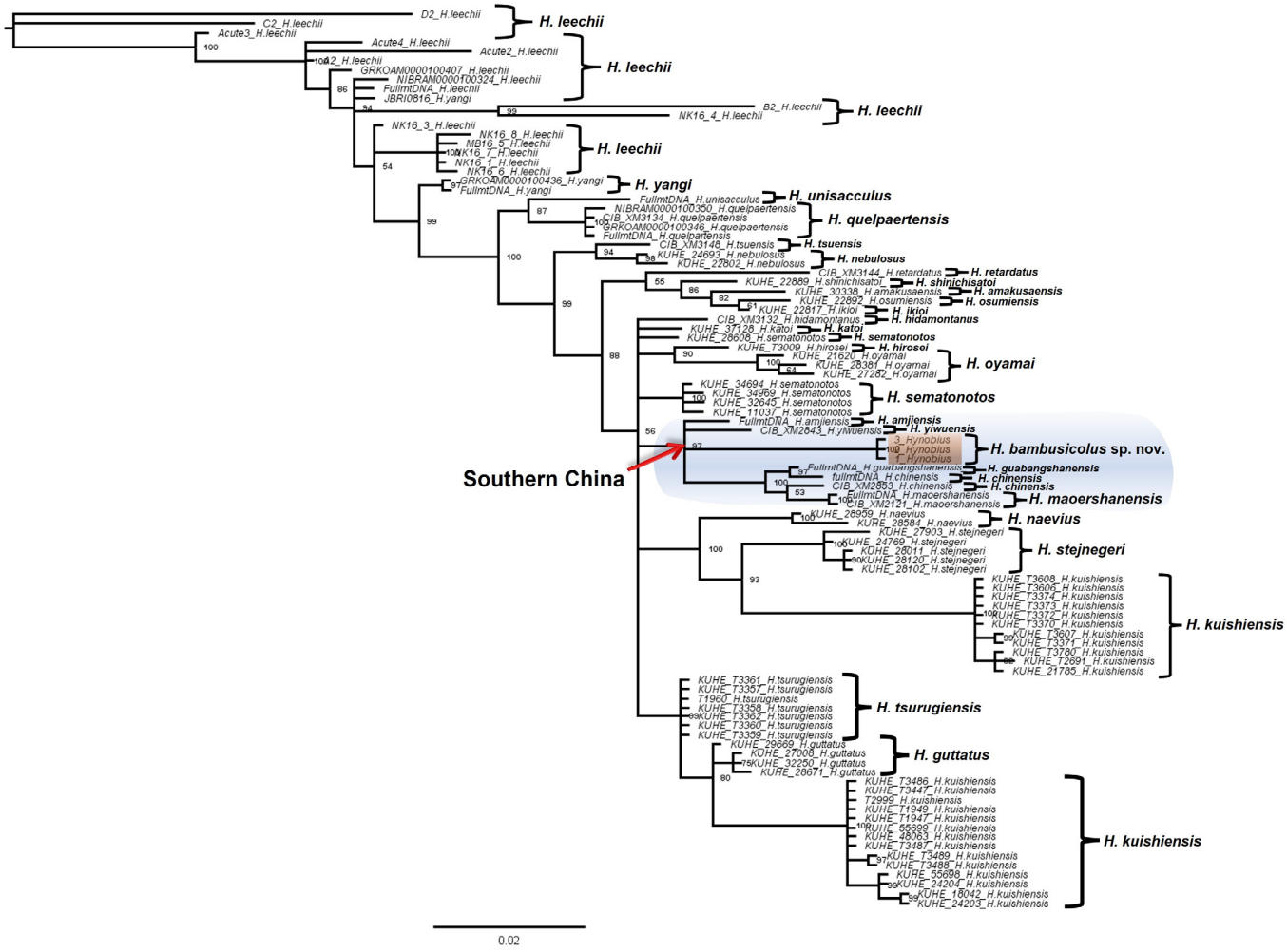

Figure 1. Bayesian Inference tree based on a 528 bp-long 16S rRNA fragment for 97 Hynobiidae salamanders in East Asia. Our results recovered *Hynobius bambusicolus* sp. nov. (orange shade) as monophyletic and sharing a sister relationship with the congeneric Hynobius species distributed in southern China (blue shade).

Ideally, a species description should include a morphological comparison between the new species and the most closely related one. Here, it would be a two-by-two comparison between the species described in this paper and *H. amjiensis* (see phylogenetic results). However, due to (1) the rarity of *H. amjiensis*, for instance, Chen et al. [44] found 16 individuals over eight years of surveys, (2) the urgency to describe the species to be able to protect it, (3) the robustness of the phylogenetic analyses (see phylogenetic results), and (4) the presence of great morphological variations; we provide a morphological description for the two adult individuals found, and a morphological key enabling the identification of the species. We do note the presence of morphological data in the literature for *H. maoershanensis* [43] and two Taiwanese species [21]; however, a two-by-two comparison with these species only would not be clarifying the taxonomy as these species are not phylogenetically or geographically close.

The species within the *H. chinensis* species complex, and ranging on the Chinese mainland, are *H. amjiensis*, *H. guabangshanensis*, *H. chinensis*, *H. maoershanensis*, and *H. yiwuensis*. We did not include individuals from northeast China, Taiwan Island, or the Japanese archipelago in the morphological comparisons as they were not closely related in the phy-

logenetic analyses. We then created a dichotomic key to enable the morphological identification of the species and the determination of discrete characters.

In addition, with the development of "technoecology" [45], 3D printing is becoming widely used, and 3D printed replicas can also benefit taxonomy and systematics [46]. Here, we provide a printable 3D model that can be downloaded online. We photographed an adult individual using a high-resolution camera (Nikon Z9 with a Nikkor MC 50 mm f 2.8 lens) in the wild but on a hard, permeable, and smooth substrate. We took multiple photographs from different angles, with a focus on capturing the characteristics and features of the species. The photos were imported into Autodesk Maya 2019 (Autodesk; San Francisco, CA, USA) as image planes, which were then used as reference images to ensure accuracy and consistency in the details of the model. Using the reference images as a guide, we created a base mesh using a polygon modeling technique. The base mesh was adjusted until it matched the overall shape of the salamander. The sculpting tools were then used to accurately refine and detail the features of the species. We then created a UV map and applied textures to the model, using photos of the specimen's skin as a reference to create a realistic representation. The skeleton of the model was created by using rigging tools. The mesh was then skinned to the skeleton, allowing for a realistic pose of the model. The completed 3D model was exported from Maya in an OBJ format.

2.6. Egg and Larval Development

To ascertain that the development of the species does not deviate from that of the *H. chinensis* species complex, we documented the development of the species before the eggs hatched and after hatching at 16, 69, 74, 77, and 87 days. We aimed to document development between stages 29 and 37 when embryos are in the egg sac; tadpoles between stages 40 and 49 when free swimming with balancers; between stages 50 and 59, when the larvae are not yet using their developing hind legs and once after stage 60 when the larvae use their hind legs [47]. The salamanders were kept at 26 °C, in water boiled, and aged for at least 24 h in open-top buckets. Photographs were taken with a Z9 camera (Nikon, Tokyo, Japan) and a Z MC 50 mm f 2.8 lens (Nikkor, Nikon).

2.7. Acoustic Signal

When probed, the individual captured in January sometimes emitted a vocalization. We managed to record the individual only once with a linear PCM recorder (Tascam DR-40; Santa Fe Springs, CA, USA) using the built-in microphone. The call was recorded at a sampling rate of 44.1 kHz with a 16-bit resolution. We analyzed the call properties following the method in Prasad et al. [48] using Raven Pro bioacoustics v.1.5 (Center for conservation bioacoustics; Ithaca, NY, USA).

3. Results

3.1. Molecular Analyses

The rates of evolutionary divergences between sequence pairs provided support for the significant variation between the candidate species and all 18 other species. The highest variation in the average base substitutions per site for sequence pairs was between the candidate species and *Hynobius amjiensis* (average rate = 0.098; marked with * in Table 3). The average divergence rate was comparable to eight other sequence pairs (bolded values in Table 3). Most importantly, the average divergence rate was comparatively higher than the rates of base substitution for the four following pairs: (i) *H. arisanensis* vs. *H. formosanus*, mean = 0.008; (ii) *H. maoershanensis* vs. *H. guabangshanensis*, mean = 0.021; (iii) *H. chinensis* vs. *H. guabangshanensis*, mean = 0.032; and (iv) *H. maoershanensis* vs. *H. chinensis* (mean: 0.035; Table 3).

Table 3. Matrix of best substitution rates for evolutionary divergence between the candidate *Hynobius* species and congeneric species. The congeneric *Hynobius* species used in this study are related Hynobiid salamanders distributed in East Asia. We inferred the genetic distance from mitochondrial 16S rRNA, Cyt*b*, and *COI* (1451 bp) from 29 taxa representing 18 species. The rate of base substitution provides support to the evolutionary divergence between sequences of *Hynobius bambusicolus* sp. nov. and all 17 species compared. The lowest genetic distance is between the focal species and *H. amjiensis* (0.098; marked with *), but it is higher than the value from four species-pair comparisons (marked with #). All genetic distance values lower than the mean of pairwise difference for *H. bambusicolus* sp. nov. (0.098) are in bold in the table.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*H. bambusicolus* sp. nov.	N/A																	
2	*P. shangchengensis*	0.166																	
3	*H. maoershanensis*	0.113	0.170																
4	*H. yiwuensis*	0.102	0.157	0.106															
5	*H. chinensis*	0.106	0.166	**0.035** #	0.096														
6	*H. hidamontanus*	0.101	0.146	**0.093**	**0.090**	0.091													
7	*H. nebulosus*	0.119	0.149	0.106	0.100	0.106	0.083												
8	*H. tokyoensis*	0.138	0.173	0.132	0.128	0.126	0.118	0.126											
9	*H. tsuensis*	0.115	0.147	0.106	0.096	0.101	0.086	**0.074**	0.126										
10	*H. amjiensis*	**0.098** *	0.161	**0.092**	0.096	0.090	0.095	0.102	0.132	0.099									
11	*H. arisanensis*	0.118	0.145	0.121	0.110	0.121	0.109	0.104	0.121	0.098	0.122								
12	*H. dunni*	0.122	0.156	0.109	0.107	0.106	0.091	**0.060**	0.121	**0.077**	0.099	0.105							
13	*H. formosanus*	0.115	0.144	0.121	0.106	0.122	0.108	0.104	0.117	0.100	0.123	**0.008** #	0.104						
14	*H. guabangshanensis*	0.106	0.161	**0.032** #	**0.093**	**0.021** #	**0.083**	0.103	0.127	0.098	0.091	0.117	0.106	0.118					
15	*H. quelpaertensis*	0.114	0.146	0.105	0.099	0.100	**0.085**	0.092	0.123	**0.088**	0.095	0.111	**0.094**	0.107	0.095				
16	*H. unisacculus*	0.116	0.141	0.105	0.096	0.102	0.095	0.093	0.120	0.092	0.096	0.113	**0.096**	0.110	0.102	**0.062**			
17	*H. yangi*	0.104	0.147	0.114	0.101	0.107	0.083	**0.088**	0.122	**0.088**	0.099	0.105	**0.096**	0.104	0.108	**0.075**	**0.073**		
18	*H. leechii*	0.100	0.148	0.107	0.099	0.104	0.081	0.089	0.121	0.089	0.098	0.105	0.097	0.104	0.107	0.066	0.070	0.036	N/A

Apart from the significant divergence in DNA sequences, our phylogeny recovered the candidate species as a deeply divergent clade amongst the East Asian Hynobiid (Figures 1–4). All the BI trees, for single genes and concatenated fragments, coherently recovered the candidate species as monophyletic within the Southern Chinese group (posterior probability (PP): between 59% to 99%; Figures 1–4). For the *COI* and concatenated trees, we recovered the candidate species as sister to *H. amjiensis* (PP: 97% and 65%; Figure 1).

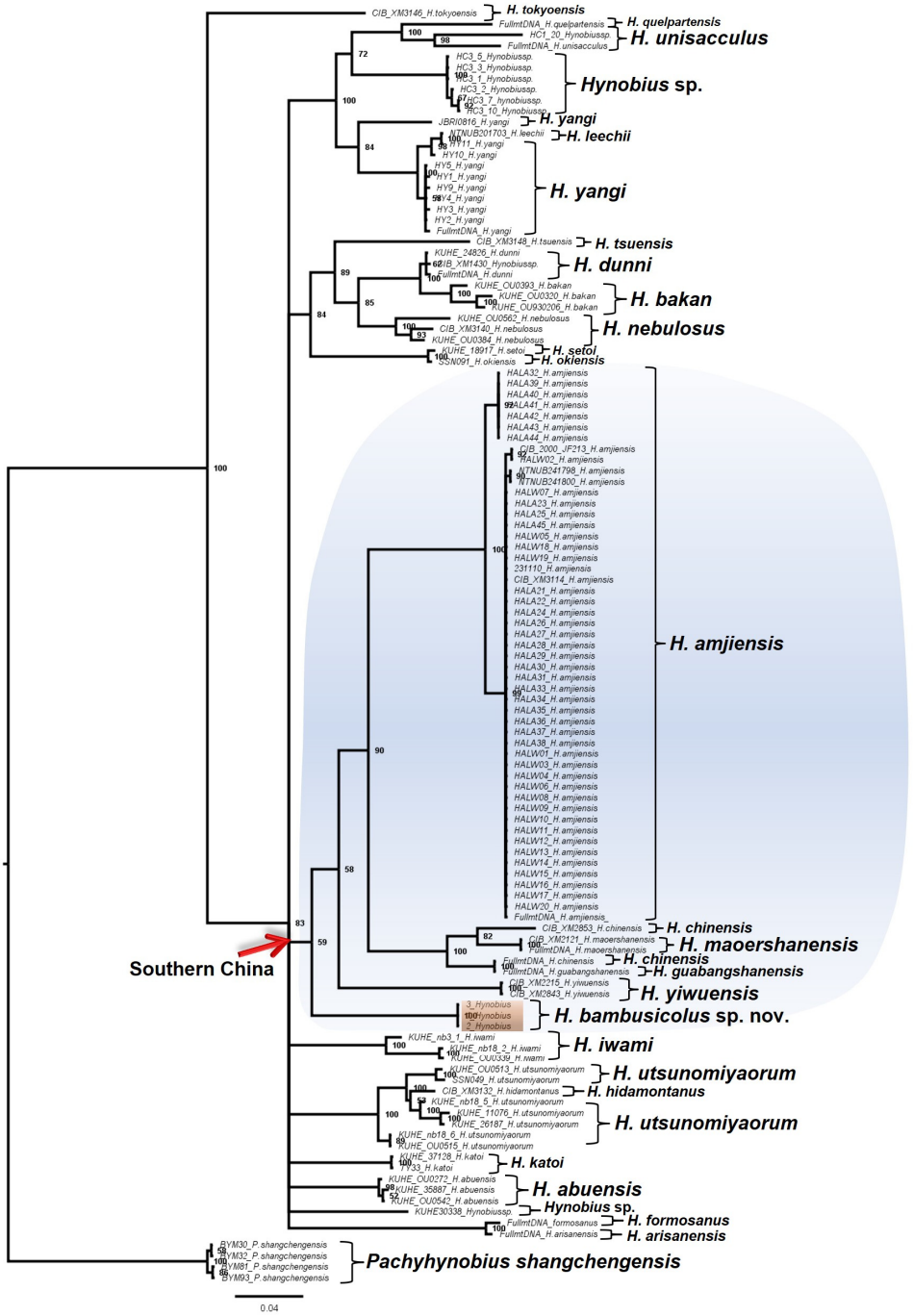

Figure 2. Bayesian Inference tree reconstructed from a 630 bp-long Cyt*b* gene fragment for 117 Hynobid salamanders distributed across East Asia. Our results recovered *Hynobius bambusicolus* sp. nov. (orange shade) as monophyletic and basal to the congeneric clades of *Hynobius* species distributed in southern China (blue shade).

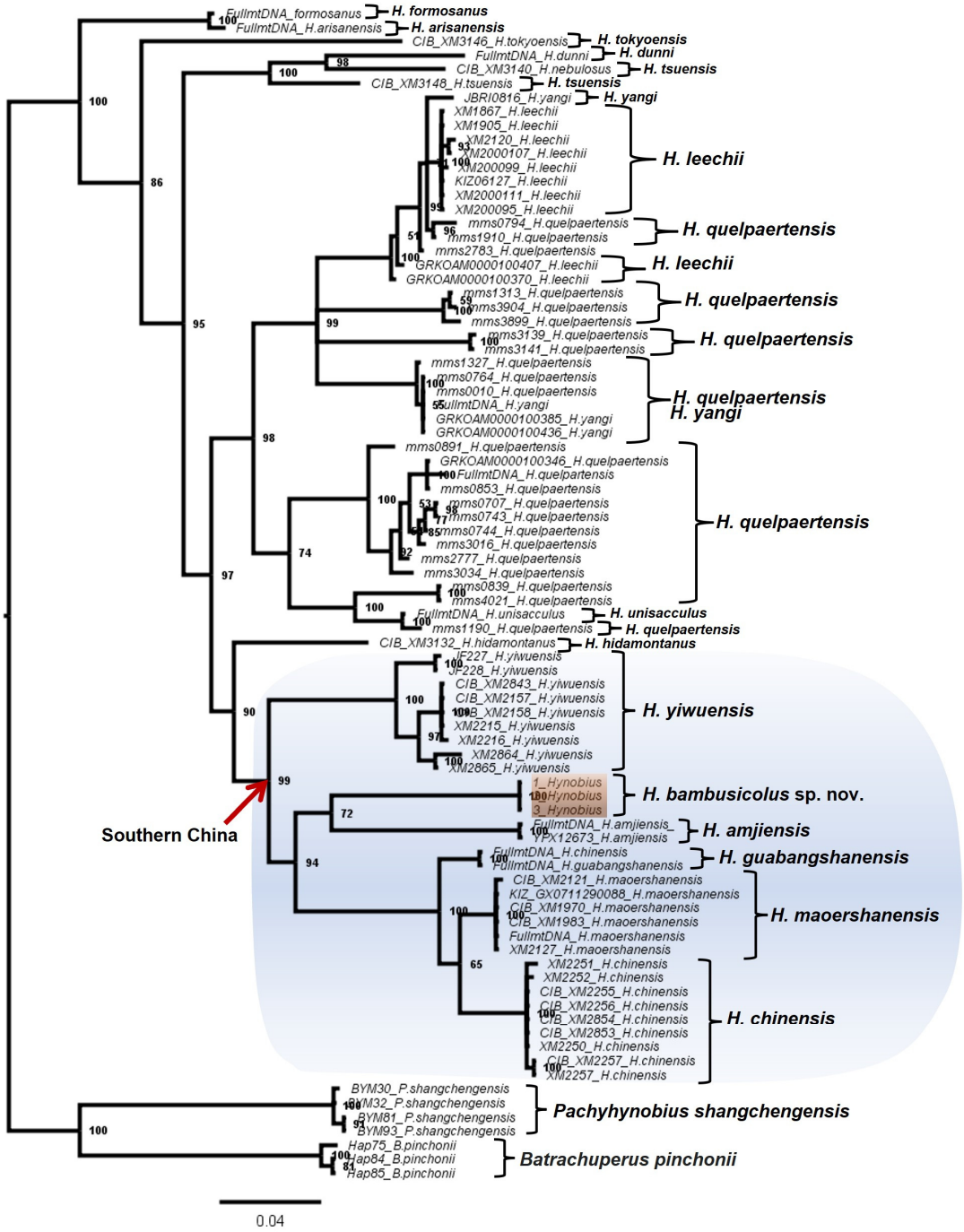

Figure 3. Bayesian Inference tree based on a 567 bp-long *COI* gene fragment for 84 Hynobid salamanders distributed across East Asia. Our results recovered *Hynobius bambusicolus* sp. nov. (orange shade) as monophyletic within the southern China group (blue shade) and sharing a sister relationship with *Hynobius amjiensis*.

Figure 4. Bayesian Inference tree inferred from 1451 bp of 16S rRNA, Cyt*b,* and *COI* gene fragments of Hynobiid salamanders distributed across East Asia. Our analyses recovered *Hynobius bambusicolus* sp. nov. (orange shade) as clustered with the congeneric *Hynobius* distributed in southern China (blue shade).

Based on the gene fragment for *COI* (*n* taxa = 84), we obtained 55 haplotypes (h) from 567 sites representing 16 species of Hynobiid salamanders distributed across East Asia, with a haplotype diversity (Hd) of 0.98 (Figure 5). The haplotype distribution further revealed a shared relationship between the haplotype group of the candidate species and the geographically related haplotypes of Southern Chinese *Hynobius*. The haplotype network and distribution also clarified the deep genetic and geographic isolation between the candidate species and the related *H. amjiensis* and *H. maoershanensis*.

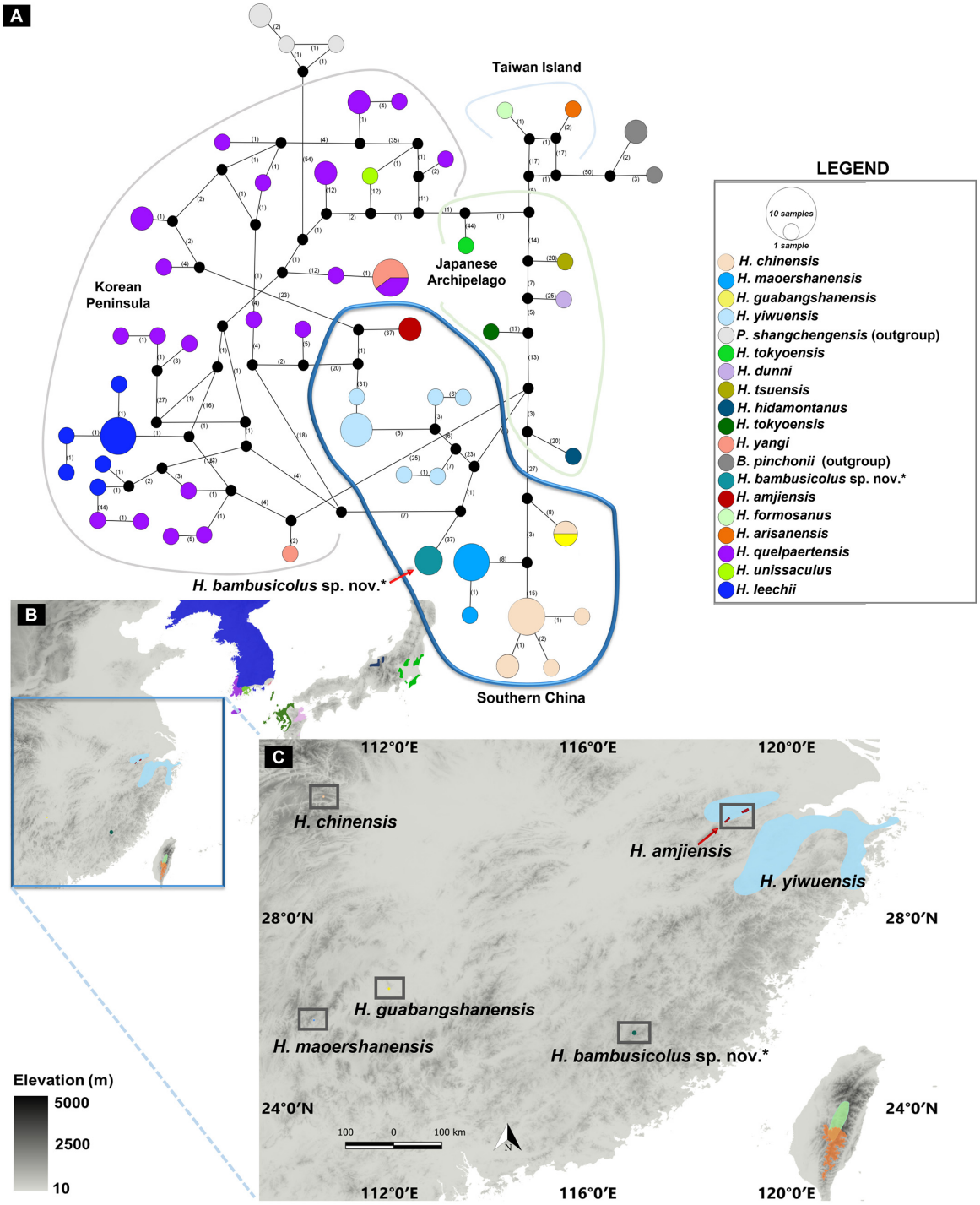

Figure 5. Haplotype network and distribution of Hynobiid salamanders distributed in East Asia. The haplotype analysis involved 84 Hynobiids individuals (*n* taxa = 18) inferred from the *COI* gene fragment (Figure 3). (**A**) TSC network and distribution of 55 haplotypes based on their geographic distribution. The number along each branch connecting haplotypes indicates the number of mutations. The asterisk (*) in the figure marks the focal *Hynobius* species described in this study, *Hynobius bambusicolus* sp. nov. (**B**) Distribution of all 18 *Hynobius* species in East Asia, matching with the haplotype network. The blue box highlights the southern Chinese clade of *Hynobius*. (**C**) Distribution of the southern Chinese clade of *Hynobius* species in which *Hynobius bambusicolus* sp. nov. is clustered. The small grey boxes highlight the restricted ranges of four *Hynobius* species within the clade.

3.2. Morphometric results

The measurements of the adults highlighted the large size of the species (Table 4), surpassed only by *H. chinensis*, but a low number of coastal grooves, similar to *H. yiwuensis*. Finally, the toe formula was similar to that of three other species in the area (Figure 6). We found a variable number of vomeral teeth (four individuals), likely due to the partial development of the voucher specimen. The holotype was characterized by 12 pairs of vomeral teeth on the right and 13 on the left, the largest individual by 18 and 19 pairs, and the two other individuals by 17 and 16, and 12 and 13 pairs (Figure 7). As this character does not seem fixed yet in our voucher specimen, we do not use it for species identification. For the morphological identification key based on non-invasive identification of the species, we determined the number of costal grooves to be the first character of importance, enabling the assignment of the individual examined into either one of three categories: 10 or fewer grooves, 11 or 12 grooves, or 13 or more grooves (Figure 6). Once assigned to one of these categories, we could develop the following identification key:

- The combination of 10 or fewer grooves with the following:
  - A total length < 151 mm assigns the individual to *H. yiwuensis*;
  - A total length > 180 mm assigns the individual to *H. bambusicolus* sp. nov;
- The combination of 11 or 12 grooves with the following:
  - A total length < 180 mm assigns the individual to *H. maoershanensis*;
  - A total length > 180 mm assigns the individual to *H. chinensis*;
- The combination of 13 or more grooves with the following:
  - A total length < 151 mm assigns the individual to *H. guabangshanensis*;
  - A total length > 152 mm assigns the individual to *H. amjiensis*.

Table 4. Morphological measurements. Morphological measurements for two adult and four juvenile *Hynobius bambusicolus* sp. nov. from Fujian, China. The measurements were taken three times per individual and averaged for consistency.

Age	**Adults**		**Juveniles**			
Type	**Wild**	**Wild**	**Paratype**	**Paratype**	**Paratype**	**Holotype**
ID	**22HyF001**	**22HyF002**	**22HyF003**	**22HyF004**	**22HyF005**	**22HyF006**
TOL	191.57	196.94	48.06	48.38	50.43	55.11
SVL	136.36	137.81	28.39	27.72	31.32	34.05
TL	55.21	59.13	19.57	18.74	20.32	21.73
HL	22.78	21.86	7.27	8.48	8.25	10.32
HW	18.57	15.84	6.22	6.80	6.82	7.82
IOD	6.19	5.25	2.67	2.92	2.83	3.44
IND	6.29	5.77	3.16	3.29	2.72	3.16
BW	17.75	16.08	5.24	6.24	6.83	7.09
AG	52.07	51.06	16.46	16.14	17.95	21.04
FOL	21.13	20.77	6.49	6.73	5.89	8.08
HIL	25.53	23.08	6.38	6.30	8.73	7.77
COS	9	10	9	9	10	9
SL	19.78	20.82	4.72	5.47	4.44	5.37
TH	10.12	10.52	3.26	4.02	3.06	3.49
TW	11.03	10.90	2.21	2.29	2.83	2.48
HH	10.50	10.99	3.81	3.91	3.92	3.70
DE	5.31	4.64	2.44	2.31	2.80	2.54

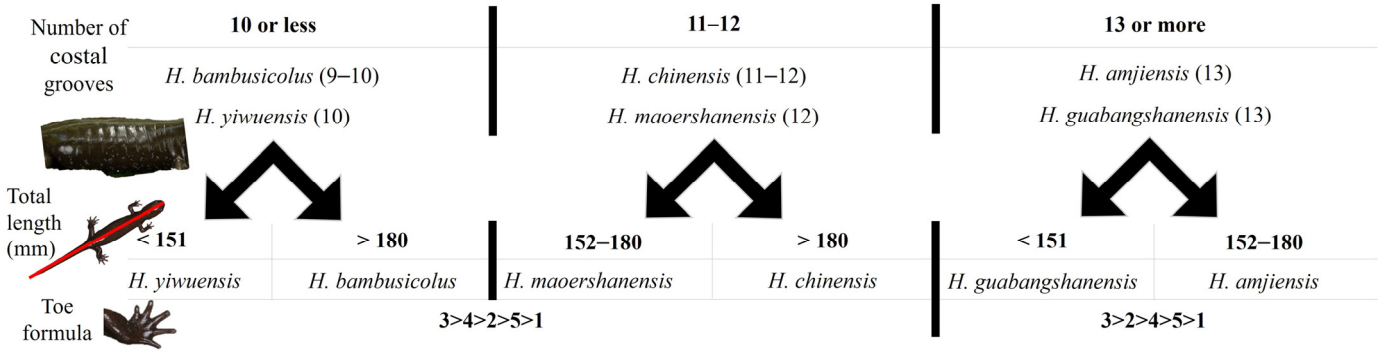

Figure 6. Species identification key for southern Chinese *Hynobius* species. With this morphological identification key, it is possible to identify adult *Hynobius* individuals from southern China, based on morphology and without invasive procedures. The data used are from AmphibiaChina.org and our study.

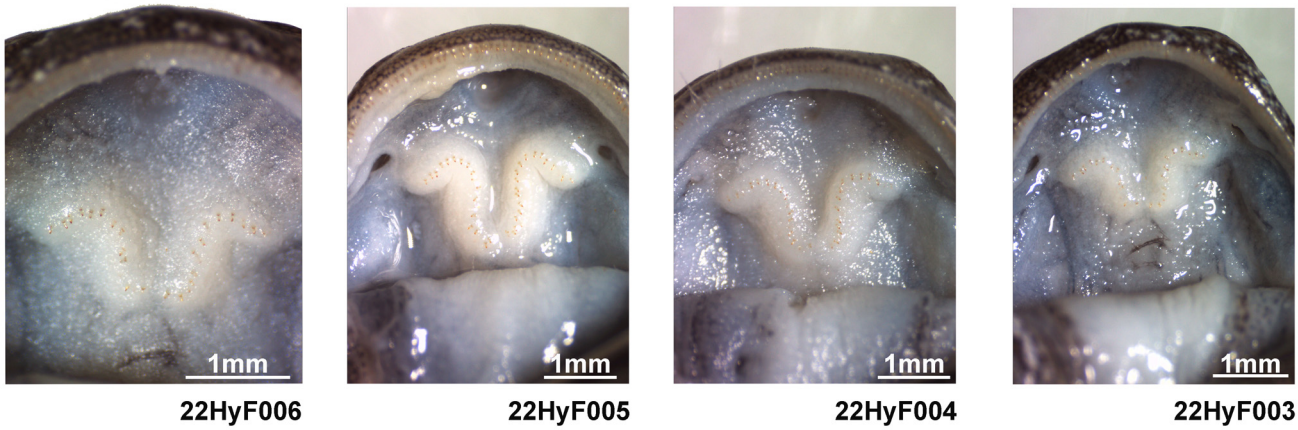

Figure 7. Vomeral teeth details for four juvenile *Hynobius bambusicolus* sp. nov. preserved in 70% alcohol. Vouchers were collected in Quxi village, Liancheng county, People's Republic of China (25.566° N, 116.938° E).

3.3. Species Description

Following the line of evidence based on molecular analyses for species-level divergence with other southern Chinese *Hynobius* species and a clearly different morphology evidenced by discrete characters, we formally describe the new species. Measurements are summarized in Table 4.

Nomenclature History

Among the species of *Hynobius* currently recognized [49], *Hynobius turkestanicus* Nikolskii is the only enigmatic taxon [50], and it is unlikely to be a member of the *Hynobius* genus [51]. Our focal taxa are also different from *Hynobius yunanicus*, which was invalidated [52] and later re-established [53], but as a synonym of *Pachyhynobius shangchengensis* [49]. The new species belongs to the group of lentic habitat breeders distributed in China and the Korean Peninsula, and morphological comparisons with the closest related species, *H. amjiensis*, and the geographically related species are presented in Figure 5.

Hynobius bambusicolus sp. nov. Wang, Othman, Qiu and Borzée

Synonymy:

"*Hynobius chinensis* (partim): [27]" pp. 218–229.

- Holotype

Voucher 22HyF006; sub-adult collected by Zhenqi Wang and Zhixin Qiu on 26 January 2022 in Quxi village, Liancheng county, People's Republic of China (25.566° N, 116.938° E; Figure 5). Measurements and counts are in Table 4 and Figure 8.

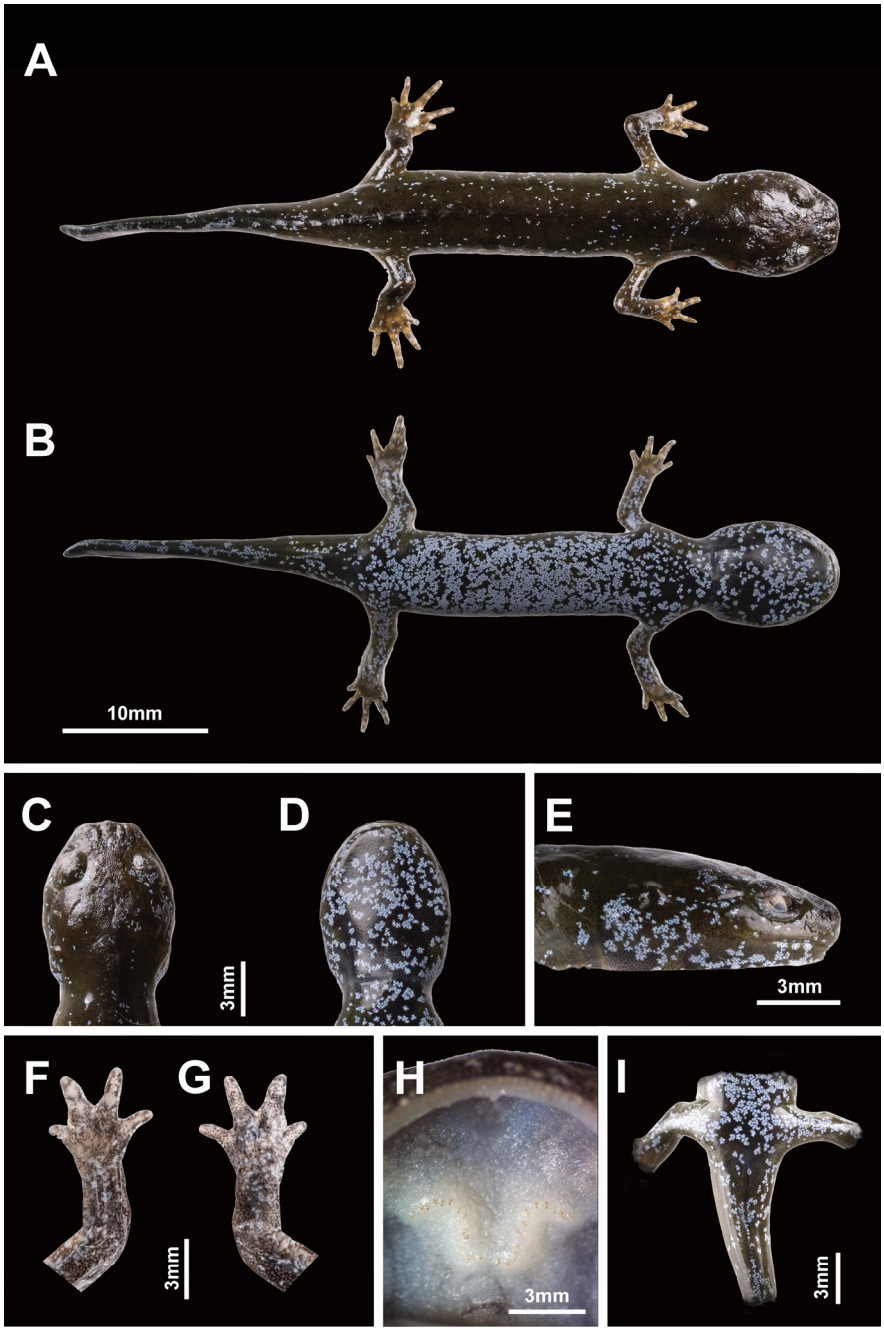

Figure 8. Holotype of *Hynobius bambusicolus* sp. nov. preserved in 70% alcohol. Voucher 22HyF006 collected in Quxi village, Liancheng county, People's Republic of China (25.566° N, 116.938° E). Measurements and counts in Table 4. (**A**) Dorsal view. (**B**) Ventral view. (**C**) Head in dorsal view. (**D**) Head in ventral view. (**E**) Head in lateral view. (**F**) Opisthenar view of left hand. (**G**) Volar view of left hand. (**H**) Palatal region showing the vomerine tooth series (pale orange vertical structures on the white background at the center of the palate). (**I**) Ventral view of cloacal area.

- Paratypes

Vouchers 22HyF003, 22HyF004, 22HyF005. Sub-adults collected by Zhenqi Wang and Zhixin Qiu on 26 January 2022 in Quxi village, Liancheng county, People's Repub-

lic of China (25.566° N, 116.938° E; Figure 5). Measurements and counts are in Table 4 and Figure 9.

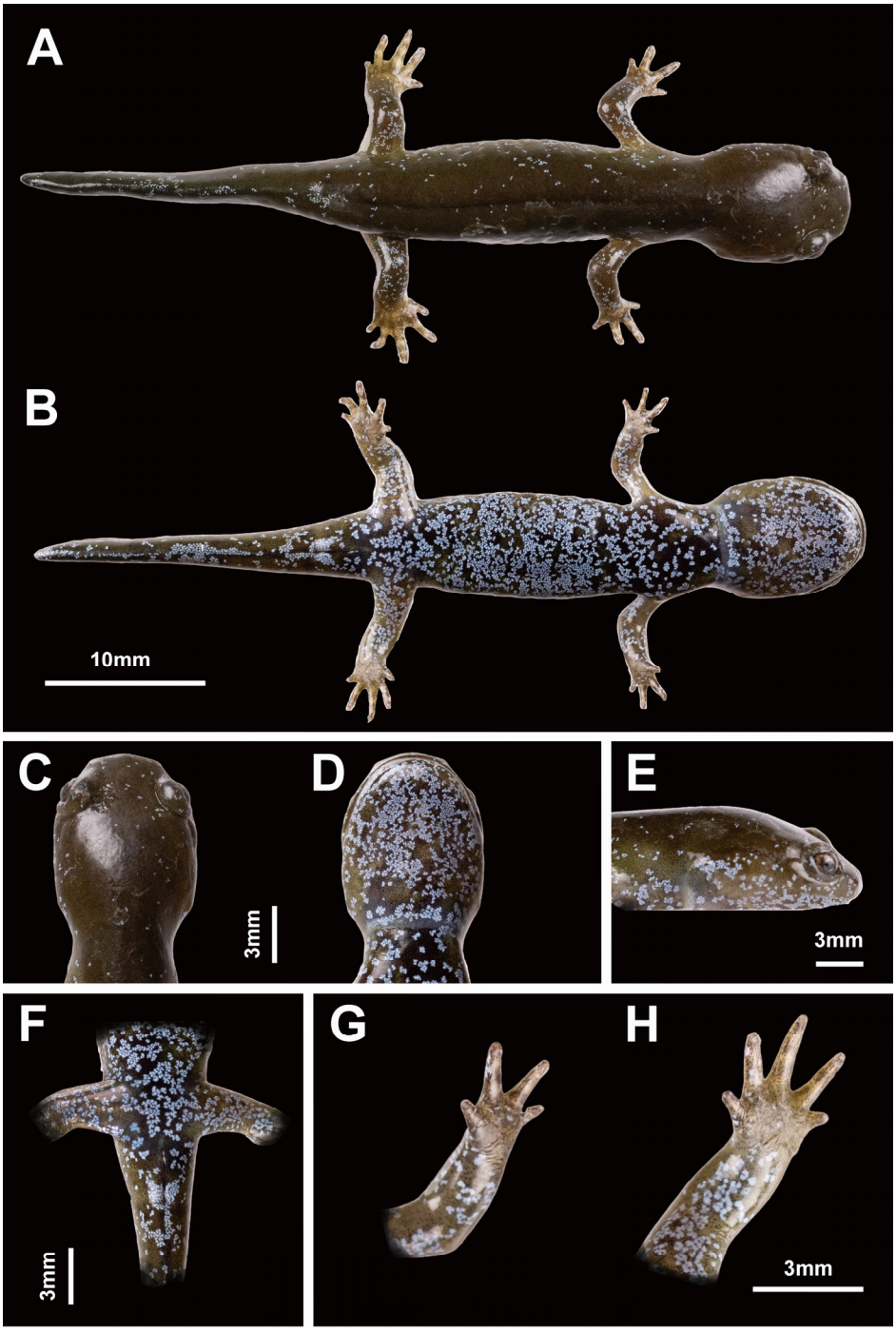

Figure 9. Paratype of *Hynobius bambusicolus* sp. nov. preserved in 70% alcohol. Voucher 22HyF003 collected in Quxi village, Liancheng county, People's Republic of China (25.5661° N, 116.9386° E; Figure 5). Measurements and counts in Table 4. (**A**) Dorsal view. (**B**) ventral view. (**C**) Head dorsal view. (**D**) Head ventral view. (**E**) Head lateral view. (**F**) Ventral view of cloacal area. (**G**) Opisthenar view of left hand. (**H**) Volar view of left hand.

- Etymology

The species was first found in Quxi village, Liancheng county, in the west of Fujian province in China. The name *H. bambusicolus* sp. nov. comes from the habitat of the holo-

type and the only known habitat type for the species: bamboo forests. The vernacular name of the species, Fujian Bamboo Salamander, reflects the scientific name of the species, as does its Chinese name: 虚竹小鲵 (pronounced: Xū Zhú Xiǎo Ní). This salamander is named after a main character from Jin Yong's swordsman fiction "The semi gods and semi devils" [54], with Xuzhu (虚竹) as the main character and where "虚" [xū] means humble, and "竹" [zhú] means bamboo. This character, Xuzhu, was an unknown Shaolin monk, but he inherited the powers of the leader of the Carefree by coincidence and started its legendary journey.

- Identity, diagnosis, and distribution

To date, the species is known from its type locality only, Quxi village, Liancheng county (Figure 5). Larvae are typical of *Hynobius* larvae in shape and color and do not differ from other *Hynobius* species in the region in their development (Figure 10). The embryos develop in egg sacs (Figure 10A), larvae first swimming freely with balancers (Figure 10B; shown at day 16), then develop non-functional hind limbs (Figure 10C; shown at day 69), which slowly become functional (Figure 10D,E; shown at day 74 and 77), and the gills regress before metamorphosis (Figure 10F; shown at day 84). Juveniles are brown, darkening with age, with a large variation in blue speckles on their dorsum, which disappears as they age (Figure 9). Adults of the species are uniform dark chocolate, with light grey and bluish speckles on the venter (Figure 11). Identification is best assessed based on location, although discrete morphological characters include the combination of 10 or fewer costal grooves with a total length > 180 mm (Figure 6). To facilitate the identification and further study, the OBJ file of this model can be downloaded (Supplementary File S1). The visual representation of the model is provided in Appendix A (Figure A1).

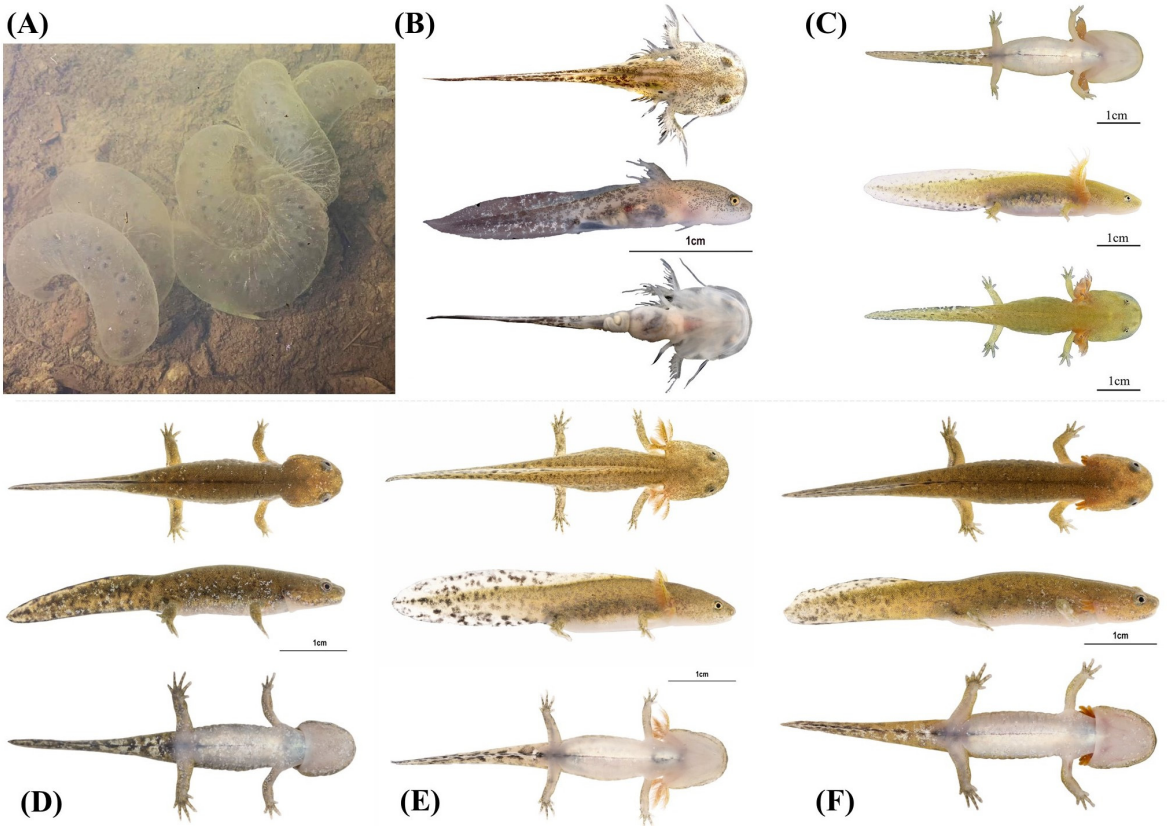

Figure 10. Representative developmental stages for eggs and larvae of *Hynobius bambusicolus* sp. nov. from Fujian, China. (**A**) pre-hatching; (**B**) 16 days old. (**C**) 69 days old. (**D**) 87 days old. (**E**) 74 days old. (**F**) 77 days old.

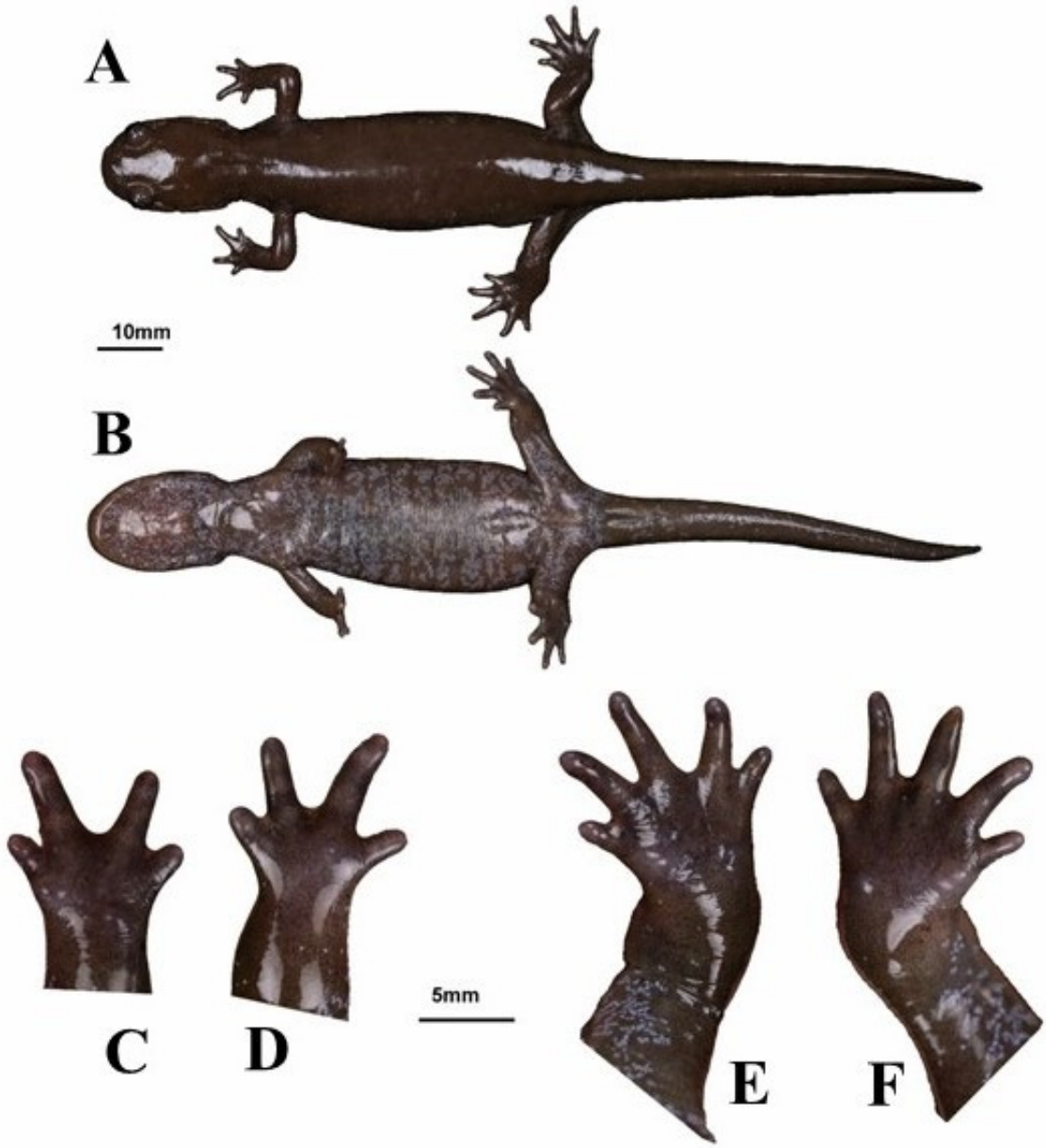

Figure 11. Example of *Hynobius bambusicolus* sp. nov. adult in life. Voucher 22HyF001 from Quxi village, Liancheng county, People's Republic of China (25.5661° N, 116.9386° E; Figure 5). Measurements and counts in Table 4. (**A**) Dorsal view. (**B**) ventral view. (**C**) Opisthenar view of left hand. (**D**) Opisthenar view of right hand. (**E**) Opisthenar view of left foot. (**F**) Opisthenar view of right foot.

- ZooBank registration

We hereby state that the present paper has been registered to the Official Register of Zoological Nomenclature (ZooBank) under LSID: urn:lsid:zoobank.org:pub: 9047D736-3394-4B36-AE97-CEEB3359B36D. The new species name *Hynobius bambusicolus* sp. nov., has been registered under LSID: urn:lsid:zoobank.org:act:18CAEC61-DF60-4401-91C5-FEF5614FA08C.

- Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that code from the electronic edition of this article. This published work and the nomenclatural act it contains have been registered in ZooBank, the

online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved, and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is urn:lsid:zoobank.org:pub:9047D736-3394-4B36-AE97-CEEB3359B36D. The electronic edition of this work was published in a journal with an ISSN and has been archived and is available from the following digital repositories: PubMed Central.

- Habitat and behavior

Hynobius bambusicolus breeds in shallow pools in bamboo forests (Figure 12) above 1400 m above sea level. All eggs were observed in ruts made from tire tracks. The puddles were 5 to 12 cm deep, and most egg sacs were laid about 10 cm deep without being attached to the substrate, containing between 21 and 27 eggs each. Adult salamanders were found under logs, stones, and dead leaves, in wet soil and humus. These shelters were surrounded by weeds and dry branches in waterlogged areas, and we did not find any individuals on the surface, even at night (Figure 12). Larvae were fed with bloodworms, but some individuals still cannibalized their kin (Figure 13). Once metamorphosed, recently metamorphosed individuals raised their tails when startled, a behavior to divert predation from vital organs [55].

Figure 12. Natural habitat and oviposition site for *Hynobius bambusicolus* sp. nov. The site is in Quxi village, Liancheng county, People's Republic of China (25.5661° N, 116.9386° E).

Figure 13. Example of cannibalism in *Hynobius bambusicolus* sp. nov. larvae.

The individual recorded produced a single type of call, with a very short duration (153.40 ms) and of low frequency (peak frequency of 129.20 Hz). The vocalization was composed of four strong harmonics (Figure 14), with a peak amplitude of 403 U and a maximum entropy of 3.473 bits. While highly unusual, underwater vocalizations have been reported in salamanders, such as *Siren intermedia* [56]. In this case, the vocalizations were produced by a male being probed, suggesting it could be submissive or alarm calls. In our recording, the individual emitted a similar "alarm call"/"squeak" while half submerged in the water, maybe as an agonistic signal.

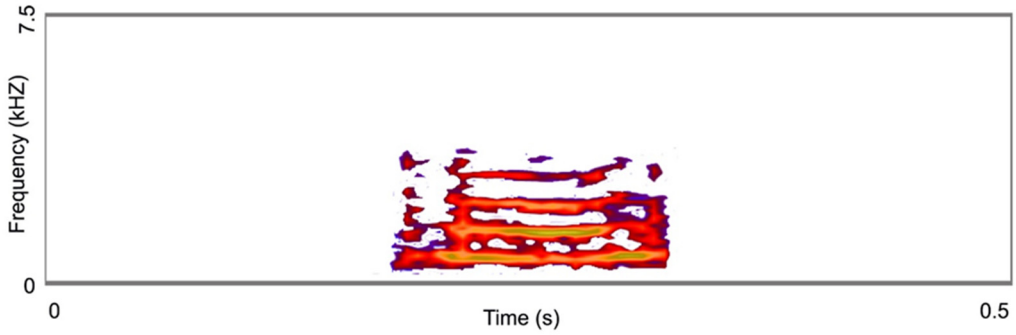

Figure 14. Spectrogram of the acoustic signal release by *Hynobius bambusicolus* sp. nov. Dense yellow-orange colour displays the harmonic-like pattern of the acoustic signal. Schematic illustrations based on the data extracted with FFT size = 1024 pts. (Hanning window, 43.1 Hz resolution).

4. Discussion and Conclusions

Here we described a Hynobiid salamander species, *Hynobius bambusicolus*, the Fujian Bamboo Salamander, based on deeply divergent molecular analyses, non-overlapping morphological characters, and vastly segregated distribution. Field identification of *Hynobius* individuals based on morphology is not always easy [17], but discrete morphological characters are present to identify *H. bambusicolus* (Figure 6). The easiest character to identify the species perhaps remains the geographic location as the range of the species does not overlap with that of the other *Hynobius* species, with a gap of several hundred kilometers (Figure 5).

Our results, through the phylogenetic trees, haplotype network, and comparative pairwise genetic distance, show that *H. bambusicolus* is alternatively the most divergent and earliest-branching species among Southern Chinese *Hynobius* clades (Cyt*b* tree; Figure 2),

nested with the other Southern Chinese *Hynobius* clades (16S rRNA and concatenated mtDNA trees; Figures 1 and 4) and only the sister taxon to *H. amjiensis* (*COI* tree; Figure 3). The stem emergence of Southern Chinese *Hynobius* clades (*H. amjiensis* and *H. chinensis*) is dated to c. 20 mya [57], and *H. bambusicolus* is, therefore, an ancient lineage, likely to have seen its distribution regularly shift, expand, and contract with paleogeographic and climatic variations [58]. The area is inhabited by other Caudata, and competition between *H. bambusicolus* and other genera, such as *Pachytriton* and *Paramesotriton*, is not impossible.

The restricted distribution of *H. bambusicolus* is also a negative point to the survival of the species. The species is micro-endemic, presumably composed of a single known relic population with an apparently incredibly small population size, similar to other south Chinese *Hynobius* species (e.g., [44]). The genus is likely to have distributed south broadly since the early Miocene [59], and withdrawn to higher elevations, similarly to other amphibians shifting distribution [60], due to climatic variations as most representatives of the genus are cold-adapted, and the mid-Miocene vegetation in Fujian was tropical [61]. For instance, specific genes in *H. chinensis* are upregulated as a response to cold temperatures [62]. *Hynobius bambusicolus*, however, is adapted to sub-tropical bamboo forests, although cold preference or tolerance for spawning is still a trait in the species, as two egg masses were found in January 2023 at the same site on a snowy day.

The species is known from a single locality, and surveys in 2023 of all water bodies in the area where the species could potentially spawn did not result in more than five extremely small water bodies. As there were eight egg sacks at the known site (therefore four females), even considering this number as a constant for each water body, it would be a maximum of 20 breeding females, and therefore a population size likely to be well below 200 breeding individuals, matching with the criteria B1, B2, and C2 for Critically Endangered following the recommendation of the IUCN Red List of Threatened Species [63]. Due to the threatened status of the species, establishing an ex situ population could help prevent the extinction of the species in the face of growing climate instability and stochastic extinction risks.

Due to the rarity of this new species, we urge all hobbyists to avoid collecting this salamander or divulging local information and resisting any trade. The breeding site is within a planted bamboo forest, surrounded by mixed forest, and the distribution of the species is likely to have been impacted by bamboo plantation and harvest over the last decades or centuries. A site where the species was found about 10 years ago by a ranger on the opposite side of the valley is now entirely dry and unlikely to support a breeding population, making habitat loss the main threat to the species. Climate change is also most likely to impact the species, as seen in other Hynobiids [64], and we can expect the distribution of the species to be contracting. It is, therefore, important to lower other stresses as amphibians can cope with a low number of conservation pressures, but populations crash when there are too many stress factors [65]. As the species is occurring in bamboo forests exploited by humans, we recommend reducing the use of herbicides and restricting water pumping in the area. In addition, restoring the habitat through the creation of additional artificial shallow ponds or rehabilitating old water reservoirs found throughout the bamboo plantation will help boost population growth [66].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13101661/s1, Table S1: GenBank accession numbers; File S1: obj 3D model.

Author Contributions: Conceptualization, Z.W., S.N.O. and A.B.; methodology, S.N.O., V.K.P., Y.D. and A.B.; software, S.N.O., V.K.P. and A.B.; validation, Z.W., S.N.O., Z.Q., Y.L., Y.D., C.-H.L. and A.B.; formal analysis, S.N.O., V.K.P. and A.B.; investigation, Z.W., S.N.O., Y.L. and A.B.; resources, A.B.; writing—original draft preparation, Z.W., S.N.O., Y.L., V.K.P. and A.B.; writing—review and editing, Z.W., S.N.O., Z.Q., Y.D. and A.B.; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All applicable international, national, and/or institutional guidelines for the care and use of animals were strictly followed. All animal sample collection protocols complied with the current laws of the People's Republic of China. All observations and experiments conducted in this study are in agreement with the ethical recommendations of the College of Biology and the Environment at Nanjing Forestry University (AICUC approval number 2022014). We did not collect adult individuals as voucher specimens, in line with the IUCN recommendation on research involving species at risk of extinction (IUCN 1989). Instead, we relied on one of the individuals raised from the egg mass.

Informed Consent Statement: Not applicable.

Data Availability Statement: The morphological data are in Table 4, and the genetic sequences are in Supplementary Table S1. Accession numbers and references for all DNA sequences used for the bioinformatic analyses. DNA sequences of the candidate *Hynobius* species generated in this study and homologous sequences are retrieved from Genbank.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

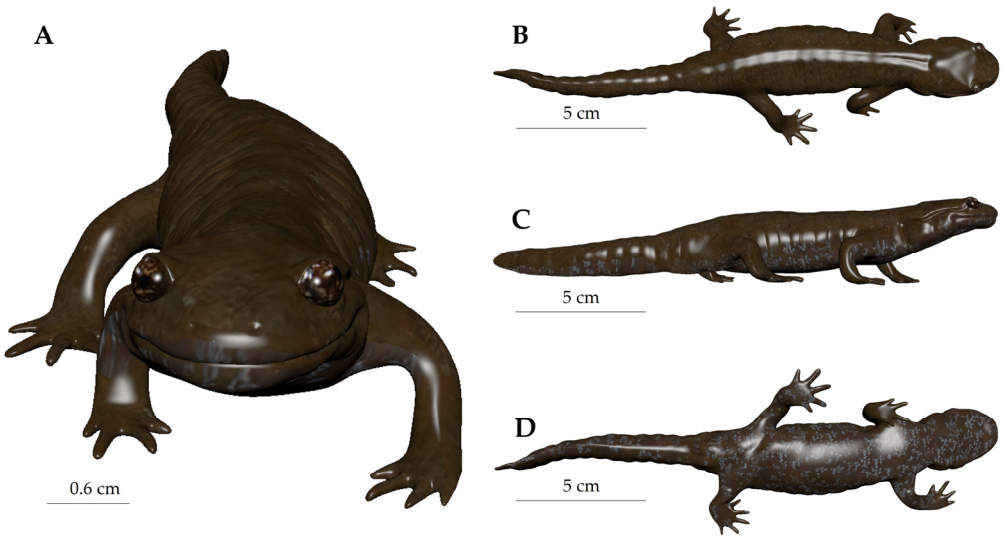

Figure A1. Representation of the 3D model. (**A**) Frontal view. (**B**) Dorsal view. (**C**) lateral view. (**D**) Ventral view.

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Survived the Glaciations, Will They Survive the Fish? Allochthonous Ichthyofauna and Alpine Endemic Newts: A Road Map for a Conservation Strategy

Ilaria Bernabò [1,*], Mattia Iannella [2,*], Viviana Cittadino [1,2], Anna Corapi [1], Antonio Romano [3], Franco Andreone [4], Maurizio Biondi [2], Marcellino Gallo Splendore [5] and Sandro Tripepi [1]

[1] Department of Biology, Ecology and Earth Science, University of Calabria, Via P. Bucci 4/B, I-87036 Rende, Italy
[2] Department of Life, Health & Environmental Sciences, University of L'Aquila, Via Vetoio—Coppito, I-67100 L'Aquila, Italy
[3] Consiglio Nazionale delle Ricerche—Istituto per la BioEconomia, Via dei Taurini 19, I-00100 Roma, Italy
[4] Museo Regionale di Scienze Naturali, Via G. Giolitti 36, I-10123 Torino, Italy
[5] Museo del Castagno, Via V. Emanuele, I-87013 Fagnano Castello, Italy
* Correspondence: ilaria.bernabo@unical.it (I.B.); mattia.iannella@univaq.it (M.I.)

Simple Summary: In Italy, there is a growing concern over the survival of an endemic subspecies of newt, *Ichthyosaura alpestris inexpectata*, commonly known as the Calabrian Alpine newt, due to the recent fish introduction and acclimatisation into three of the few localised lentic habitats where this glacial relict occurs. Results of a recent survey focused on this rare endemic taxon are presented. We provide updated information on the Calabrian Alpine newt distribution, adding two new localities, documenting local extinction at a few historical sites, providing a rough estimation of population size, and giving a description of breeding habitat features. The results of our pilot study will facilitate future research activities, conservation measures for the amphibian assemblage, and habitat management after fish introduction in the Natura 2000 site. We also pinpoint some actions useful to avoid the extinction of this remarkable taxon.

Abstract: The Calabrian Alpine newt (*Ichthyosaura alpestris inexpectata*) is a glacial relict with small and extremely localised populations in the Catena Costiera (Calabria, Southern Italy) and is considered to be "Endangered" by the Italian IUCN assessment. Climate-induced habitat loss and recent fish introductions in three lakes of the Special Area of Conservation (SAC) Laghi di Fagnano threaten the subspecies' survival in the core of its restricted range. Considering these challenges, understanding the distribution and abundance of this newt is crucial. We surveyed the spatially clustered wetlands in the SAC and neighbouring areas. First, we provide the updated distribution of this subspecies, highlighting fish-invaded and fishless sites historically known to host Calabrian Alpine newt populations and two new breeding sites that have been recently colonised. Then, we provide a rough estimate of the abundance, body size and body condition of breeding adults and habitat characteristics in fish-invaded and fishless ponds. We did not detect Calabrian Alpine newts at two historically known sites now invaded by fish. Our results indicate a reduction in occupied sites and small-size populations. These observations highlight the need for future strategies, such as fish removal, the creation of alternative breeding habitats and captive breeding, to preserve this endemic taxon.

Keywords: amphibian; Urodela; conservation; *Ichthyosaura alpestris inexpectata*; Calabria; Italy; endemism; invasive fish; morphometrics

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1. Introduction

The biodiversity crisis is particularly dramatic in freshwater ecosystems [1,2]. This biodiversity crisis requires actions and conservation measures at multiple geographical

scales, global, regional, and local, considering both conservation priorities and limited resources [3–5]. The most often used criteria to establish conservation priorities are taxon rarity and endemism (e.g., [6,7]). IUCN criteria consider the limited number of localities of a given taxon as a key factor for assigning high-risk categories [8], while other conservation projects (e.g., the EDGE of Existence programme) involve other evaluation frameworks, such as including endangered species that embody a substantial portion of distinct evolutionary heritage. The insular nature of lentic freshwater habitats has produced a high rate of endemism with small geographic ranges [2] (and reference therein).

Amphibians are nowadays subject to a plethora of possible threats and pressures, ranging from alien species' introduction to pollution, emerging infectious diseases and habitat loss, which sometimes act in synergy and result in detrimental effects at both individual and community levels (e.g., [9–15]). Alien species in freshwater habitats are among the greatest threats to native biodiversity [9]. Indeed, for amphibians in general and newts in particular, fish introduction is considered a determining factor of pond-breeding populations' decline and extinction [12,16–20]. Naturally, the impact of alien species is particularly alarming for native taxa with a restricted range.

Ichthyosaura alpestris inexpectata (Dubois & Breuil, 1983), known as the Calabrian Alpine newt, is an endemic taxon of Southern Italy considered to be a post-glacial relict with small and fragmented populations [21,22]. It represents a unicum of high biogeographical and conservation value, with populations exhibiting genetic variation likely originating from the Middle Pleistocene and possessing limited genetic diversity, which supports a long-standing isolation hypothesis [21]. Since the early 1980s, the newt has been known in five localities in the north of the Catena Costiera (a coastal chain representing the first mountain range of the Calabrian Apennines), in ponds and lakes of varying depths localised in clearings within beech forests at an altitude ranging from 850 to 1135 m a.s.l. [22–27]. All these sites currently fall within the Natura 2000 Network established under the EU Habitats Directive (92/43/EEC) (Table 1).

Table 1. Summary of the data related to known sites. The last year of observation of *Ichthyosaura alpestris inexpectata* for each site is reported. Maximum number of adults captured or observed is based on information retrieved from the available literature or collected ante 2022 in the DiBEST database. Trifoglietti: TR = large pond Trifoglietti; TRIF = small pond Trifoglietti Inferiore. Fonnente: FO = Lake Fonnente; FA = Fosso Armando.

Site Code N2000	Municipality	Locality	Year of Last Observation	Maximum Number Captured/Observed (1983–2018)	References
IT9310058 Pantano della Giumenta	Malvito	Stagno C/Pantano dorato	1983	4	[24,25]
IT9310060 Laghi di Fagnano	Fagnano Castello	Trifoglietti (pond TR and pond TRIF)	2018 (TR) and 2022 (TRIF)	23	[22–26]
		Due Uomini	2018	21	
		Fonnente (lake FO and pond FA)	2022	25	[25,26]
IT9310061 Laghicello	San Benedetto Ullano	Laghicello	2022	69	[22–25,27]

The Calabrian Alpine newt is assessed as "Endangered (EN)" in the Red List of Italian Vertebrates [28] because of the low number of occurrence localities, the continuous degradation and loss of the few sites due to the impact of human activity, and the recent introduction of allochthonous ichthyofauna in its core range. Since 2017, in fact, three fish species, *Cyprinus carpio*, *Gambusia holbrooki* and *Carassius* sp., have been observed in three newt breeding sites in the Special Area of Conservation (SAC) "IT9310060 Laghi

di Fagnano", originally devoid of fish fauna [23,24]. Moreover, other natural pressures, such as the transformation of habitats due to silting phenomena and drought, represent worrying critical issues [23,24], as was also found for another peninsular Italian amphibian species [5] (and references therein).

The aims of this study are threefold. First, we aim to provide an updated framework of the presence/distribution, status and threats of the Calabrian Alpine newt. Second, we aim to supply baseline ecological data for each inhabited site derived from recent systematic monitoring in the SAC Laghi di Fagnano. Third, we aim to provide the first demographic and population parameters. Specifically, we surveyed all spatially clustered wetlands in the protected area where the focal species was previously observed and the two recently discovered sites, thus giving the most updated information about *I. a. inexpectata* breeding habitats (e.g., habitat features and aquatic macroinvertebrate assemblages). Moreover, we collected data regarding abundance, body size and body condition in four sites (two new and two historical), expanding the information on morphometrics and sex ratio to obtain the first perceptions of demography.

2. Materials and Methods

2.1. Study Taxon

The Alpine newt *Ichthyosaura alpestris* (Laurenti, 1768) is widely distributed in most of western and central Europe and the Balkans [29]. In Italy, this urodele has a discontinuous distribution with three subspecies, two of which have a large distribution area and one of which is extremely localised. *Ichthyosaura a. alpestris* (Laurenti, 1768) is widespread in the Alpine area, *I. a. apuana* (Bonaparte, 1839) occurs in the Langhe hill system, Turin hills, Oltrepo' Pavese (Northern Italy), the Tuscan-Emilian Apennines, the Maritime and Apuan Alps and a restricted and disjointed area within the Laga Mountains (Central Italy); conversely, *I. a. inexpectata* has a very limited range in Southern Italy [22–25,30–33] (Figure 1).

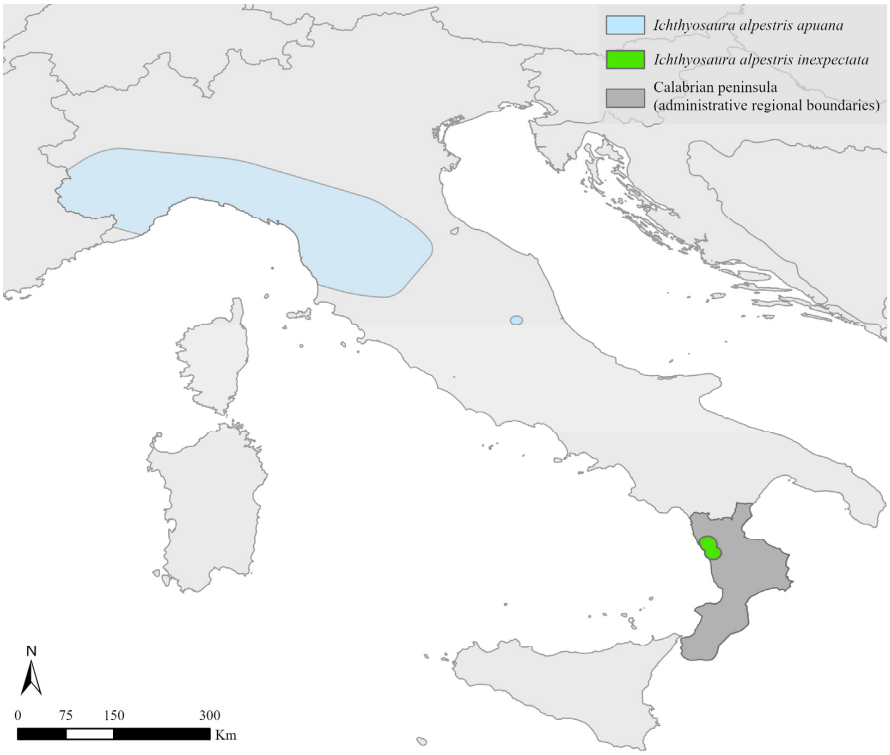

Figure 1. Italian ranges (minimum convex polygons based on [30,31]) of the two Italian endemic subspecies of the Alpine newt *Ichthyosaura alpestris*, namely, *I. a. apuana* (light blue) and *I. a. inexpectata* (green); in dark grey, the Calabria region, where our target subspecies (*I. a. inexpectata*) occurs.

Adults of this medium-sized newt present an aquatic lifestyle in spring, enabling them to breed in ponds with aquatic and riparian vegetation, transparent waters and short periods of winter frost. Reproduction occurs mainly in May and June, and no autumn breeding events are known to date [31]. Paedomorphic phenotypes were observed in Lago Due Uomini and Laghicello [31]. Available data on ecological requirements, phenology and life history traits, and demographic information for *I. a. inexpectata* are scarce or outdated. Knowledge of feeding habits only refers to the population of Laghicello [34]. In addition, for this site, the rough estimation of a few hundred individuals of *I. a. inexpectata* (210–300) is the only one reported in the literature [27]. The same authors assumed that the total number of mature individuals in all populations (from Laghicello and Fagnano lakes) could be estimated between 840 and 1200 [27]. Much less is known about populations in Laghi di Fagnano, which have never been assessed.

2.2. Study Area and Site Description

We surveyed seven lentic habitats, ranging from temporary ponds to permanent lakes (Figure 2 and Table 2). Five sites fall within the Natura 2000 site "Laghi di Fagnano", which protects a natural system of lakes and ponds with an elevation range from 1000 to 1100 m a.s.l. There are nine other amphibian species within this network of wetlands, in addition to *I. a. inexpectata* (Table 2). Due to this species diversity, the site was recognised as an Area of National Herpetological Relevance by the Societas Herpetologica Italica in 1998 (AREN—ITA022CAL002) [35].

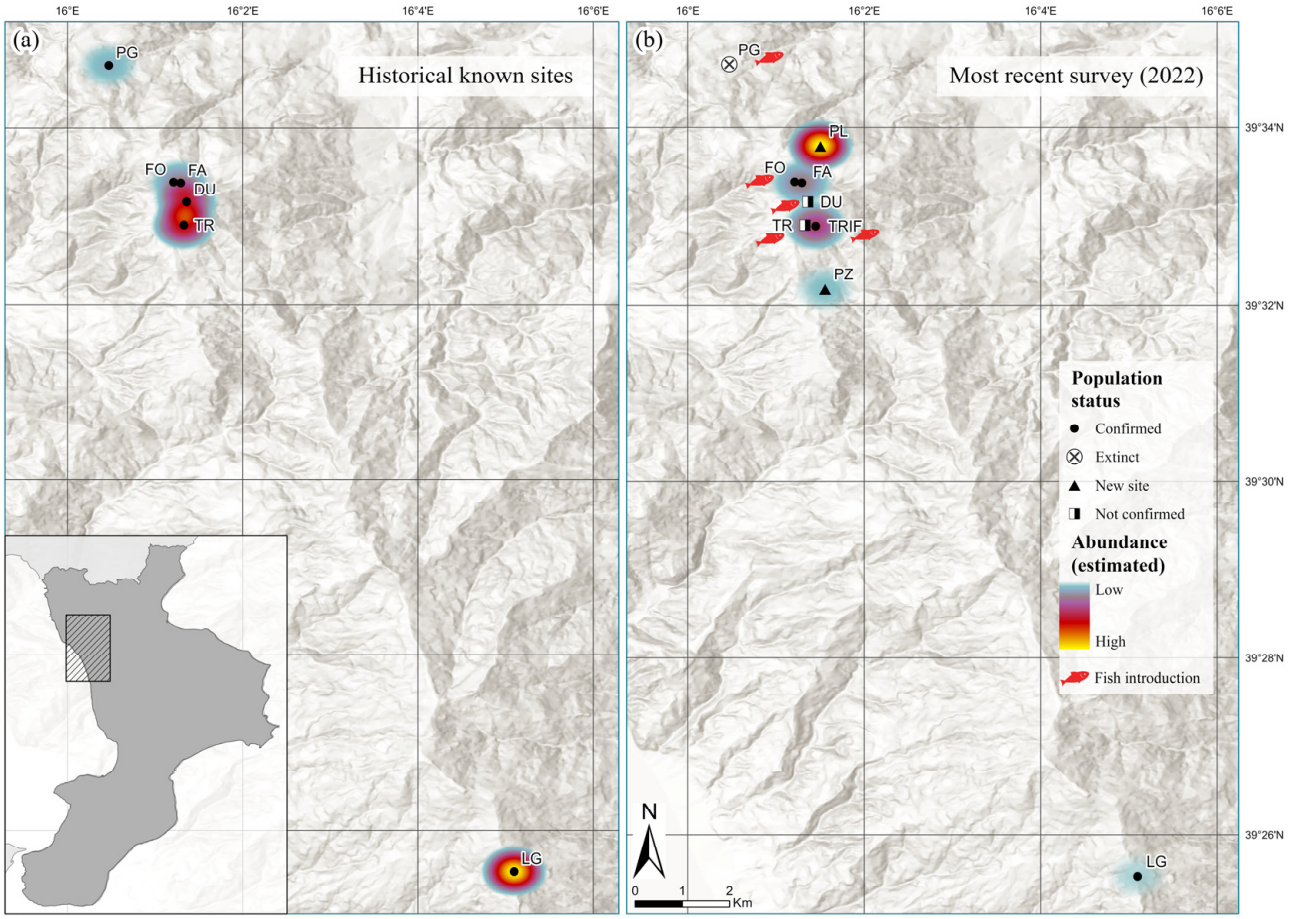

Figure 2. (**a**) Distribution and putative population sizes in the known localities of *Ichthyosaura alpestris inexpectata* based on literature data and historical observations; see Table 1 for details; (**b**) Results from recent surveys.

Table 2. Geographical locations, morphometric parameters and environmental characteristics measured in the study ponds. Site name: Fonnente (FO), Due Uomini (DU), Trifoglietti (TR), Trifoglietti inferiore (TRIF), Fosso Armando (FA), Piano di Zanche (PZ), Pantano Lungo (PL). The approximate area was calculated using Google Earth Pro satellite images (May 2022). Pond water depth (considering the maximum seasonal depths measured in April–May 2022), pond regime (P: permanent; T: temporary pond subject to drying out at least during a single survey), shading (i.e., the extent of shading of the site during the surveys) and presence or absence of fish are reported. Life stage: adults = A, larvae = L; overwintering larvae = OL; paedomorphs = P. Species abbreviations: Tla: *Typha latifolia*; Pna: *Potamogeton natans*; Pau: *Phragmites australis*; Cve: *Carex vesicaria*; Sau: *Sphagnum auriculatum*; Cro: *Carex rostrata*; Cpa: *C. paniculata*; Jef: *Juncus effusus*; Lvu: *Lysimachia vulgaris*; Eca: *Eupatorium cannabinum*; Epa: *Eleocharis palustris*; Lit: *Lissotriton italicus*; Tca: *Triturus carnifex*; Ssa: *Salamandra salamandra*; Hin: *Hyla intermedia*; Pes: *Pelophylax* sinkl *esculentus*; Rda: *Rana dalmatina*; Rit: *R. italica*; Bbu: *Bufo bufo*.

	Site Name						
	FO	**DU**	**TR**	**TRIF**	**FA**	**PZ**	**PL**
Lat. N Long. E	39°33′24″ 16°1′13″	39°33′9″ 16°1′22″	39°32′54″ 16°1′2″	39°32′53″ 16°1′27″	39°33′23″ 16°1′18″	39°32′11″ 16°1′34″	39°33′48″ 16°1′30″
Altitude (m a.s.l.)	1050	1077	1048	1045	1055	880	1010
Area (m^2)	3561	20,679	10,800	520	827	742 [1]	235 [1]
Depth (m)	1	-	1.5 [2]	0.9	1.2	0.7	1.1
Permanence	P/T [3]	P	P	P	T	T	P
Shading (%)	20	20	30	80	20	90	90
Fish presence	*Carassius* sp.	*Cyprinus carpio*	*G. holbrooki*	*G. holbrooki*	-	-	-
Aquatic vegetation	Tla, Pna	Pna, Pau, Cve	Sau, Cro, Cpa, Jef, Lvu, Eca, Epa, Pna	Cve, Jef	Jef	absent	absent
I. a. inexpectata	L	-	-	A, L, P	A, L	A, L	A, L, OL
Other breeding species	Tca, Lit, Pes, Rda, Hin	Tca, Bbu, Pes, Rda	Hin, Pes, Rda	Lit, Tca, Ssa, Pes, Rda, Rit, Bbu	Lit, Tca, Ssa, Hin, Pes, Rda, Rit	Lit, Tca, Bbu, Rda	Lit, Ssa, Rda, Rit

[1] Area calculated by measuring the maximum length and width and assuming an elliptical shape. [2] From [36]. [3] Temporary as referred to the adjacent flooded meadow.

The Special Area of Conservation covers a surface area of around 19 ha, and it extends mainly along the slopes of Monte Caloria (Fagnano Castello) along a sub-plateau belt in which high-grade metamorphic rocks outcrop. The local climate is strongly influenced by the warm and humid air masses from the Tyrrhenian Sea, which give rise to thick fog formations and frequent and abundant rainfall that help mitigate the long, dry summer [37]. The site falls within the lower supratemperate bioclimatic belt with upper subhumid ombrotype [38]. The ponds and lakes network are within beech forest formations (*Fagus sylvatica*), included in habitat 9210*, with a rich shrub and herb layer. The climatic conditions and soil characteristics allowed the formation of peat bogs consisting of a layer of sphagnum and a mosaic of different plants of humid and marshy environments linked with dynamic successions due to variations in water level [37]. A detailed description of the habitats and vegetation in the SAC is available at http://retenatura2000.regione.calabria.it/, (accessed on 12 January 2023).

Briefly, a higher and constant water depth of up to 2 m characterises "Lago Due Uomini" (hereinafter referred to as DU), which hosts submerged vegetation of *Potamogeton natans*

and a massive presence of *Phragmites australis*. "Lago Trifoglietti" (TR) is a silting peat bog consisting of a thick moss layer of *Sphagnum auriculatum* with various species of *Carex rostrata*, *C. paniculata*, *Juncus effusus*, *Lysimachia vulgaris*, *Eupatorium cannabinum* and, in the most flooded areas, *P. natans* and *Eleocharis palustris*. Moreover, the lake holds a unique palynological archive that has yielded important information on the dynamics of Holocene vegetation and climate in southern Italy [36]. A spring flows into the lake from the north, whereas an outflow runs southward. To prevent dry-up during summer and complete infilling, the municipality of Fagnano Castello built a small earthen dam in 2000 that led to the formation of a small underlying pond [36] called "Trifoglietti inferiore" (TRIF). "Lago Fonnente" (FO) refers to an area including the main lake and an adjacent flooded meadow, which dries up in the dry season, and a pond, historically called "Fosso Armando" (FA) [39,40] which is adjacent but not connected with the other wetlands. FO shows well-preserved marsh vegetation with sedges and rushes and is characterised by *Typha latifolia* and *P. natans*. During the study period, the contiguous flooded meadow appeared almost completely dry in June, when numerous dead larvae of all three species of newts and tadpoles of *Hyla intermedia* were found. Therefore, the site was not visited further in subsequent samplings.

The new breeding sites of the Calabrian Alpine newt are constituted by two woodland ponds found in the localities of Piano di Zanche (PZ) and Pantano Lungo (PL) (municipality of Fagnano Castello) during a herpetological survey in 2015 and, after discovering, visited sporadically. The description of these habitats is given in Section 3.

2.3. Sampling Procedures

We sampled the five sites in the SAC Laghi di Fagnano and the two new ponds from April to October 2022, during and after the newts' breeding season (May–July), once a month. PZ and PL were visited six times (except in May) due to a weather change (sudden thick fog) preventing sampling. All visits were conducted during daylight hours.

Newt sampling techniques differed across sites due to the intrinsic features of each. Sites TR and DU are the most extensive, and due to the morphology and presence of dense aquatic vegetation, these cannot be easily and completely surveyed. Therefore, the visual encounter survey method (VES) was used for the lakes TR and DU by walking across the entire wadable surface of the wetlands for at least 30 min, so as to cover, in that time, the entire pond perimeter (~14 m/min). While searching for newts, we also reported the detection of fish. For FO, FA, TRIF, PZ and PL sites, survey protocol required at least two observers to enter the wetland and capture animals using dip netting. At least three removal passes lasting 20 min were made during a single visit, removing the individuals captured at each pass to avoid multiple counts and applying the removal sampling scheme to estimate population abundance (see below).

All the captured newts were handled with latex gloves and temporarily kept in plastic containers filled with water from the pond and measured for body mass (to the nearest 0.01 g using digital scales), snout-vent length (SVL) and total length (with a ruler to the nearest 0.1 cm). A randomly selected subset of captured adults (n = 25) for PL in June was measured, so as to make the captured individuals' number homogeneous. The sex was determined by typical secondary sexual characters. Recognition of newt species during larval stages was based on the comparative staging tables of Bernabò and Brunelli [41]. Paedomorphic newts were distinguished by gill slits and three pairs of external gills, and evident secondary sexual characteristics [39]. Once all measurements and data had been collected, newts were released into the capture pond.

At each sampling session, other amphibian species occurring and breeding in the investigated aquatic sites were also recorded.

2.4. Habitats' Characteristics and Macroinvertebrates Assemblages

We described the features of the seven sampled aquatic habitats with different hydrological balances and subjected to different pressures (fish-containing and fishless ponds) by

recording the size, the water permanence, the distances between each pond and the water's physical–chemical characteristics.

To evaluate the biodiversity levels and trophic availabilities of the four breeding water bodies where adult newts currently occur, we investigated the structure of macroinvertebrate assemblages. Therefore, macroinvertebrate sampling was conducted in June and October, following a time–space-standardised quantitative procedure. In each monitoring site, we sampled three main mesohabitats in fordable zones (emergent and submerged aquatic vegetation, littoral and central sediments) using a Surber-type sampler (square opening: 25 cm × 25 cm) (Scubla s.r.l., Remanzacco UD, Italy) equipped with a 60 cm net of 250 µmesh and a plastic gathering glass. All samples were sieved through a 0.45 µm sieve (Endecotts Ltd., London, UK) and immediately stored in refrigerated containers with 80% ethanol. Once in the laboratory, macroinvertebrates were separated from sediments through a stereomicroscope. All organisms were counted and identified at the family level [42–44] to estimate the following parameters and indices: abundance (N), taxonomic richness (TR), Shannon–Wiener Index (H′) [45] and Margalef Index (D) [46].

Water physical–chemical parameters were assayed twice during the study, in the early summer and autumn, with three measurements along a transect at about 10–20 cm below the surface water. Specifically, temperature, conductivity, pH and total dissolved solids were determined with a multiparameter probe (Hanna Instruments, model HI991300). Dissolved O_2 and relative saturation percentage were measured with a portable oxygen meter (Hanna Instruments, model HI98193).

2.5. Data Analysis

We analysed the dataset, collecting information on adults of the Calabrian Alpine newts captured in TRIF, FA, PZ and PL (Table 2). The sample size from the four populations in several months was too small since we captured only larvae and <3 individuals; therefore, for each population, we pooled the total number of captured males and females during the sampling period.

Operational sex ratio in each sampling was expressed as the proportion of males over the total adult number (males/(males + females)); deviations from equality were assessed via a two-tailed binomial test when more than ten newts were caught.

To assess the body condition (a proxy for physical condition, overall health and fitness [47]) of the four populations of Calabrian Alpine newts, we calculated the scaled mass index (SMI) [48]. This index provides a comparable measure across individuals of different sizes by rescaling body mass measurements to a standard length (here calculated for SVL) accounting for allometric growth [48]. We calculated SMI separately for males and females within each site, thus avoiding the scaling issue that results when comparing BCIs across groups known to differ in size [49]. SMI is computed as follows (with *Mi* and *Li* being the body mass and the linear body length, respectively, *bSMA* the scaling exponent estimated via the SMA regression of *M* on *L*, and *L*0 the mean length of the study population):

$$\hat{M}i = Mi\left[\frac{L0}{Li}\right]^{bSMA}$$

We quantified the scaling exponent bSMA in R [50] by using 'smatr' package [51] from ln-transformed data.

The normality of morphometric data (body mass, SVL, total length and SMI) was analysed via the Shapiro–Wilk test and the homogeneity of variances was analysed via the Brown–Forsythe test. Data fit normal distributions and resulted homoscedastic; therefore, we used a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test to determine whether there was a difference in body size and body condition between sex within populations and comparing females and males, respectively, among populations.

We also tested the effect of fish presence, macroinvertebrate diversity (in terms of Shannon index), site and month on the SMI using a linear model.

We used OriginPro, Version 2022b (OriginLab Corporation, Northampton, MA, USA) to produce plots.

We estimated newt abundance through the most recent field capture data to obtain updated information about TRIF, FA and PL (sites where sufficient numbers of adult newts were found). Considering that the sampling method we used can be ascribed to the constant probability of capture (each pass does not lower or increase the probability of capture of the subsequent pass, and the chance of sampling each individual is constant, both for their detectability and/or population stability) and the removal of captured individuals was performed at each pass, we used the whole capture dataset, setting the removal method = 'Zippin' [52–54]. The population size was estimated through the 'FSA' package [54] in R. We considered the highest population estimate in the month of greater detectability of adult individuals (present in the water during the breeding season).

3. Results

3.1. Current Distribution Framework and Habitats' Characteristics

Based on our multi-year observations (1984–1992, 2005, 2015–2018) and during a sampling visit in June 2022, as well as from information gathered from locals and landowners, the occurrence of the Calabrian Alpine newt population reported in the SAC "Pantano della Giumenta" is seriously questionable, since the last observation refers to the early 1980s (Table 1 and Figure 2). In Laghicello, the presence of Alpine newts was verified during a single visit in April 2022; no fish were introduced, but the site appeared subjected to advanced natural silting phenomena (I. Bernabò pers. obs.).

During surveys conducted in 2022 in three study sites in the SAC Laghi di Fagnano (FO, TRIF and FA), Calabrian Alpine newts were observed at least once for each life stage, whereas in DU and TR we did not observe any (Table 2 and Figure 2b). A true absence was not proven, and we could not exclude its presence in the deep central areas of the lakes, which cannot be sampled except with methods other than those used. Individuals of the common carp (Cyprinus carpio) and of the Eastern mosquitofish (Gambusia holbrooki) were highly abundant in the lakes DU and TR, respectively; the presence of cyprinid fish (Carassius sp.) and G. holbrooki was also recorded in FO and TRIF, respectively, but at a low density (Table 2).

The two new records were confirmed to be breeding sites (Figure S1): PZ is a temporary pond, and the water comes almost exclusively from rainfall; conversely, PL is a large woodland puddle that presents a mostly stable hydroperiod thanks to a water flow from a spring. Both sites have significant tree cover and low O_2 levels due to a high plant debris load; woods of Fagus sylvatica dominate the vegetation around the two wetlands, and no water vegetation (macrophyte or algae) is present. Water bodies' environmental features (measured during the last field campaigns) are summarised in Table 2 and in Table S1.

Considering the linear distances among clustered wetlands in the SAC of Laghi di Fagnano and the new sites, we found that the nearest was PL (835 m away from FA), whereas PZ occurs at a greater distance (1321 m from TRIF) (Figure 3).

About the amphibian community, among the ten species occurring in the SAC, only *Bombina pachypus* was not found. *Rana dalmatina* was the most common species (in 100% of the sites), followed by *Lissotriton italicus* and *Triturus carnifex*, and then *Pelophylax* sinkl *esculentus* (71%). *Salamandra salamandra*, *Rana italica*, *Hyla intermedia* and *Bufo bufo* were detected in 43% of the aquatic sites (Table 2). Syntopy of *I. a. inexpectata* with *L. italicus* occurred in five sites (FO, TRIF, FA, PL and PZ), and it also lived with *T. carnifex* in four ponds (FO, TRIF, FA and PZ).

Regarding aquatic macroinvertebrate assemblages, a total of 43 families, 15 orders, and 7 classes of invertebrates, cumulatively, were identified in the four study sites (TRIF, FA, PZ and PL). The taxonomic list of all organisms collected at each monitoring site is reported in Table S2. All ponds in both sampling campaigns showed high diversity indices, indicating stable habitats for aquatic invertebrates. In particular, PL showed the highest values of diversity indices (TR: 25, H′: 3.056, D: 3.050), followed by FA (TR: 15, H′: 2.727,

D: 2.261) and TRIF (TR: 17, H′: 2.555, D: 2.119), whereas PZ had the lowest ones (TR: 12, H′: 2.439, D: 1.591). Moreover, the macroinvertebrate community biodiversity increased in terms of number of taxa from June to October, due to the significant input of trophic resources associated with woody/leaf litter, with an overall autumnal increase of 15.7%.

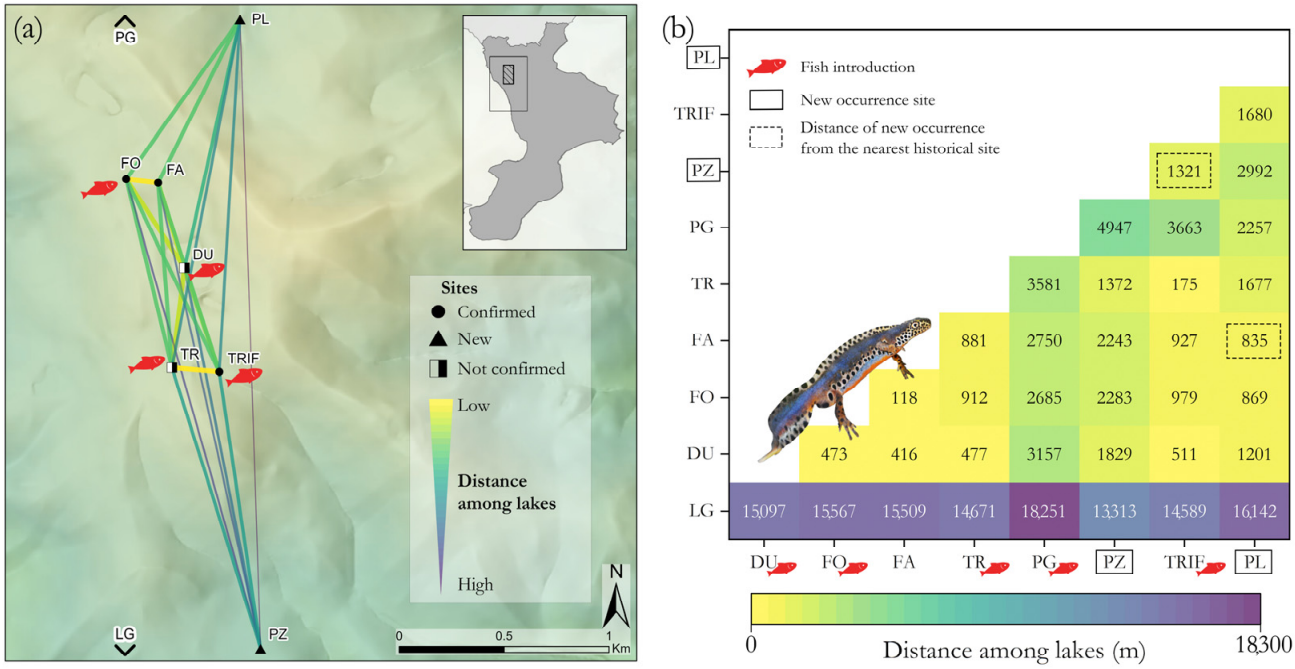

Figure 3. (**a**) Water bodies' network within the SAC "Laghi di Fagnano" (Laghicello and Pantano della Giumenta sites are excluded from the view) and (**b**) the distances between each site (including all); the two new sites and the correspondingly shorter distances from which the Calabrian Alpine newt possibly colonised them are highlighted through boxes (full and dashed lines, respectively).

3.2. Population parameters

In TRIF, we captured 53 adult Calabrian Alpine newts from May to September, with a maximum of 34 individuals captured in June (20 males and 14 females; estimated abundance 37 ± 3, 95% C.I. = 30–44) (Figure 4), whereas we observed numerous larvae from late summer to autumn, with many of these showing evidence of predation (i.e., tails and legs partially nipped). TRIF was the only site where we detected paedomorphic individuals (four males and three females); paedomorphic males and females had an average SVL (± SE) of 38.8 ± 0.4 mm and 41.8 ± 0.5 mm, respectively. In FA, 30 adult newts were captured from April to June, with a maximum of 15 captured adults in April (10 males and 5 females; estimated abundance 20 ± 8, 95% C.I. = 5–35) when the pond, characterised by a highly variable water level, presented its maximum water level (Figure 4). The pond was almost completely dry (level of about 20 cm) from the end of July until October, and only abundant larvae were observed. In PZ, we collected eight adults (five males and three females) of Calabrian Alpine newts in June, whereas in September, we found one female with a few larvae (Figure 4). The complete drying of the pond was detected in July and August. In PL, we detected adult Calabrian Alpine newts every month except May (see Materials and Methods); larvae were observed from the end of July to October, including overwintering larvae. Altogether, we captured 129 adults; the highest number of individuals was observed in June (33 males and 35 females) (Figure 4). The estimated maximum abundance of newts was 88 ± 14 (estimate ± SE; 95% C.I. = 62–115).

In individual samples with N ≥ 10, our results showed a male-biased sex ratio of 0.59 (p = 0.256) in TRIF. In FA, the proportion significantly differed from 0.5 both in April (0.67) and June (0.73) (all $p < 0.05$), whereas, in PL, a significant deviation from 50:50 toward males (0.65) occurred only in April ($p < 0.05$).

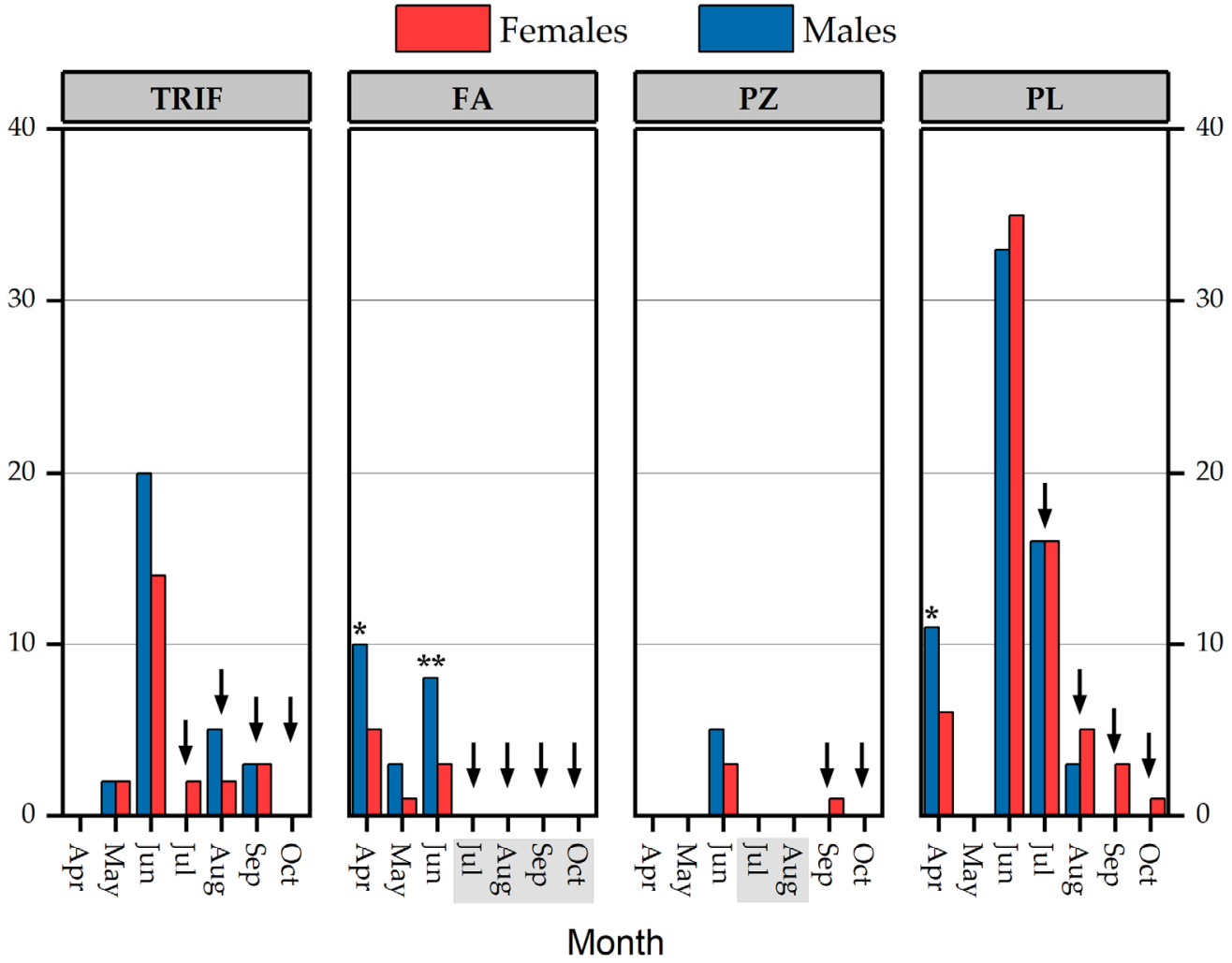

Figure 4. Captures of adults of *Ichthyosaura alpestris inexpectata* at four of the study sites during the sampling period. The months with no water are highlighted in grey. Asterisks mark a significant deviation from evenness, * $p < 0.05$, ** $p < 0.01$ (Fisher's exact probability test); arrows indicate when larvae were found.

In all populations except for PZ (due to its small size), females were confirmed to have larger body sizes than males ($F_{7,116} = 10.50$, Tukey's multiple comparisons test, $p < 0.001$) (Figure 5 and Table S3). Overall, the average (±SD) SVL was 47 ± 3.5 mm in males and 53 ± 4.2 mm in females, whereas the average total length was 81 ± 5.4 mm and 95 ± 5.5 mm, respectively. Sex differed statistically in body mass within sites ($F_{7,116} = 17.86$, Tukey's multiple comparisons test, $p < 0.001$). The mean body mass was 2.6 ± 0.5 g in males and 4.4 ± 1.1 g in females, showing no intra-sexual divergence between populations ($F_{7,116} = 0.55$, Tukey's multiple comparisons test, $p > 0.05$). There were no differences among populations in females' or males' body mass, SVL or total length (Figure 5 and Table S3). SMI differed statistically between sexes in each site, and males exhibited the lowest body condition index (Tukey's multiple comparisons test, all $p < 0.001$); neither male nor female body conditions varied significantly among the four populations (Figure 5 and Table S3). Moreover, we found no significant effect of fish presence, macroinvertebrate diversity, site or month on the SMI (R^2 adj. = 0.073, $p > 0.05$ for all of the factors considered).

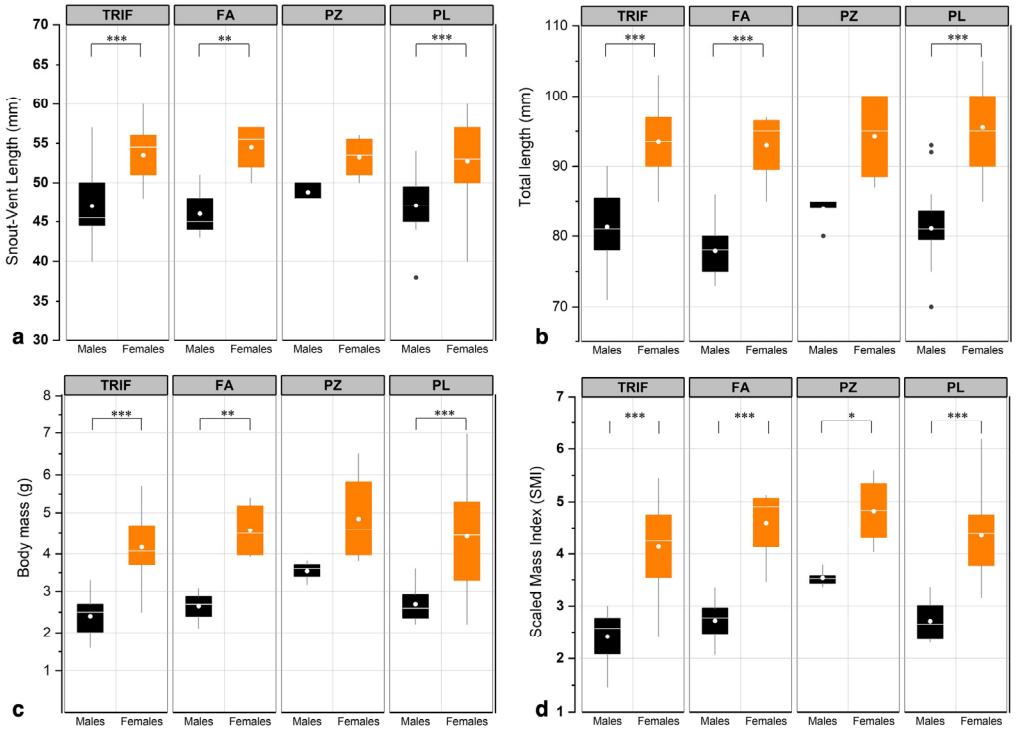

Figure 5. Boxplots of: SVL (**a**), total length (**b**), body mass (**c**), and SMI (**d**) for *I. a. inexpectata* males and females in the four sites. The horizontal white bars and circles represent median and mean values, respectively. The box is determined by the 25th and 75th percentiles, and the whiskers refer to the minimum and maximum values observed within data for each population. Outliers are shown as grey circles. One-way ANOVA with post hoc Tukey tests: * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

4.1. Updated Distribution, Species Occurrence and Habitat Characteristics

In recent years, no effort has been directed towards surveying and monitoring the Calabrian Alpine newt. Our results update the information on the newt distribution, providing records of local disappearance from three historical sites and the discovery of two new breeding ponds.

The Calabrian Alpine newt was traditionally considered to occur in five localities (see Table 1): three in the SAC Laghi di Fagnano (FO, DU, TR), one in the locality Laghicello and one at the SAC "Pantano della Giumenta". Their occurrence in this protected area, where a goldfish population (*Carassius auratus*) has been stable for almost twenty years, has not been confirmed since 1984, and the population has to be considered extinct. In Laghicello, the endemic newt still occurs. In contrast, in two of the three historically known breeding sites in the SAC Laghi di Fagnano, DU and TR, the newts were not recorded after fish introduction in 2017 and 2019, respectively. However, past surveys (until 2018) applying VES, when the two lakes were fishless, allowed the detection of all newt species. Thus, VES may not be the most efficient way to detect newts in these two studied lakes and could lead to false absences. Furthermore, to avoid predation risk from fish, amphibians can increase shelter use and reduce activities [55,56], resulting in a lower probability of being detected and, likewise, reduced foraging activities and reproduction, two essential fitness components [57]. On the other hand, it is also well known that newts avoid fish-invaded habitats [58,59]. Another Alpine newt subspecies, the Bosnian Alpine newt *I. a. reiseri* (Werner, 1902) from the Prokoško Lake, was dramatically reduced or, more probably, disappeared from the lake following fish introductions [60].

In our case, if the event is not a local extinction, it is a demographic reduction since the detection probability is also a function of the abundance [61].

The progressive modification or loss (early desiccation and silting phenomena) of the few suitable habitats in which *I. a. inexpectata* breeds and the substantial threat posed by the introduction of invasive fish may lead to reproductive failure. This could result in the extinction of the endemic subspecies and those of other newts' populations of community and conservation concern. Fish eradication would also bring benefits to the two other newt species that are syntopic with the Calabrian Alpine newt, i.e., *Triturus carnifex* (Annex II and IV) and *Lissotriton italicus* (Annex IV), which are protected under the Habitats Directive and categorised as NT and LC by the IUCN, respectively.

Knowledge of taxon distribution in a given area underpins most conservation efforts and planning [62]. In the context of the severe threat to the survival of the Calabrian Alpine newt, the discovery of two new occurrence localities (PZ and PL) is crucial for the newts' persistence and highlights the importance of constant field monitoring to assess the species distribution at a local scale [63,64], which is a dynamic trait, determined by local extinction and new colonisation phenomena. These new breeding sites are small woodland ponds formed on natural depressions in well-preserved beech forests, away from the main roads and excessive tourism. Local landscape features include forest continuity and a network of ponds that allow the permeability of matrix habitats for dispersion. The linear distance and the narrow differences in altitude (about 200 m) separating the new ponds from the known ones of Laghi di Fagnano suggest that these sites may have been colonised through the dispersal of newts from the nearby locations. Indeed, the closest connection pathway may have been about 0.8 km from FO and FA toward the northeast for PL and about 1.3 km from TR and TRIF toward the southeast for PZ. Although fidelity to the breeding site is reported in the Alpine newt [65], movement capabilities over long distances, exceeding 1 km and up to 4 km, have also been reported [66,67]. Future studies should map all wetlands in the vicinity of the SAC and consider inter-pond movements, migrations and dispersal to disentangle newt population dynamics and better understand how to plan adequate conservation measures, such as pond creation. In addition, conducting investigations with new approaches, such as eDNA, could be helpful in detecting newts' occurrence in suitable habitats.

At high altitudes (i.e., in the Alps), *I. alpestris* usually lives in oligotrophic sites where the water is very clear. In the Apennines, on the other hand, it inhabits low- and mid-altitude sites where water is frequently turbid, the maximum temperature is high and the environment is generally unpredictable [30]. We provided the first information on water parameters where the subspecies *I. a. inexpectata* breeds. The new ponds can be considered oligotrophic environments based on physical–chemical measurements and total hardness (the total amount of Ca and Mg ranged between 3.29 and 11.4 mg/L) [68]. Dissolved oxygen values are very low, suggesting a strong insaturation, most likely due to the scarce primary productivity and the high oxygen consumption triggered by a large amount of autochthonous and allochthonous plant detritus in the ponds. Here, we describe the first data on the potential feeding resources available for the Calabrian Alpine newt. Overall, the four studied ponds showed high diversity and a well-structured macroinvertebrate assemblage. Our biodiversity indices values are comparable to those calculated for other natural lakes and ponds [69–71] and higher than those measured in artificial, garden and urban ponds [72,73]. In October, the macroinvertebrate assemblages showed, in each pond, an increase in shredding invertebrates feeding on organic detritus derived from the riparian vegetation [74]. With regard to hydrological variation, there was a drop in the water level in October at all sites, particularly in FA and PZ. However, this lower volume of water did not negatively affect the macroinvertebrate assemblages and seemed adequate to sustain the ecological niches occupied by these organisms. Most invertebrates found in breeding ponds are involved in the diet, during the aquatic phase, of adults, paedomorphs and metamorphs of any subspecies of *I. alpestris* [34,75–77]. Typically, the Alpine newt shows generalist dietary habits and exhibits seasonal plasticity; it exploits temporary resources and differing prey taxa in relation to their cycles of availability and local abundance [34,75,76]. However, an individual shift towards a more specialist feeding strategy in artificial sites in response to

increasing prey diversity has been reported [78]. In general, the abundance of invertebrate prey in the aquatic breeding sites allows newts not to leave these habitats to forage, a relevant factor for their conservation status [79]. Our preliminary analysis reported no significant correlation between the body condition index, prey species availability and fish presence. The contribution of the newts to the feeding ecology may be elucidated only with dietary studies that may also clarify the trophic strategy of the Calabrian Alpine newt. Newts act as predators in naturally fishless ponds [80,81]; therefore, it is also essential to investigate if the unavoidable trophic competition with introduced fish may become a decisive limiting factor. Indeed, knowledge of the trophic ecology of threatened and protected newts is crucial to evaluating possible threats [82], especially when dealing with the effects of fish introduction, and thus to adopting appropriate conservation policies [83].

4.2. Population Parameters

The ecology and demographic parameters of the Calabrian Alpine newt are scanty or outdated. Although long-term monitoring programmes are required for more robust estimations of trends in their distribution and abundance, our survey confirmed that the overall population of the Calabrian Alpine newt in its core range might be much smaller than hitherto assumed. Indeed, the only available study by Dubois and Ohler in 2009 [27] on the population of Laghicello roughly estimated counts of between 210 and 300 individuals. The authors supposed that the total number of mature individuals at the four historically known sites (Table 1) is probably much less than four times the estimated population size of Laghicello. Altogether, 90 adult newts were caught in the ponds within the SAC Laghi di Fagnano, whereas 138 adults were sampled at the two new sites. The highest number was found in permanent ponds. A few adults and larvae were found in PZ; this pond may completely dry out in the warm season and is replenished by late summer rainfall. The detection of larvae of the three newts at the end of September confirmed a late summer reproduction in this water body. However, its interannual hydroperiod is not well-studied, and it remains unclear what reproduction and metamorphosis success occurs at this pond. PL was confirmed to be a suitable breeding habitat of high conservation value for the Calabrian Alpine newt and other amphibians; here, the most abundant population is present.

Population monitoring and demographic estimates in the breeding sites of Laghi di Fagnano are pivotal for the persistence of the Calabrian Alpine newt after fish introduction. To ensure accurate population size estimates, we plan to adopt a monitoring scheme using aquatic funnel traps in lieu of dip-netting and a capture–mark–recapture approach.

Throughout the breeding season, we found statistically significant sex bias in caught newts towards males in FA (every sampling) and PL (in April). In each sampling event, males outnumbered females. The observation of distorted sex ratios in amphibians using counts or captures may reflect an actual ecological trait of the studied populations but may also be an artefact due to different capture probabilities between sexes [84], and references therein]. In other populations of Alpine newts, sex ratios have been reported to be either male- or female-biased [27,85–87]. According to Lanza and coauthors [31], both sexes remain in the water throughout the breeding season. However, males were more numerous than females at the breeding sites early in the year, and females outnumbering males in late spring and early summer represents a weaker trend in many newt species, including the Alpine newt [88]. Given our data, we can speculate that the sex ratio in the studied ponds during the breeding season is quite dynamic and is determined by locally specific factors. Further studies are needed on such dynamics, which may vary between different types of water bodies (e.g., temporary vs. permanent, temperature, food availability and habitat quality), influencing mating behaviour and the duration of the breeding season.

Body condition is a measure of an animal's foraging success and physiological state [47,89,90]. In our estimations of body condition, remarkable intersexual differences were found within each population. Males' and females' body conditions of Calabrian Alpine newts did not vary significantly among the four study ponds, which were both

with and without fish and had differences in their hydrological regime. Further long-term monitoring studies are required to determine the seasonal and inter-annual variation in SMI values for each population and the effects of fish presence on newts' body condition.

Our results on body size are comparable with those available in the literature on *inexpectata* and other subspecies. Sexual size dimorphism was detected in our study populations, with females being longer and heavier than males, as previously reported in the literature [21,31].

4.3. Threats, Conservation Measures and Active Management

We suppose that within a few years, fish may cause the extirpation of the Calabrian Alpine newt and other native amphibian species from otherwise viable habitats. Meanwhile, the temporary ponds, although protected from the introduction of fish due to their hydroperiod, could likely disappear or become unsuitable habitats for amphibians in a changing climate. Omnivorous fish, such as carp, can cause an increase in water turbidity by damaging plant communities and accelerating eutrophication processes, thus compromising the habitat choice and reproduction of several amphibian species [91,92]. Predatory fish cause numerous adverse effects, such as direct predation on eggs and larvae, injuries due to predation attempts, resource competition, and changes in activity patterns and micro-habitat use [19,55,93–96]. Furthermore, both fish categories may damage native amphibian populations by pathogen transference [97].

We found paedomorphic Calabrian Alpine newts only in TRIF, and to date, paedomorphs have been reported to occur in two localities: Lago Due Uomini and Laghicello [31]. Facultative paedomorphosis is frequent in several Italian populations of *I. alpestris* [22,39,98–100] and is favoured in permanent aquatic habitats and where prey are abundant [77,99]. Evaluating the occurrence and incidence of alternative paedomorphic phenotypes should be of interest, considering the impacts of competition and predation. Indeed, it has been documented that introduced fish have determined the decline of metamorphs and the disappearance of paedomorphs from many newt sites in Europe [100]. A recent study showed several detrimental effects of the invasive *G. holbrooki* on paedomorphs of *Lissotriton graecus*, which exhibited avoidance behaviour and higher metamorphosis rates in the presence of fish [101].

The eradication or control of non-native invasive fish species in the lakes of Fagnano are undoubtedly unique and powerful conservation tools to improve the status of endemic and protected newts as well as the whole amphibian community. Given the different ecological and biological conditions of each water body, a targeted strategy combining several fish management techniques is necessary. Eradication programmes should include physical removal and chemical approaches [13,102–104] after a risk analysis to decide which strategy should be chosen and what the likelihood of success is. Considering the relatively high financial costs of eradication, the prioritisation of fish removal from ponds of higher suitability for newts could buffer the impact of fish presence [19]. Frog and salamander populations are expected to recover within a few years after fish removal from lakes [13,81,104–106]. The proximity of stable newt-breeding populations that can act as both sources and sinks may strongly favour the long-term persistence and newt recolonisation of lakes that have been returned to a fishless condition [12,19,107]. Therefore, in this emergency context, it is mandatory to create a network of small satellite artificial and semi-natural ponds, spatially connected to the historical sites, where to maintain newt populations until programmes to remove fish from lakes can be carried out. The identification of sites where to create suitable habitats should be realised through field surveys and the application of the most recent ecological modelling techniques to indicate the areas with major potential for retaining water, and also predicting changes in temperature and precipitation.

Regarding the newly discovered localities, both sites deserve regular interventions to guarantee water permanence and reduce silting phenomena by enlarging and deepen-

ing the ponds and removing excessive biomass accumulation. Furthermore, continuous surveillance is desirable to quickly notice any introduction of fish.

Finally, to deal with the 'emergency' situation, priority conservation action should be taken to preserve the Calabrian Alpine newt by implementing an *ex situ* conservation programme with research and conservation facilities in scientific institutions or zoos in Italy and Europe. The necessary *ex situ* initiatives will complement and support the *in situ* conservation of this highly endangered taxon, also considering that its disjunct distribution and its narrow range make this Alpine newt, by far, the most relevant biogeographic and conservation-related taxon compared to all of the other five *Ichthyosaura alpestris* subspecies [108]. Thus, the conservation of the Calabrian Alpine newt is not only important in terms of protecting taxonomic diversity but, being an evolutionarily significant unit, it assumes relevance for the purpose of better conserving the whole species' genetic diversity [109].

5. Conclusions

Updated distribution information and ecological data are mandatory to start a specific management plan for an endemic taxon on the brink of extinction, which is deserving of urgent conservation actions. Our observations facilitate conservation and management activities in the SAC Laghi di Fagnano and the surrounding area. The radical ecological upheaval following fish introductions undermines the survival of the Calabrian Alpine newt and the other two syntopic pond-breeding newts of community and conservation interest. An effective long-term management strategy to restore disturbed habitats by removing fish cannot be further postponed, including pond creation to provide all the amphibian species with alternative habitats. Local biodiversity and ecological functions greatly benefit from the eradication or control of non-native fish. Intensive monitoring and a systematic collection of data to ascertain more in-depth key ecological requirements and population status over a long period are also essential.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13050871/s1, Figure S1: Representative images of the new breeding sites of *Ichthyosaura alpestris inexpectata*; Table S1: Water physical–chemical parameters measured in the study ponds; Table S2: Macroinvertebrates taxonomic list and relative abundance; Table S3: Morphometric parameters of *Ichthyosaura alpestris inexpectata* from the study sites.

Author Contributions: Conceptualisation, I.B.; methodology and formal analysis, I.B., V.C., A.R., A.C. and M.I.; investigation, I.B., V.C., A.C., M.G.S. and S.T.; data curation, I.B. and V.C.; writing—original draft preparation, I.B., A.R., V.C. and M.I.; writing—review and editing, I.B., V.C., M.I., F.A., A.R., A.C., M.B. and S.T.; supervision, M.B. and S.T. All authors have read and agreed to the published version of the manuscript.

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Article

Monitoring of the Endangered Cave Salamander *Speleomantes sarrabusensis*

Roberto Cogoni [1], Milos Di Gregorio [2,*], Fabio Cianferoni [3,4] and Enrico Lunghi [5,6,7]

1 Unione Speleologica Cagliaritana, Quartu Sant'Elena, 09045 Cagliari, Italy
2 Dipartimento di Biologia, Università degli Studi di Pisa, 56126 Pisa, Italy
3 Istituto di Ricerca sugli Ecosistemi Terrestri (IRET), Consiglio Nazionale delle Ricerche (CNR), Sesto Fiorentino, 50019 Firenze, Italy
4 Museo di Storia Naturale dell'Università degli Studi di Firenze, "La Specola", 50125 Firenze, Italy
5 Dipartimento di Medicina Clinica, Sanità Pubblica, Scienze Della Vita e dell'ambiente (MeSVA), Universià degli Studi dell'Aquila, 67100 L'Aquila, Italy
6 Associazione Natural Oasis, 59100 Prato, Italy
7 Unione Speleologica Calenzano, 50041 Calenzano, Italy
* Correspondence: m.digregorio4@studenti.unipi.it

Simple Summary: Here, we provide the information derived from the first monitoring activities performed on the endangered Sette Fratelli cave salamander, *Speleomantes sarrabusensis*. Adopting two different monitoring schemes, we estimated the abundance of four populations of *S. sarrabusensis*, providing important data for future status assessments of the species.

Abstract: In this study, we performed the first monitoring activities on one of the most endangered amphibians in Europe, the Sette Fratelli cave salamander *Speleomantes sarrabusensis*. The data presented here are derived from two monitoring activities aiming to assess the status and abundance of four populations of *S. sarrabusensis*. With the first monitoring, we surveyed the well-known population occurring within artificial springs during the period 2015–2018, providing monthly data on the number of active individuals. With the second monitoring performed during spring to early summer of 2022, we surveyed four populations at three time points (the one from artificial springs and three from forested areas) and we provided the first estimation of the populations' abundance. Furthermore, we analyzed for the first time the stomach contents from a population of *S. sarrabusensis* only occurring in forested environments. With our study, we provided the first information on the abundance of different populations of *S. sarrabusensis*, representing the starting point for future status assessments for this endangered species.

Keywords: *Hydromantes*; Plethodontidae; abundance; wildlife; amphibian; conservation

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1. Introduction

The Sette Fratelli cave salamander, *Speleomantes sarrabusensis* Lanza et al., 2001 (Figure 1), is one of the European amphibian species with the smallest distribution ($\leq$70 km^2); it can be found only in the most south-eastern area of Sardinia (Italy) [1].

Before 2001, *S. sarrabusensis* was considered a sub-species of *S. imperialis* [1]. Although called "cave salamanders", all the European plethodontid salamanders of the genus *Speleomantes* are epigean species that occur in caves and other subterranean environments to avoid unsuitable climatic conditions [2,3]. However, *S. sarrabusensis* is distributed over a granitic area where no caves exist, and thus it can be found only in surface environments or within anthropic structures characterised by a suitable inner microclimate [1,4]. The very small distribution and the lack of potential subterranean refuges are within the characteristics that make *S. sarrabusensis* highly sensitive to extinction risk [5].

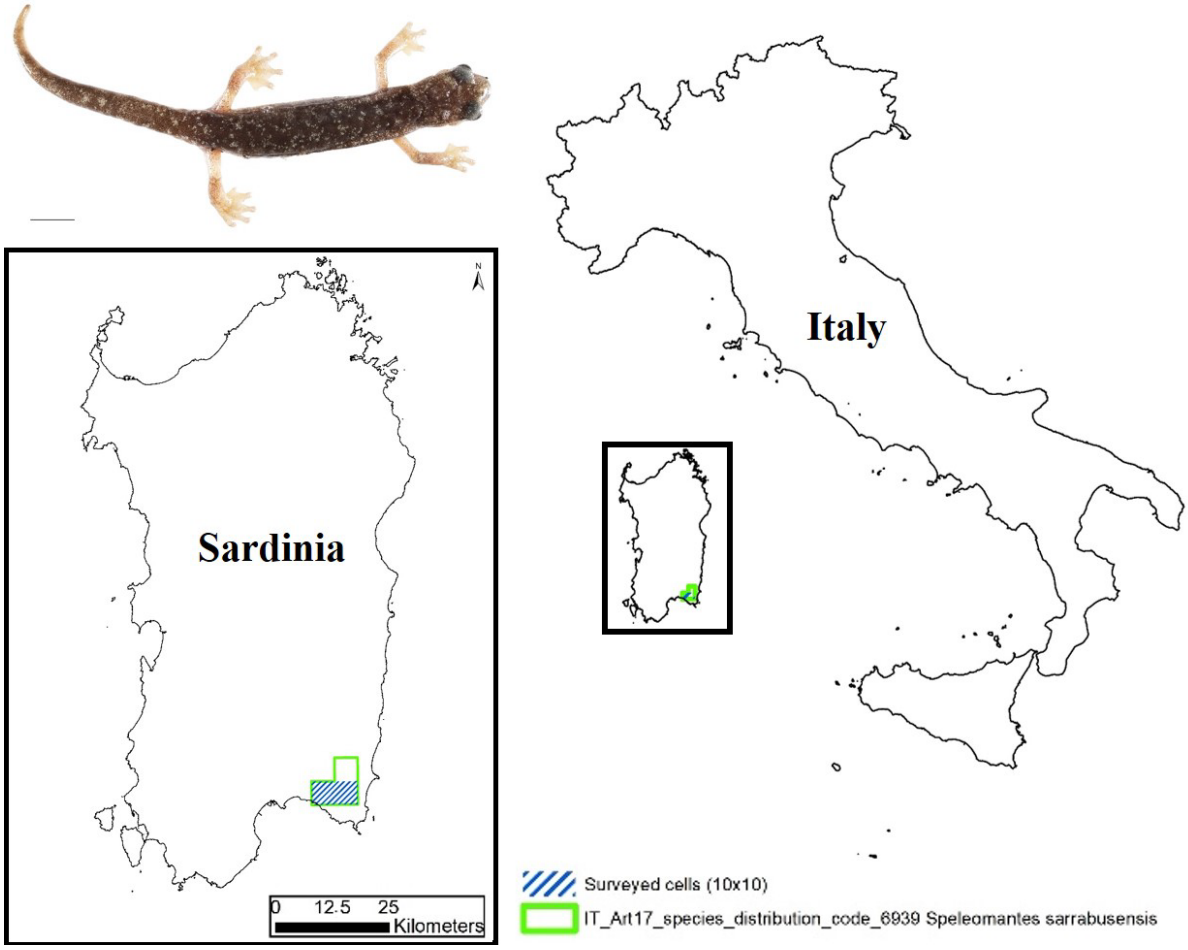

Figure 1. Map indicating the area of interest in our monitoring activity. In the upper-left corner, an adult of *Speleomantes sarrabusensis* (scale bar = 10 mm).

The Sette Fratelli cave salamander is the *Speleomantes* species for which the available knowledge is the poorest and the most anecdotal. For example, observing captive individuals, Lanza and Leo [6] hypothesised that the species might be viviparous; however, recent studies confirmed its oviparity as for the rest of the genus [7]. Most of our lack of knowledge is probably due to the difficulties in observing the species when it hides in its refuges. Subterranean *Speleomantes* usually show a high detection probability, as individuals can be easily observed clinging onto cave walls [8–10]. On the contrary, detecting *Speleomantes* in a forested area is more challenging as individuals have plenty of places to hide, and they can be confused with the background [11]. The only well-known population of *S. sarrabusensis* is the one that occurs inside the artificial springs located on the Sette Fratelli Mountain (squared structures made of concrete with water tanks that cover about 80% of the inner surface; Figure 2) [1], where salamanders cling onto the flat walls and experience microclimatic conditions that are comparable with those found in subterranean environments [4]. Consequently, our knowledge of *S. sarrabusensis* is only based on studies performed on the population from artificial springs [7,12–14].

Figure 2. (**A**,**B**) show the two artificial springs in which *Speleomantes sarrabusensis* occurs. In (**C**), it is possible to see part of the inner environment of one spring; arrows are pointing to a few individuals that are clinging on the concrete walls.

The present study provides new information on the distribution and life history of the strictly protected *S. sarrabusensis*. Specifically, we provide data that originates from two different monitoring schemes. The first was long-term monitoring which was interested in the well-known artificial springs aiming to estimate the population trend over a period of four years [15]. Although in this part of the study we provide the number of individuals observed within each spring, we should remark that the two structures are not far enough from each other to assume that they hold two independent populations [16]. In the second part, we performed repeated surveys in both the artificial springs (considered as a single population) and in three different forested areas to provide the first estimation of their abundance (i.e., the abundance of active individuals at that time). The occurrence of these three independent epigean populations was previously assessed from literature and due to novel discoveries. The need for improving the information on this poorly known species was recently boosted by a large death toll observed in the summer of 2021, where 14 individuals were found dead. We still do not know the cause of such a death toll, but this dramatic event was one of the reasons behind the change in the species conservation status to critically endangered [5].

2. Materials and Methods

The first long-term monitoring of the artificial springs (Figure 2A,B) involved monthly surveys from March 2015 until June 2018. During each survey, one operator counted the number of active individuals of *S. sarrabusensis* which are clearly visible clinging onto the flat concrete walls (Figure 2C).

At the end of the survey, at about 5 m from the entrance, air temperature and humidity were recorded at the ground level using a thermohygrometer (accuracy ± 0.5 °C, $\pm 2.5\%$). We used Linear Models (LM) [17] to evaluate the potential variation of the number of observed individuals during the period of monitoring. The number of observed individuals was used as the dependent variable, the month and the year as fixed factors, and the spring identity as a random factor. The likelihood ratio test was used to test the significance of variables.

The second monitoring occurred in 2022. Besides the well-known population from the artificial springs, we surveyed three additional epigean populations aiming to perform the first estimation of their abundance and to provide much-improved information on species

status. Two sites were characterized by forested areas mainly composed of evergreen oak *Quercus ilex* L., 1753 and the strawberry tree *Arbutus unedo* L., 1753, while one was characterized by Mediterranean scrub. The exact coordinates of the populations are not provided due to conservation concerns [18]. The three epigean populations were surveyed in March, while the one occurring inside the artificial springs was surveyed in June. These periods were chosen based on the general knowledge of *Speleomantes* phenology, where individuals are more active in surface environments during periods characterized by higher precipitation and relatively low temperatures, while during hot and dry periods the subterranean abundance should be the highest [9,11,19]; see also Table 1. Each population was surveyed three times within a period of 30 days, to guarantee population closure and to avoid strong microclimatic fluctuations [9,20]. During each survey, individuals were searched within the defined area and counted without providing any further disturbance. In each site, we selected a defined area to monitor according to the local characteristics of the territory. For the first site (forest #1) we selected a transect of 962 m, for the second site (forest #2) we selected a transect of 219 m; both transects were defined using GPS tracks. In the third site (scrub #1) we defined a plot of 320 m^2 in which we adopted a serpentine (bustrophedic) search of salamanders on the ground and under the stones, avoiding inaccessible dense bushes. The overall surveyed area of the artificial springs is 70 m^2 of building footprint (about 4 $\times$ 7.5 m the first and 4 $\times$ 10 m the second). Inside the artificial springs, a single operator searched for *Speleomantes* on the flat walls, with a constant sampling effort of 7.5 min per 3 linear meters [19]. For the epigean populations, two operators simultaneously searched for individuals on the ground (also lifting logs/stones) and on the trees [21] within the defined area. The two forested areas were surveyed with an average sampling effort of 1 h/1270 m^2 per person (1 h/1227 m^2 for the first and 1 h/1314 m^2 for the second site), while for the scrub we dedicated 1 h for the whole plot (320 m^2). During the third survey on each site, we measured temperature and humidity at the ground with a thermohygrometer (accuracy $\pm$0.5 °C, $\pm$2.5%). Estimations of the populations' abundance were performed using *N*-mixture models [22].

Considering the large number of individuals observed from site forest #1 (see Results), we selected this population to perform an analysis on the stomach contents of individuals. During the last survey, individuals were captured and then we proceeded as follows: each individual was weighed using a digital scale (0.01 g), photographed for post hoc estimation of both their snout-vent length (SVL) and total length (TL) both SVL and TL; [23,24], and stomach flushed to collect the residuals of their last foraging activity [25]. Adult salamanders showing male's sexual characteristics (mental chin, conical shape of the head) were considered males [1]. The SVL was then used to distinguish between adults females ($\geq$55 mm) and juveniles (<55 mm) [7]. Individuals were then stabled in situ inside fauna boxes; this allowed us to perform a second round of capture–photograph–stomach-flushing the following night, without the risk of recapturing the same individuals. The recognised prey items were counted and divided following Lunghi, et al. [26]. In brief, consumed prey were divided according to their taxonomic order and possibly also according to their life stage (larva vs. adult). In two circumstances, the family Staphylinidae (Coleoptera) and the family Formicidae (Hymenoptera) were considered separately. Considering the limited number of individuals and the single-season sampling, we simply describe the seasonal diet of this population, comparing this information with those available for the population from the artificial spring [26], avoiding performing weak statistical analyses. The full data related to salamanders' stomach contents is provided as supplementary material (Table S1).

3. Results

During the first long-term monitoring, we performed a total of 929 observations of *Speleomantes sarrabusensis* within the two artificial springs (737 in the first and 102 in the second). A detailed summary of the observed individuals is shown in Table 1.

Table 1. Monthly data from the long-term monitoring of *Speleomantes sarrabusensis*. In the upper part of the table, we show the monthly count performed within the two artificial springs coded following Lunghi, et al. [27]. The symbol (-) indicates that the survey has not been performed. In the lower part, we show the average monthly microclimatic conditions recorded inside the two artificial springs during the monitoring period. For each month, we show the average (±SD) temperature (°C) and relative humidity (%) recorded during the period 2015–2018. When the standard deviation is not shown, it means that the relative microclimatic variable has been recorded only once.

						Number of Observed Individuals							
Site	**Year**	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
Spring 1	2015	-	-	4	40	72	79	66	50	83	16	1	-
	2016	3	-	10	70	84	90	36	39	42	22	0	1
	2017	1	1	5	29	18	23	36	33	20	35	2	1
	2018	2	6	9	21	39	59	-	-	-	-	-	-
Spring 2	2015	-	-	0	6	10	2	5	5	7	18	3	-
	2016	3	-	8	8	5	3	3	1	1	4	5	3
	2017	2	2	8	12	6	5	6	5	3	7	5	3
	2018	3	5	6	0	8	6	-	-	-	-	-	-
						Data on the inner microclimate							
Site		January	February	March	April	May	June	July	August	September	October	November	December
Spring 1	Temperature	10.2 (±0.8)	10.3 (±0.1)	10.3 (±0.3)	12.2 (±1.3)	13.2 (±1)	14.2	16.4 (±0.8)	17.3 (±0.8)	16.5	15.2 (±0.3)	13.0 (±0.7)	9.8
	Humidity	81.2 (±2.2)	81.8 (±0.3)	82.7 (±0.9)	83.4 (±2.1)	84.1 (±3)	81.0	78.8 (±1.4)	79.7 (±2.6)	79.5	82.2 (±1)	82.4 (±1.5)	81.6
Spring 2	Temperature	10.0 (±1.3)	9.9 (±0.3)	10.9 (±0.1)	11.9 (±0.8)	12.6 (±0.7)	13.9	15.4 (±0.6)	16.0 (±0.9)	15.7	14.2 (±0.1)	12.8 (±1.3)	9.3
	Humidity	82.7 (±1)	82.6 (±1.1)	82.6 (±0.9)	84.1 (±2.8)	84.5 (±3.4)	82.6	82.2 (±0.4)	81.8 (±1)	81.6	82.9 (±1.2)	80.0 (±2.8)	82.4

The number of observed individuals was significantly affected by both the month ($F_{11,61}$ = 3.17, p = 0.002) and the year of the survey ($F_{1,61}$ = 4.72, p = 0.034). Overall, in June, the number of observed individuals was the highest, while in November, it was the lowest. The number of individuals significantly declined over the monitoring period. In Table 1 are also shown data on the monthly average (±SD) of the air temperature and humidity recorded within the two springs.

With the second part of our study, we were able to provide the first estimation of the abundance for four different populations of *S. sarrabusensis* (Table 2).

Table 2. Data related to the monitored populations of *Speleomantes sarrabusensis*. Along with general information on the location of the site, the surveyed areas, and the local microclimate, for each population we provide the number of observed individuals during each survey and the estimation of the population abundance. The elevation represents the average elevation of the monitored area. The abundance (mode) is accompanied by the confidence interval (2.5–97.5%).

Population	Latitude	Longitude	Elevation (m a.s.l.)	Surveyed Area (m^2)	Microclimate	Survey #1	Survey #2	Survey #3	Estimated Abundance	2.5%	97.5%
Springs	39°29′	9°44′	783	70	15.4 °C—79.2%	21	14	3	34	28	41
Forest #1	39°12′	9°28′	444	2454	10.3 °C—80%	10	12	28	41	35	48
Forest #2	39°14′	9°28′	569	2628	10.3 °C—80.4%	2	0	1	9	5	16
Scrub #1	39°15′	9°23′	620	320	8.7 °C—79.9%	0	2	0	9	4	15

The species detection probability was relatively low (0.328 ± 0.081 SE). For the population inhabiting the two artificial springs on Sette Fratelli mountain, we estimated 34 individuals, while the estimated abundance for the other three epigean populations was 41, 9, and 9, respectively (Table 2).

In site forest #1 we captured a total of 37 individuals (24 + 15) of which 11 were males, seven females, and 21 juveniles (Table S1). Six juveniles were too small and we did not perform stomach flushing; among the other individuals, none had an empty stomach. We recognised a total of 356 prey items belonging to 31 different prey categories; three categories accounted for more than 43% of the consumed prey (Entomobryomorpha, 25%; Araneae and Diptera both >9%) (Table S1).

4. Discussion

With this study we provided the results of the first monitoring performed on *Speleomantes sarrabusensis*. Our results provided quantitative information on the monitored populations, data that represents the starting point for future conservation assessments. During the systematic monitoring performed in this study (2015–2018), we noticed a significant decline in the number of active individuals inside the springs. This can be considered just a red flag that should stimulate further monitoring because we have no additional information that helps us in understanding the potential causes. According to the data on the microclimate recorded inside the springs throughout the monitoring period (Table 1), we can exclude a drastic change in the inner microclimatic conditions as a potential cause for the reduction of the number of active individuals [8]. Indeed, from our data we can notice a yearly natural fluctuation of the inner microclimate correlated to the external climatic conditions [9]. Furthermore, when averaging the monthly data of the inner microclimate recorded throughout the survey period, we obtain a standard deviation whose maximum reaches 1.3 °C and 3.4% for temperature and humidity, respectively, a condition that maintains the inner microclimate within the preferred range for the species [4]. It is possible that some human-induced factors negatively affected the populations causing a sensible decline, but we also cannot exclude the possibility that we simply recorded a natural contraction of the population abundance; further monitoring activities are therefore necessary to better comprehend the causes of such decline.

In the second part of this study we provided the first quantitative information on the abundance of different populations of *S. sarrabusensis*. However, we must say that the

observed individuals and the related estimation of abundance may be lower than expected. According to the studies performed on different subterranean populations of *Speleomantes*, during the period April–May, the number of active individuals was the highest [8,9,19], a condition that allows to produce estimations that are the closest to the real population size [22]. Considering the fully epigean habits of *S. sarrabusensis*, we chose to perform our surveys within the forested area in March, a month in which we expected suitable environmental conditions [8]. Unfortunately, the microclimatic conditions registered at the sites during our surveys were sub-optimal for the species [4] (Table 2), a circumstance that strongly affects the activity of individuals [8,9]. Therefore, our data should be interpreted as a snapshot taken in a sub-optimal period when the number of active individuals was very low, producing a highly underestimated estimation. Similarly, the surveys within artificial springs were performed in June, a period in which the subterranean activity of *Speleomantes* is the highest [4]. However, the extremely harsh climatic conditions occurred in that period likely induced an earlier aestivation of the population [1], allowing us to observe only a small number of active individuals, and therefore produced unexpected underestimated abundances.

The trophic spectrum of this population was larger than that of the population from artificial springs (31 vs. 19 recognized prey categories) [26], suggesting that resource availability may play an important role in defining the diet and the foraging behaviour of these generalist salamanders [13,28,29]. According to the optimal forage theory, individuals should adopt a foraging strategy that maximises their food intake with the least effort [30]. Aggregative behaviour is often adopted by prey to dilute predation risk but, at the same time, it may allow predators to easily locate a very fruitful target. Considering the > 3000 prey items recognised from the *S. sarrabusensis* population occurring in the artificial spring [26], it can be noted that about 94% of the consumed prey belongs to just two categories: Diptera (76,45%) and Coleoptera Staphylinidae (17.42%). These relatively small prey are often observed in very dense clumps inside those artificial springs, giving insights on the reasons promoting the poorly diverse diet of salamanders occurring therein [13]. Indeed, if we only consider individuals that consumed these two types of prey, we observe that the average number ($\pm$SD) of consumed prey is 20.36 $\pm$ 25.29 for Diptera and 10.62 $\pm$ 14.3 for Coleoptera Staphylinidae. On the other hand, the individuals from the epigean population studied here showed more variability in their diet (Table S1), and the most consumed prey was Entomobryomorpha, which represents the most diverse group of Collembola [31]. Although these Collembola can reach high densities [up to 1800 individuals per dm^3; 31], in our case, each individual did not consume a very high number of Entomobryomorpha (3.42 $\pm$ 2.96), meaning that these prey may be locally common but do not show particularly gregarious behaviour.

5. Conclusions

Our study provided new information on the poorly known *Speleomantes sarrabusensis* through standardized methodologies that should be systematically adopted in the future to monitor the conservation status of this endangered amphibian species. Monitoring of epigean populations of *Speleomantes* is more challenging compared to subterranean ones [9,11,19], but they also require more attention as they have fewer opportunities to oppose human-induced alterations of the environment [32,33]. Furthermore, monitoring both epigean and hypogean populations may allow us to discover potential divergences in life traits between conspecific populations [34]. In conclusion, *S. sarrabusensis* needs particular attention and we are willing to continue with our data collection to provide important information useful for the conservation of this highly endangered species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13030391/s1, Table S1: Data on the recognized stomach contents in the epigean population of *Speleomantes sarrabusensis*. The columns represent: (A) the site code; (B–D) the coordinates and elevation of the site; (E,F) Region and Province; (G,H) the month and year of the survey; (I) the sex of individuals (male, female, and juvenile); (J,K) weight and total length of

individuals; (L) stomach condition (1 = empty; 0 = full); (M) recognizable contents (yes = 0, no = 1); (N-AR) the number of recognized prey items for each prey category. NA = not available data.

Author Contributions: R.C. and E.L. conceived the study; R.C., M.D.G. and E.L. performed data collection; M.D.G. and F.C. recognized prey from stomach contents; E.L. analyzed the data and drafted the manuscript. All authors reviewed the manuscript and accepted its final form. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data for *Speleomantes sarrabusensis* stomach contents are provided as supplementary material (Table S1).

Conflicts of Interest: The authors declare no conflict of interest.

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Different Traits, Different Evolutionary Pathways: Insights from *Salamandrina* (Amphibia, Caudata)

Claudio Angelini [1,*], Francesca Antonucci [2], Jacopo Aguzzi [3] and Corrado Costa [2]

[1] Salamandrina Sezzese Search Society, Via G. Marconi 30, 04018 Sezze, Italy
[2] Consiglio per la ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Centro di Ricerca Ingegneria e Trasformazioni Agroalimentari, Via della Pascolare 16, 00015 Monterotondo, Italy
[3] Instituto de Ciencias del Mar (ICM-CSIC), Paseo Marítimo de la Barceloneta, 37–49, 08003 Barcelona, Spain
* Correspondence: oppela@gmail.com

Simple Summary: In this paper we studied the body and colour features of the endemic amphibian *Salamandrina* across its range. Using a multimodel approach and machine learning clustering, we found much difference relative to body size and shape, as well as colour, among populations and between the habitats in which the individuals dwell, while differences between the two species of *Salamandrina* genus are lower. We suppose that selective pressures are more similar across species' ranges than within them. Taking into account also previous knowledge on this genus, here we point out that different traits of an organism could result from different evolutionary routes, something not unexpected but often neglected.

Abstract: Species delimitation is often based on a single or very few genetic or phenetic traits, something which leads to misinterpretations and often does not provide information about evolutionary processes. Here, we investigated the diversity pattern of multiple phenetic traits of the two extant species of *Salamandrina*, a genus split only after molecular traits had been studied but the two species of which are phenetically very similar. The phenetic traits we studied are size, external body shape and head colour pattern, in a model comparison framework using non-linear mixed models and unsupervised and supervised clustering. Overall, we found high levels of intra-specific variability for body size and shape, depending on population belonging and habitat, while differences between species were generally lower. The habitat the salamanders dwell in also seems important for colour pattern. Basing on our findings, from the methodological point of view, we suggest (i) to take into account the variability at population level when testing for higher level variability, and (ii) a semi-supervised learning approach to high dimensional data. We also showed that different phenotypic traits of the same organism could result from different evolutionary routes. Local adaptation is likely responsible for body size and shape variability, with selective pressures more similar across species than within them. Head colour pattern also depends on habitat, differently from ventral colour pattern (not studied in this paper) which likely evolved under genetic drift.

Keywords: phenetic characters; multivariate clustering; image analysis; local adaptation; evolutionary processes

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1. Introduction

Whichever species concept is used, the taxonomic assignment of individuals to a species relies on the (combined) analysis of phenotypic and genetic characters [1,2]. Thanks to spectacular technological advancements in molecular biology, genetics has become the backbone of evolutionary studies more and more during the previous decades. However, researchers have become aware that "gene trees and species trees are not the same" [3], and multilocus approaches are advisable. Furthermore, genetic surveys require well-equipped and funding-sustained laboratories, a combination of conditions not always available. In

practice, the phenotype is largely the way by which species are still recognised, at least as the first step, and genetic studies are usually carried out for deeper and further investigation. Therefore, most field ecologists primarily rely on the phenetic species concept.

On the other hand, genetic evidence of speciation stimulates the study of differences among species, and differences or resemblances in turn stimulate evolutionary hypotheses, i.e., speciation pathways. Usually, searching for differences among species leads one to think in terms of "there is no difference" (null hypothesis, H_0) or "there is a difference" (a single alternative hypothesis, H_1), which is null hypothesis testing. However, while the difference (or resemblance) detected by hypothesis testing can be true, it is not necessarily the stronger signal the dataset bears, it could even be the weaker. In fact, any dataset brings several signals, and while it is very difficult to detect the true information (i.e., the strongest signal), it is possible to search for and likely find various signals (i.e., patterns). On the other hand, not all signals have the same strength. Instead, multimodel inference and model selection [4] allow to compare the strength of different signals, and thus each of multiple hypotheses (H_i). Thus, model selection is a natural companion for finding discrete clusters within an apparently homogenous group of organisms or for comparing the partitions within a group based on different biological rationales (species, populations, sexes, ecotypes, etc.).

In this paper, multimodel inference was applied to investigate if any phenetic trait differs between the two only extant species of the genus *Salamandrina*, the Northern Spectacled Salamander *S. perspicillata* (Savi, 1821), occurring from Liguria to northern Campania, and the Southern Spectacled *S. terdigitata* (Bonnaterre, 1789), from Campania to Calabria, in the Italian peninsula. The species are clearly separated taxa based on allozymes [5] and mitochondrial DNA [6,7]. However, nuclear DNA reveals incomplete lineage sorting and a hybrid zone [7–9]. The split between the two species happened between 11 and 2.2 mya [6,8], likely as the result of a climatic cooling trend and geographical segregation [8]. It is known that differences in colouration and morphology allow for a percentage of correct species assignment larger than 90% when using discriminant statistics [10,11]. Furthermore, it is known that salamanders' body size is affected by the type of breeding site, and it shows high intra-specific variability, at least for *S. perspicillata* [12,13].

The main goal of this study is to analyse multiple phenetic traits to evaluate if differences between the two species exist, and if differences due to the habitat are stronger than differences between species. In doing this, we also took into account the contribution to phenetic variability attributable to intra-specific differences at a population level, a factor often underestimated or even neglected when investigating differences between species, which instead turned out to be important.

2. Materials and Methods

Three populations of the Northern spectacled salamander *S. perspicillata* and two populations of the Southern spectacled salamander *S. terdigitata* were compared (Table 1). All populations inhabited mixed deciduous forests. The northern species populations were sampled in the Lepini mountains (about 800 km^2, Lazio region) as this area harbours the largest body size individuals and populations discovered in this species [12,13]. By doing so, we maximized body size differences between species, a caution which allowed us to evaluate the role of species belonging versus habitat and population belonging in shaping salamanders' bodies (in size). We are aware the study design is incomplete, because a southern pond population is missing (as a matter of fact, very few *terdigitata* populations oviposit in lentic water), however, this is not a limitation for the study, as will result from our findings. Data for both species were collected during oviposition seasons. Only data collected from females were analysed.

Table 1. Summary information about the sites of *Salamandrina perspicillata* and *S. terdigitata* used in this study; site codes are the same as in supplementary Table S1.

Species	Site (Code)	Location	Habitat Features	Sampling Year and Size
S. perspicillata	Acqua della chiesa (AC)	Lepini Mountains, Latium	Trough, 900 m a.s.l.	2007 $n = 28$
S. perspicillata	Ciccopano nuove (CN)	Lepini Mountains, Latium	Rocky spring ponds, 700 m a.s.l.	2006, 2007 $n = 19$
S. perspicillata	Sant'Angelo (SA)	Lepini Mountains, Latium	Brook, 940 m a.s.l.	2007 $n = 27$
S. terdigitata	Torrente Cerasuolo (TC)	Picentini Mountains, Campania	Brook, 750 m a.s.l.	2007 $n = 41$
S. terdigitata	Torrente Rosa (TR)	Pollino, Calabria	Brook, 550 m a.s.l.	2007 $n = 41$

2.1. Biometries

Animals were captured on sight. We measured the following body features: AG: distance between axilla and groin, CL: cloaca length, HL: head length, HW: head width, No: distance between nostrils, RU: radius-ulna (forearm) length, SVL: snout-vent length, TF: tibia-fibula (leg) length, TL: total length, tL: tail length and tW: tail width (Figure 1, Supplementary Table S1). They were measured directly on the individuals after anaesthesia (by using a 0.01% solution of MS-222-Sandoz) with a calliper (to the nearest 0.1 mm), but HL, HW and No were measured from photographs using the software tpsDig2 [14]. SVL and TL showed high collinearity with AG ($r_{Pearson} = 0.94$ and 0.9 respectively, $p < 0.001$ in both cases) and thus were not used for the main analyses. All salamanders were released at their site after they recovered from the anaesthesia.

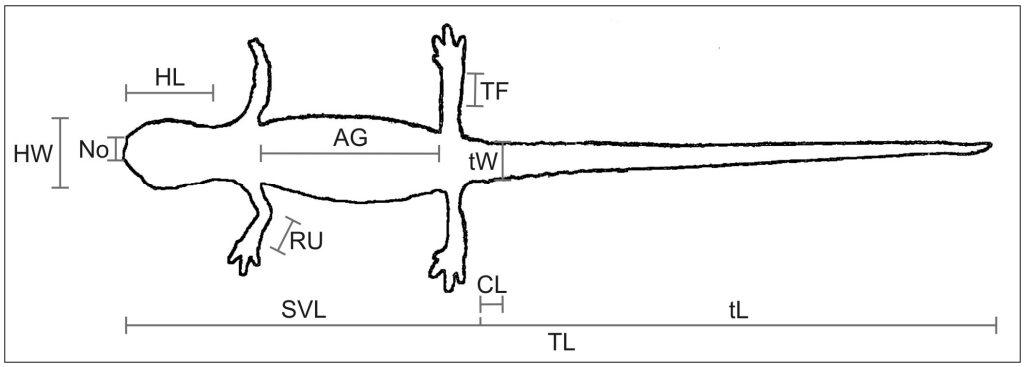

Figure 1. Biometric features of *Salamandrina perspicillata* and *S. terdigitata* used for the study: AG: distance between axilla and groin, CL: cloaca length, HL: head length, HW: head width, No: distance between nostrils, RU: radius-ulna (forearm) length, SVL: snout-vent length, TF: tibia-fibula (leg) length, TL: total length, tL: tail length, tW: tail width.

We investigated how biometric variation was related to species (*S. perspicillata* or *S. terdigitata*), type of oviposition site (brooks versus lentic water) and population belonging, by using model selection based on an information theoretic approach [15]. Namely, linear mixed-effects models were fitted to evaluate how the three factors explain size (AG) and multivariate morphological variation. Both "species" and "site typology" predictors were used as fixed factors separately and never crossed each other; "population" was always used as a random factor, either nested within "species" and "site typology" or alone. For AG we compared five models (Table 2) (since SVL and TL are often used as

proxies for body size, we also show the results of the same analysis for these features in Supplementary Tables S2 and S3). For the analysis of the multivariate morphological variation, we considered all (eight) body features, except AG, and then compared five models built as described above, plus the same five models also including AG as a covariate, as well as a further model with only AG as a continuous predictor, thus obtaining 11 candidate models in total (Table 3). Model comparisons were done using Akaike's information criterion for small sample size (AICc) and AICc weight, the likelihood that a given model is actually the best among the candidate ones [15]. Analyses were performed in the R environment [16], using the packages lme4 (version 1.1-31, [17]) for fitting the models: lmerTest (version 3.1-3, [18]) was used to obtain the (multivariate) analysis of variance [(M)ANOVA] output from the linear mixed model fitted with lme4, and MuMIn (version 1.9.13, [19]) for the computation of model weight.

Table 2. Model ranking of candidate models used to analyse the dependence of axilla-groin distance of salamanders based on the species (*Salamandrina perspicillata* or *S. terdigitata*), the type of breeding site (brook or lentic water) and their population (five populations). Species and site type were used as fixed factors, population as a random factor (when together, the random factor is nested in the fixed one). AICc is the Akaike's information criterion for small sample size, w is the model weight. *p*-value refers to the fixed factor for the given ANOVA model.

Model	AICc	W	*p*-Value
site typology:population	609.25	0.74	0.018
species:population	612.38	0.16	0.1
population	613.27	0.1	
site typology	644.94	0	<0.001
species	668.81	0	<0.001

Table 3. Model ranking of candidate models used to analyse the multivariate dependence of body features (see text for details) of salamanders based on the species (*Salamandrina perspicillata* or *S. terdigitata*), the type of breeding site (brook or lentic water), their population (five populations) and the individual axilla-groin distance (AG; "*" denote the use of AG as covariate). Species, site type and axilla-groin distance were been used as fixed factors, population as random factor (when together, the random factor is nested in the fixed one). AICc is the Akaike's information criterion for the small sample size, w is the model weight. *p*-value refers to the categorical fixed factor species or site typology for the given MAN(C)OVA model.

Model	AIC	W	*p*-Values
population*AG	4260.98	0.986	
species*AG:population	4270.87	0.008	=0.04
site typology*AG:population	4271.35	0.006	=0.04
species*AG	4448.34	0	<0.001
site typology*AG	4470.28	0	<0.001
AG	4722.57	0	
population	4851.38	0	
site typology:population	4862.03	0	=0.03
species:population	4863.34	0	=0.14
site typology	5509.29	0	<0.001
species	5718.67	0	<0.001

2.2. Head Colour Topographical Analysis

Salamandrina is characterized by a yellow V-shaped patch on the head. Analyses were based on digital photographs (1200 × 1600 pixels; 72 dpi) of the dorsal view of salamander heads. Animals were placed horizontally, as much as possible following the main body axis, the camera was parallel to the animals and a metric reference was placed aside. Then, a geometric morphometric analysis was performed on each picture [20–22]. Nine landmarks

and 24 equidistant points along the outline (semi-landmarks) [21] from the head of each salamander were digitized using the software TpsDig2 (Ver. 2.17 [14]). All landmark and semi-landmark configurations for each specimen were aligned, translated, rotated and scaled to a unit centroid size by the Generalized Procrustes Analysis (GPA [23]), using the consensus configuration of all specimens as the starting form. Residuals from the fitting were modelled with the thin-plate spline interpolating function [21–23]. The shape and head colour pattern of each individual were morphologically adjusted to a standard view by means of the consensus configuration. Before the morphometric standardization, the head colour pattern was manually segmented into pure black and white. The final grey-scale images were 2288 × 1712 pixels at 96 ppi, for an amount of 26,748 pixels constituting the head's area.

We used the information on position and colour (black or white) of every single pixel of each individual to cluster salamanders on the basis of their head colour pattern by applying both unsupervised and supervised clustering. For unsupervised clustering we compared all the models with a candidate number of clusters from two to six. Candidate supervised clustering was based on: partition from the best model from unsupervised clustering (two clusters), species (two clusters), population belonging (five clusters) and habitat (two clusters). This has been done in the R environment [16] by using the package MixAll (version 1.5.1 [24]). MixAll allows for model-based clustering and classification. Notably, we used multivariate categorical mixture models. For model selection we used the Integrated Completed Likelihood (ICL) criterion [25], a model selection criterion specifically introduced for model-based clustering. Since MixAll does not allow for reducing dimensionality and does not show the main features underlining differences, we applied the Partial Least Square Discriminant Analysis for this purpose. Details and results from this analysis are reported in the Supplementary Material S4.

3. Results

3.1. Biometries

Model selection shows that the effect of both the type of oviposition site and species on salamanders' body sizes (AG) appear evident only when accounting for intra-specific differences at the population level (Table 2). Based on models' weight, population belonging is the main determinant for body size, then it depended on site typology (salamanders from brooks are smaller than ones from lentic waters; Supplementary Table S1) and finally, there is poorly if any effect from salamander species (second best model, *S. perspicillata* are larger; supplementary Table S1). Nevertheless, the fixed factors always obtained significant *p*-values (marginally so in one case). We want also to stress that our findings are not invalidated by the absence of a *S. terdigitata* pond population from the dataset: since salamanders from ponds tended to be larger, a *terdigitata* pond population would have further made the size of *S. terdigitata* similar to *perspicillata*.

Model selection for multivariate morphological variation clearly indicated that it depended on intra-specific population variability and body size (all body features are positively correlated with AG, with r_P ranging from 0.37 to 0.84, $p < 0.001$), and the role of both species and oviposition site is poor and only detectable when accounting for body size and population variability (Table 3). At both the univariate (size) and multivariate (morphology) level, species is the predictor that performed the worst. However, fixed factors again achieved significant *p*-values, except in one case.

3.2. Head Colour Topographical Analysis

Unsupervised clustering indicates that individuals group in two clusters (ICL = 2,382,622), since the ICL from three clusters (2,393,146) onward increasingly augment. There were associations between these two clusters and habitat (chi-square $p < 0.01$) and population ($p < 0.001$), but not with species ($p = 0.28$). Supervised clustering ranked as best the partition obtained from unsupervised clustering (ICL = 5,441,850), then the partition based on site typology (Table 4).

Table 4. Model ranking of candidate models for supervised clustering. Unsupervised partition refers to the best clustering obtained from unsupervised clustering. Accuracy is the classification accuracy of the model.

Model	ΔICL	Accuracy
unsupervised partition	0	0.97
site typology	32,919	0.72
species	44,959	0.73
population	116,985	0.52

4. Discussion

Both kinds of analyses show that, even though *Salamandrina* species differ, interspecific difference is not the stronger signal the data bear. A one-way (M)ANOVA approach would have detected a significant effect of the fixed factors species and site typology on morphometry for (almost) all alternative models (Tables 2 and 3), which is hardly useful. On the other hand, testing only one hypothesis (e.g., difference between species) would have led us to neglect other, more likely, hypothesis (e.g., difference between site typology taking into account population, see Table 2). Model selection allowed us to figure out: (i) the importance of population belonging for both univariate and multivariate variability; (ii) the partial importance of the site typology for body size variation (when accounting for population belonging); (iii) the dependence of multivariate variability on population belonging and on body size (two factors that being related reinforce their signal in explaining the multivariate information).

The unsupervised analysis of the head colour pattern found two clusters. These clusters are not randomly associated with site typology and population, but reveal a further, more important, even though not interpretable, pattern. Apart from these clusters, supervised clustering ranked as the best the partition between site typologies, showing that local factors are determinants also for the shape of head spots. Despite the very high difference among the information criterion values, the accuracies of the classifications are rather good for site typology, species and population (taking into account that the probability of random assignment among populations is 20%). Again, also for the head colour pattern, as for body shape and size, testing only one model could have led us to a spurious conclusion, as well as testing more models without an estimator of their relative quality would have been uninformative (see Supplementary Material S4). However, it depends on the objective of the investigation: if it is finding a way to distinguish groups, supervised learning based on only one hypothesis can be useful. For example, when applied to *Salamandrina*, it actually allows for a percentage of correct species assignment larger than 90% [10,11]. However, if the issue is to find the most relevant biological pattern, model selection among unsupervised learning and/or supervised models should be the choice.

The problem of statistically approaching unknown classification patterns is emerging with the evolution of machine learning approaches; consequently, clear protocols on whether using supervised or unsupervised techniques are not yet clearly established [26]. Unsupervised learning methods significantly outperform the supervised ones when the number of clusters [27] or the observation attributions [28] to classes are unknown or unclear. On the other hand, supervised methods could produce a more informative output, e.g., finding out the latent dimensions responsible for a given clustering or identifying the main features underlining differences. The combination of supervised and unsupervised approaches is called semi-supervised learning and offers a promising direction of future research [26,29]. We suggest as an optimal workflow, especially when dealing with high multidimensional data, such as data from image analysis: first to use unsupervised modelling, then compare hypotheses by supervising learning and finally, apply to the best clustering a statistical tool which reduces dimensionality and shows the features responsible for differences among clusters. As an example, in the supplementary material (S4) we show the implementation of Partial Least Square Discriminant Analysis on our models.

Apart from methodological hints, our analyses yielded also some biological findings. It was already known that *S. terdigitata* is smaller than the northern species [10] as well as that the typology of the oviposition site is even more important in determining the body size [13]. We add to this information the key role played by population belonging in determining both size and shape of salamanders. Despite the fact we opportunistically introduced a bias in the analysis by sampling the largest body size populations of *S. perspicillata*, we found a high level of biometric and morphometric diversity within both *Salamandrina* species compared to diversity between them. This confirms that the selective pressures acting on these traits are more similar across ranges than at a local scale. The split between the two species happened in an allopatric speciation scenario [8], which is expected to cause slow diversification [30–32]. In fact, if the physical barrier does not produce any ecological difference between the new-species' ranges, the species continue to share the same kind of environment, they will still be under the same selective pressures, and traits under such pressures will differ little between species. In this framework, local pressures could become the main ones. Since the *perspicillata* populations we studied are closely related (same mitochondrial haplogroup and microsatellite cluster [8]) and are no more than 4 km away from each other, we are prone to attribute population differences to very local factors (i.e., excluding climate). Based on our results, we suppose that local topography is a candidate to take into consideration. This is in accordance with what was previously suggested, that salamanders from brook populations experience a lower survival rate due to floods, thus leading to a smaller body size [12]. However, lower survival could cause smaller body size just as a demographic by-product (younger individuals are smaller) and/or by adaptative reaction leading to earlier sexual maturity [33] and thus smaller adult size ([34] and reference therein). It is known that *Salamandrina* shows high phenotypic plasticity, with close populations having different oviposition periods [35,36], however, only knowledge on individual ages can disentangle the role of demography and reaction norms on body size and shape variability, as well as the validation of our hypothesis requires studies involving (local) environmental factors.

The V-shaped patch on the head depends mostly on the type of oviposition site, little if any on species and even less on population. It is difficult to explain this finding, because no hypothesis exists about the role of the yellow head patch (we could just speculate it is a disruptive or distractive colouration). Differently than the head yellow patch, the extension of red colouration on the tail and the ventral colour pattern proved to efficiently discriminate between *Salamandrina* species [10,11]. The discriminatory performances of such colour features suggest that they are not subjected to the same selective pressures as the morphometric and colouration traits we analysed in the current study. Likely, the anterior part of the ventral colouration is involved in intra-specific communication [11,37–39], as well as the tail [40]. The speciation scenarios suggested for *Salamandrina* [8,9] involve the reduction of population size in separated refugial areas, from which both species colonized the extant ranges. In particular, both studies pointed out that the *perspicillata* lineage survived in a single refugial area. In the small, ancestral population, the importance of genetic drift was likely higher than natural selection. For any colour pattern involved in intraspecific communication, the random process would have been positively reinforced by the efficacy of signalling [41], thus leading to fixation of colour differences between the two *Salamandrina* species. Something that did not happen for the head V-patch, or that has been blurred later by any selective pressures.

5. Conclusions

Our findings confirm that within-species variability is not negligible when studying phenetic and functional differences between species, and that such variability can even challenge the discrimination between species [42,43]; thus, taking into account differentiation at population level is an important caution. We also showed how different phenotypic traits of the same organism could follow different evolutionary routes. On one hand, genetic drift probably led to strong differentiation of some, but not all, colour patterns between *Salamandrina* species. On the other hand, natural selection seems to act on size and shape

of individuals in similar ways across the genus' range, but depending on local factors, it produces ecotypes that differ at least as much as the species and are shared between them. Focussing the analysis only on species discrimination would have hidden more important differences within a genus, as well as intriguing insights.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12233326/s1, Table S1: Data summary for the measured body features; Table S2: Snout-vent length model comparison; Table S3: Total length model comparison; Supplementary material S4: Example of second-step further analysis. References [44–51] are cited in the supplementary materials.

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The Trophic Niche of Two Sympatric Species of Salamanders (Plethodontidae and Salamandridae) from Italy

Enrico Lunghi [1,2,3,4,*], Claudia Corti [5], Marta Biaggini [5], Yahui Zhao [1,*] and Fabio Cianferoni [5,6]

[1] Key Laboratory of the Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[2] Division of Molecular Biology, Institut Ruđer Bošković, 10000 Zagreb, Croatia
[3] Natural Oasis, 59100 Prato, Italy
[4] Unione Speleologica Calenzano, 50041 Florence, Italy
[5] Museo di Storia Naturale dell'Università degli Studi di Firenze, Museo "La Specola", 50125 Florence, Italy
[6] Istituto di Ricerca sugli Ecosistemi Terrestri (IRET), Consiglio Nazionale delle Ricerche (CNR), 50019 Florence, Italy
* Correspondence: enrico.arti@gmail.com (E.L.); zhaoyh@ioz.ac.cn (Y.Z.)

Simple Summary: Studies on species' trophic niches are essential to understand the characteristics of species' ecology and life traits, as well as to improve conservation strategies. In the absence of competitors, species realize their trophic niche including in their diet the most profitable food resources. In the presence of competitors, species modify their preferences to reduce competition and maintain the highest benefits at the same time. In this study, we assessed the trophic niche of two species of salamanders coexisting in a forested area of Italy and evaluated which might be the mechanisms that these two species adopted to reduce competition. We found that the Italian cave salamander (*Speleomantes italicus*) mostly consumed flying prey with a hard cuticle, while the fire salamander (*Salamandra salamandra*) preferred worm-like and soft-bodied prey. In conclusion, we hypothesize that in our case, the two species of salamanders did not have to change their prey preference in order to avoid competition, but divergences in metabolism and behavioral traits likely worked as natural deterrent.

Abstract: The trophic niche of a species is one of the fundamental traits of species biology. The ideal trophic niche of a species is realized in the absence of interspecific competition, targeting the most profitable and easy-to-handle food resources. However, when a competitor is present, species adopt different strategies to reduce competition and promote coexistence. In this study, we assessed the potential mechanisms that allow the coexistence of two generalist salamanders: the Italian cave salamander (*Speleomantes italicus*) and the fire salamander (*Salamandra salamandra*). We surveyed, in April 2021, a forested area of Emilia-Romagna (Italy) during rainy nights. Analyzing the stomach contents of the captured individuals, we obtained information on the trophic niche of these two sympatric populations. Comparing our results with those of previous studies, we found that the two species did not modify their trophic niche, but that alternative mechanisms allowed their coexistence. Specifically, different prey preferences and predator metabolisms were likely the major factors allowing reduced competition between these two generalist predators.

Keywords: *Speleomantes*; *Hydromantes*; *Salamandra*; diet; forest; competition; prey selection

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1. Introduction

The trophic niche is one of the fundamental traits of species biology [1]. The study of the trophic niche provides important information on multiple species traits such as behavior (e.g., foraging strategy and prey selection), physiology (e.g., specific nutritional requirements and metabolism), and its trophic position in the local community [2–5]. Nonetheless, this type of study may be pivotal to implement conservation strategies for

species that need particular protection [6]. In the absence of heterospecific competitors, a species tends to develop its trophic niche by targeting the most profitable resources in terms of nutritional intake and handling ability [4,7]. Under this condition, the overall trophic spectrum of the population can mirror the shared preference of individuals (when they exploit the same typologies of food resources), or it can result from the combination of different preferences when intraspecific competition forces individuals to target only a subset of the food resources consumed by the entire population [8,9]. When co-occurring species compete for the same food resources, their realized trophic niche usually differs (at least for the weaker competitor) from the ideal one, as individuals have to switch to alternative resources to reduce the competition and be able to coexist [10,11].

We here assessed for the first time the trophic niche of two sympatric generalist salamanders: the Italian cave salamander *Speleomantes italicus* and the fire salamander *Salamandra salamandra*. The trophic niche of these two species has only been assessed in the absence of potential competitors e.g. [12–14], leaving unknown the mechanisms and the extent to which these species are able to modify their trophic niche to coexist with competitors. *Speleomantes italicus* is one of the eight species of Plethodontidae occurring in Europe, and its distribution encompasses the Apennine chain, from northern Tuscany to Abruzzo (Italy), where it occurs in forested areas and subterranean environments as well [15,16]. *Speleomantes* are lungless salamanders that require specific microhabitat conditions (i.e., high humidity and relatively low temperature) to maintain the high efficiency of cutaneous respiration [17,18]. *Speleomantes* are fully terrestrial amphibians that live and reproduce exclusively in subaerial environments [15]. Courtship can occur all year round, while gravid females lay their eggs twice per year (beginning of spring or autumn) in hidden places where they provide prolonged parental care ($\geq$4 months) until the hatchlings are ready to leave the nest [19–22]. The narrow microhabitat requirements and the *k*-selected reproductive strategy of these species make them deserving of special protection [18,23,24]. Although being epigean species, *Speleomantes* gained the vernacular name of "cave salamanders" because they can be easily observed in natural and artificial subterranean environments [15,25]. These species are therefore able to prey in both surface and subterranean environments [26–29]. The trophic niche of *S. italicus* was only studied in subterranean populations [12]. Researchers have observed high variability in the consumed prey among populations, with a clear predominance of Diptera, one of the most abundant prey in subterranean environments [30,31].

Salamandra salamandra is the most widespread species of Salamandridae in Europe, ranging from the Iberian Peninsula to the western part of Ukraine, including the Balkans and all central European countries [32]. Despite being a widely spread Urodela, the fire salamander recently faced a huge decline in some parts of its distribution due to infection with the fungus *Batrachochytrium salamandrivorans* [33]. The fire salamander is a typical biphasic amphibian that has aquatic larvae and terrestrial adults [34]. Courtship occurs from spring to autumn, and females usually maintain the fertilized eggs in their bodies until the following spring. When eggs are ready, the female enters into a body of water to release the newly hatched larvae, which need at least 6 months to metamorphose into the adult form [34]. Although *S. salamandra* usually reproduces in surface water, several cases of reproduction in subterranean environments are also known [35,36]. The larvae feed upon multiple aquatic invertebrate species, but they can also adopt cannibalism when prey are scarce [14,37,38]. Adults forage in terrestrial environments and they often prey upon "worm-like" prey such as annelids, diplopods (among arthropods), and "slugs", defined as apparently shell-less terrestrial gastropods (among mollusks) [13,39,40].

The present study aimed to assess whether *S. italicus* and *S. salamandra* may be potential competitors when they occur in sympatry and to determine the mechanisms preventing the competition. In order to coexist, species tend to reduce the competition by targeting different types of prey [41,42]; therefore, we expected a little overlap in the realized trophic niche of these populations when occurring in sympatry. In addition, we evaluated potential divergences in foraging behavior among conspecific individuals.

2. Materials and Methods

We carried out surveys in a forested area of the northern Apennines in the province of Bologna (Emilia-Romagna, Italy); precise information on the site is omitted to ensure species protection [43]. The surveys (six in total) were carried out in April on rainy nights (7 p.m.–2 a.m.) with random frequency, but with at least a one-day interval between two surveys. We surveyed an area of about 4000 m^2 in search of individuals of *Speleomantes italicus* and *Salamandra salamandra* on the ground, on trees, and on dry stone walls [44]. The captured salamanders were photographed using a portable photo-studio [45]. We estimated the snout-vent length (SVL, in mm) of salamanders from the images using ImageJ software [46–48]. We focused only on SVL as this measure provides more accurate information on both the age and size of the salamanders [23], as the tail can be lost (and possibly regenerate) due to predation events [49]. The images were also used to individually recognize the salamanders of both species by observing their dorsal pattern [50,51]; for *S. italicus*, an additional marking method was also employed (i.e., visual implant elastomers) [52]. Individuals of both species were sexed based on their size and the presence of distinctive sexual characters. For *S. italicus*, we used the size of 50 mm (SVL) as a threshold to distinguish adults ($\geq$) and juveniles (<) [23]. Among adults, male recognition was based on the presence of their typical secondary sexual characters (i.e., mental gland, prominent pre-maxillary teeth, and conical shape of the head) [15]; all adult individuals lacking these traits were considered females. In *S. salamandra*, we used the size of 90 mm (SVL) to distinguish between adults ($\geq$) and juveniles (<). Among adults, those with swelling at the base of the cloaca were considered to be males [49]. We weighed all the salamanders using a digital scale (accuracy 0.01 g) and then inspected their stomach residues by stomach flushing, a harmless technique that allows to obtain information on the individual's latest foraging activity [26,53]. Prey residues were recognized at the order level, and each order represents a single prey category [26]. In some cases, further distinctions were also made at the family level, or between larval stages, i.e., when these groups are morphologically distinct and characterized by different ecology (e.g., aquatic vs. subaerial) (see Table 1). Each of these groups was considered an independent category. For additional information on the method, see [26,44].

We used the analysis of similarity (ANOSIM with 10,000 permutations) to assess whether the similarity in the trophic niche between the two populations was higher than that occurring within each population [54,55]. Furthermore, we tested whether the two populations diverged in terms of multivariate dispersion of their diet (*betadispr* function, 999 permutations) [56]. We used PERMANOVA to assess potential interspecific differences in diet composition [57,58]. The nonmetric multidimensional scaling (NMDS) plot with Euclidean distance was used to show the trophic niche differences between the two populations of salamanders. Although the dataset contained data on individuals captured multiple times (see Results), we decided to maintain all the data in this analysis to have a more complete information on the species' overall trophic niche.

Generalized linear mixed models (GLMMs) [59,60] were used to assess whether differences between sex and ontogenetic stage in terms of the number and diversity of prey consumed existed. In the first GLMM, the log-transformed number of consumed prey by each individual was used as the dependent variable, while individual SVL and sex/stage (male, female, or juvenile; hereafter only referred as "sex" for the sake of brevity) were used as independent variables; the day of the survey was used as the random factor. In the second GLMM, we used the Shannon index of the prey consumed by each individual as the dependent variable; all the other variables remained the same. The analysis of the two GLMMs were separately performed for *S. italicus* and *S. salamandra*. To avoid bias due to pseudoreplication, we purged the datasets used in GLMM analyses removing the data on recaptured individuals and maintaining only the event in which the number of recognized prey was the largest. We decided not to analyze individuals with empty stomachs or with unrecognized prey, as they represented a very small percentage of the overall pool of individuals (about 6% for *S. italicus* and 3% for *S. salamandra*; see Results).

Table 1. List of the prey items found in the stomach contents of *Speleomantes italicus* and *Salamandra salamandra*. To each group of prey, we assigned a code (first column), which we used in the NMDS plot to increase its clarity. In brackets is the relative importance (%) of each group of prey within the trophic niche of each species.

Prey Code	Prey Order	Number Recognized in *Speleomantes italicus* and Relative Importance (%)	Number Recognized in *Salamandra salamandra* and Relative Importance (%)
A	Pulmonata	54 (1.86)	59 (35.12)
B	Sarcoptiformes	78 (2.69)	0
C	Mesostigmata	15 (0.52)	0
S	Trombidiformes	7 (0.24)	0
E	Araneae	359 (12.38)	9 (5.36)
F	Pseudoscorpiones	125 (4.31)	0
G	Opiliones	30 (1.03)	8 (4.76)
H	Lithobiomorpha	22 (0.76)	2 (1.19)
I	Geophilomorpha	14 (0.48)	0
J	Scolopendromorpha	5 (0.17)	0
K	Julida	16 (0.55)	7 (4.17)
L	Polydesmida	92 (3.17)	13 (7.74)
M	Isopoda	81 (2.79)	2 (1.19)
N	Symphypleona	11 (0.38)	0
O	Poduromorpha	35 (1.21)	0
P	Entomobryomorpha	288 (9.93)	0
Q	Blattodea	4 (0.14)	0
R	Hemiptera	186 (6.41)	0
S	Hymenoptera	22 (0.76)	0
T	Hymenoptera-Formicidae	121 (4.17)	1 (0.6)
U	Coleoptera	275 (9.48)	2 (1.19)
V	Coleoptera-Staphylinidae	82 (2.83)	0
W	Coleoptera-larvae	35 (1.21)	4 (2.38)
X	Trichoptera-larvae	3 (0.10)	0
Y	Plecoptera	179 (6.17)	3 (1.79)
Z	Lepidoptera	1 (0.03)	1 (0.6)
AA	Lepidoptera-larvae	24 (0.83)	0
AB	Diptera	582 (20.07)	10 (5.95)
AC	Diptera-larvae	109 (3.76)	23 (13.69)
AD	Archaeognatha	10 (0.34)	0
AE	Speleomantes-skin	5 (0.17)	0
AF	Haplotaxida	22 (0.76)	24 (14.29)
AG	Siphonaptera	3 (0.10)	0
AH	Dermaptera	4 (0.14)	0
AI	Ixodida	1 (0.03)	0

Finally, we estimated the degree of individual diet specialization that occurred in these two sympatric populations [61]. For each species, we calculated the index of individual specialization (IS) as shown in [62,63]. We focused only on IS, as this index is significantly correlated to the other diet specialization niche metrics [64]. To improve the clarity, we used the index V =1—IS proposed in Bolnick, et al. [65], where values tending to 1 indicate a high degree of individual diet specialization, while values tending to 0 indicate that the population is mostly made up of generalists [62]. Bootstrapping (repeated 9999 times) was used to test whether the observed index of individual diet specialization significantly diverged from the simulated one, i.e., a scenario in which all individuals randomly choose their prey. In this analysis, we further purged the dataset used in GLMM, removing individuals from which we recognized <3 prey items; this was a precaution to not overinflate the individual diet specialization index.

The data on *S. italicus* used here were retrieved from [44], while the data on *S. salamandra* are provided as Supplementary Materials (Table S1).

3. Results

We captured and inspected the stomach contents from 315 *Speleomantes italicus* (128 females, 146 males, and 41 juveniles) (average captured individuals ± SD per night; 52 ± 20.5) and 32 *Salamandra salamandra* (22 females, 9 males, and 1 juvenile) (5 ± 6). Individuals of *S. italicus* were captured during all six surveys, while individuals of *S. salamandra* were only captured in three. Some of them (30 *S. italicus* and 4 *S. salamandra*) were recaptured several times. In the stomachs of most of the captured salamanders, we found residuals of consumed prey; only 18 *S. italicus* had an empty stomach. In two *S. italicus* and one *S. salamandra*, the stomach contents were in an advanced state of digestion, so we were unable to recognize the prey consumed at the established taxonomic level. We recognized 2,900 prey items from *S. italicus* (average ± SD per individual; 9.83 ± 6.56) and 168 from *S. salamandra* (5.42 ± 3.08), belonging to 35 groups of prey (Table 1).

The trophic niche of *S. italicus* included all the prey categories described in Table 1, where just four (Araneae, Entomobryomorpha, Coleoptera, and Diptera) accounted for 51.86% of the consumed prey. The trophic niche of *S. salamandra* included only 15 of the prey categories, with 3 of them (Gastropoda, Diptera-larvae, and Haplotaxida) accounting for 63.1% of the consumed prey. The analysis of similarity identified a significant divergence between the trophic niche of *S. italicus* and that of *S. salamandra* (R = 0.476, $p = 0.001$) (Figure 1A); the diet of the two populations showed a significant heterogeneity of multivariate dispersion (permutation test: $p = 0.001$). The analysis of PERMANOVA confirmed the divergence of the trophic niche between the two populations ($R^2 = 0.07$, $p = 0.001$). The trophic niche of *S. italicus* was much larger than that of *S. salamandra* (Figure 1B).

In *S. italicus*, neither the SVL ($F_{1,262.13} = 1.13$, $p = 0.289$) nor the sex ($F_{2,260.75} = 1.35$, $p = 0.261$) significantly affected the number of prey consumed. Similar results were obtained for the diversity of prey consumed (SVL: $F_{1,261.43} = 0.1$, $p = 0.748$; sex: $F_{2,260.19} = 0.95$, $p = 0.387$). In *S. salamandra*, none of the variables affected the number (SVL: $F_{1,22.52} = 0.03$, $p = 0.863$; sex: $F_{2,22.99} = 0.6$, $p = 0.558$) or diversity of the prey consumed (SVL: $F_{1,23} = 0.26$, $p = 0.612$; sex: $F_{2,23} = 0.07$, $p = 0.933$). In *S. italicus*, we found a significantly high proportion of specialized individuals (V = 0.621, $p < 0.001$), while in *S. salamandra*, we observed a similar proportion of both generalist and specialist individuals (V = 0.506, $p = 0.019$).

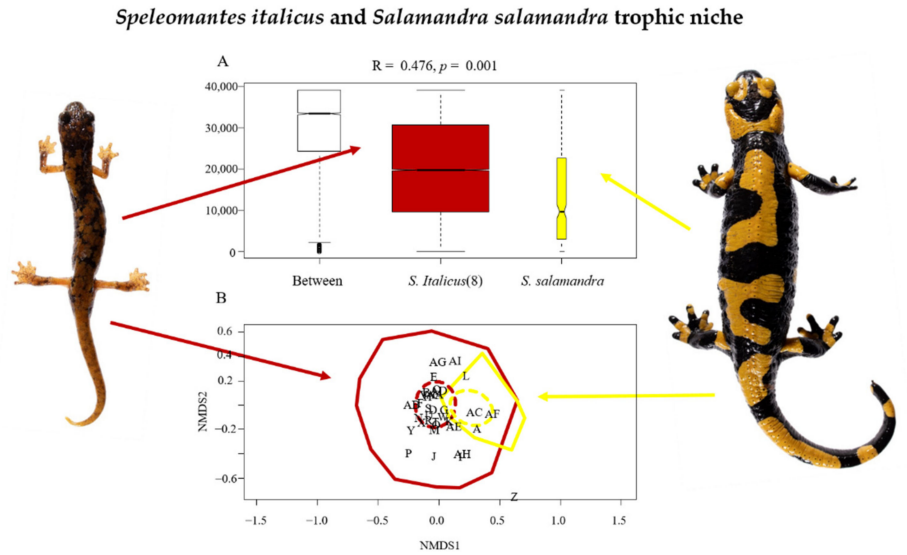

Figure 1. (**A**) Box whisker plot of ANOSIM analysis comparing the diets of *Speleomantes italicus* and *Salamandra salamandra*. Boxes indicate values from 25th (bottom) to 75th (top) percentile; horizontal black line indicates the median; box width is proportional to sample size. (**B**) Cumulative NMDS and dashed 95% confidence ellipses with relative position of each species. NMDS plot needs to be carefully interpreted due to the unbalanced datasets (stress = 0.26). The number (8) indicates the population code for *S. italicus*, which is aligned with that in [44,66]. The two sample animals (*S. italicus* on the left and *S. salamandra* on the right) are not to scale.

4. Discussion

The trophic niche of the plethodontid salamander, *Speleomantes italicus*, was the widest, including in its diet all the prey categories recognized in this study (Table 1, Figure 1B). These results are in line with what has been observed in other populations of *S. italicus* and for the entire genus, as *Speleomantes* generally show a wide variability in the prey consumed regardless of sex and age [12,26–28,44,67]. In this epigean population, most of the prey consumed are flying prey (about 43%), similar to what has been observed in subterranean populations of *Speleomantes* [4,35,68]. It may be possible that *Speleomantes*' protrusible tongue increases their ability to capture flying prey, a skill that has proven to be extremely useful when individuals cling to vertical surfaces [69–71]. Conversely, *Speleomantes* are not very attracted by slow wormlike prey [71,72], so the poor representation of these types of prey in their diet may be the result of individual choice [8,64]. Indeed, the overall worm-like prey consumed by this population of *S. italicus* did not even cover 14% of its diet (Table 1). Soft-bodied prey was usually underrepresented in previous studies on *Speleomantes*' diet, as this type of prey is likely rapidly digested without a trace [12,26,27,66]. In the studied epigean population, this type of prey (i.e., Pulmonata and Haplotaxida) represented approximately 2.5% of the prey consumed (Table 1). Notably, not only snails were consumed in this population, but also slugs, a type of prey never reported for *Speleomantes* before. It could be possible that, in our case, individuals underwent stomach flushing shortly after ingesting the prey, without having completed their digestion. The stomach contents of the subterranean *Speleomantes* populations were usually obtained during the day (9 a.m.–6 p.m.) [26,44]. *Speleomantes* come out of their subterranean shelter to feed on the surface, especially on cold and humid nights [15,73]; therefore, checking the stomach contents the day after foraging may be too late to obtain information on prey being quickly digested. No significant effect on the number of consumed prey was found in this population of *S. italicus*. In a previous study, it was observed that juvenile *Speleomantes* from subterranean populations consumed significantly less prey than adults [4]. In this study, we surveyed a fully epigean population, meaning that individuals only forage in the surface environment, where the diversity and availability of prey are remarkably different

from that found in subterranean environments [74]. A large number of prey consumed in this study were Collembola (Table 1), animals of millimetric size that can be eaten in large number also by juveniles. These prey were widely underrepresented in the stomach contents from subterranean populations [4], despite their steady presence in subterranean environments [75]. This divergence could be due to a variability in the abundance of Collembola between subterranean and surface environments rather than different prey preferences between juveniles inhabiting different environments. This hypothesis remains to be tested.

The trophic niche of *Salamandra salamandra* was narrower, as it included less than half of the overall prey consumed by *S. italicus* (Table 1, Figure 1B). *Salamandra salamandra* behaved exactly the opposite from *Speleomantes*, preferring mostly slow-moving worm-like prey [40,76]. This type of prey consumed by *S. salamandra* accounted for 79% of its diet (Table 1), corroborating similar results obtained from different populations throughout its distribution [13,39,53,77,78]. Among the stomach contents recognized from *S. salamandra*, we observed a very low frequency of prey with hard cuticles, and generally they were <1 cm in size (Table 1). In a recent study, it was shown that soft-bodied prey can be underrepresented when analyzing the species' diets through stomach flushing [13]. In our study, we observed that most (>63%) of the prey consumed by *S. salamandra* exclusively consisted of soft-bodied prey (Table 1), which means that these taxa are not necessarily underrepresented in this type of study. As we discussed for *S. italicus* (see above), this inconsistency may have been due to the delay between the foraging event and the inspection of salamanders' stomachs. In their study, Marques, Mata and Velo-Antón [13] investigated the trophic niche of *S. salamandra*, analyzing their feces. This means that the prey had undergone the entire digestive process and that the salamanders had assimilated most of the wet mass by defecating only the solid residues that they had not been able to digest. In this circumstance, it is understandable how soft-bodied prey can only be detected through DNA analysis.

Our hypothesis in which we predicted limited overlap of the trophic niche between the two syntopic species was supported by our results, as only a small portion of the 95% CI of the species trophic spectrum overlaps (Figure 1B). The divergence in prey preference, as well as their size, may play a fundamental role in reducing the competition between these two species, and those preferences may be related to different energy requirements. Lungless and relatively small salamanders such as *S. italicus* do not need a large energy supply because they have a low-energy and efficient metabolism, while larger salamanders with lungs require much more energy to maintain their relatively higher metabolism [79,80]. Soft-bodied prey is mostly composed of moist mass that can be fully digested, providing much more energy (kilojoules) than those that have hard parts that cannot be digested [79]. This may be one of the reasons why *S. salamandra* more frequently consumes soft-bodied prey. This also supports the hypothesis that *S. italicus* rather chooses to not prey on this type of prey, as it does not seem that the surveyed area was characterized by a particularly low abundance of worm-like prey. Although we lack specific data to show, Pulmonata and Haplotaxida consumed by *S. salamandra* had an impressive size, certainly unsuitable for *S. italicus*. A study focusing on six species of *Speleomantes* showed that the size of prey consumed can vary between species and between individuals of different sizes [4]. Therefore, although the overall categories of prey consumed by *S. salamandra* can also be consumed by *S. italicus* (Figure 1B), prey size can be a powerful mechanism to reduce competition. This is a hypothesis we would like to test in the near future.

In our study the analyzed dataset was quite unbalanced; the data for *Speleomantes italicus* was almost 10 times higher than that of *Salamandra salamandra*. This asymmetry may have affected the robustness of some analyses, so we recommend a careful interpretation of our results.

The two species also diverged in terms of proportion of specialized individuals: the population of *S. italicus* had a higher proportion of specialized individuals, while in *S. salamandra* the proportion of both generalists and specialist individuals were similar. Con-

sidering the co-occurrence of these two populations, we can exclude the potential effects of the environment on the frequency of specialized individuals [64]. Alternatively, intraspecific competition may play a major role here [8]. In our study, the observed density of *S. italicus* (i.e., the overall captured individuals) was about 0.07 individuals/m^2, while for *S. salamandra*, it was ten folds lower (0.007 individuals/m^2). Previous studies on *Speleomantes* did not provide straightforward information about the potential occurrence of intraspecific competition or the related effects on the diet specialization of individuals [64,81–83]. To the best of our knowledge, we are not aware of similar studies performed on adult individuals of *S. salamandra*. Therefore, this remains an open hypothesis that deserves to be tested.

5. Conclusions

Our study provides indications on the potential mechanisms used by two co-occurring generalist salamanders to avoid competition. In our case, the two generalist salamanders, although able to target similar prey, mostly chose different prey types, and when they consumed similar prey, they did target prey of different size. *Speleomantes italicus* mostly consumed flying prey with hard cuticle, while *Salamandra salamandra* did the opposite, more frequently consuming worm-like soft-bodied prey. The prey consumed by both species mostly differed in size, making morphological constraints a potential tool useful for reducing competition. In conclusion, morphological constraints, together with other characteristics such as species metabolism (i.e., digestion ability) and prey preference, might play an important role in reducing the competition between sympatric generalist predators.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12172221/s1, Table S1: Dataset used for the analysis on *Salamandra salamandra* trophic niche. The dataset contains: the site in which the study was performed together with low-resolution coordinates and elevation (m a.s.l); the region and province in which the population was located; the population code; the date of the survey; the unique individual code (Tag), together with sex and snout-vent length (SVL, in mm); the stomach condition (1 = empty; 0 = full); Not_identifiable indicates whether the prey were recognized (0) or not (1) at order level; the abundance of each prey type recognized from stomach contents.

Author Contributions: E.L. conceived the study, analyzed the data, prepared the figure and drafted the manuscript. E.L., C.C. and M.B. performed data collection. C.C. contributed in data interpretation. E.L. and F.C. recognized prey from stomach contents. E.L., C.C., M.B., Y.Z. and F.C. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data for *Speleomantes italicus* were retrieved from [44], while data for *Salamandra salamandra* are provided as Supplementary Materials (Table S1).

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A New Disease Caused by an Unidentified Etiological Agent Affects European Salamanders

Raoul Manenti [1,*], Silvia Mercurio [1,*], Andrea Melotto [2], Benedetta Barzaghi [1], Sara Epis [3], Marco Tecilla [3], Roberta Pennati [1], Giorgio Ulisse Scarì [3] and Gentile Francesco Ficetola [1,4,5]

[1] Department of Environmental Science and Policy, University of Milano, 20133 Milano, Italy; benedetta.barzaghi@unimi.it (B.B.); roberta.pennati@unimi.it (R.P.); francesco.ficetola@gmail.com (G.F.F.)
[2] Centre of Excellence for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch 7602, South Africa; mel8@hotmail.it
[3] Department of Biosciences, University of Milano, 20133 Milano, Italy; sara.epis@unimi.it (S.E.); marco.tecilla@gmail.com (M.T.); giorgio.scari@unimi.it (G.U.S.)
[4] Laboratoire d'Ecologie Alpine (LECA), University Grenoble Alpes, CNRS, 38400 Grenoble, France
[5] Laboratoire d'Ecologie Alpine (LECA), University Savoie Mont Blanc, CNRS, 38400 Grenoble, France
* Correspondence: raoul.manenti@unimi.it (R.M.); silivia.mercurio@unimi.it (S.M.); Tel.: +39-3490733107 (R.M.)

Simple Summary: In the last few years, multiple new infectious diseases have affected amphibians, causing unprecedented declines and extinctions at the global scale. Many of these diseases are caused by pathogens that have been described during recent decades. In this study we report a novel disease, affecting European amphibians. In a protected area of Northern Italy, since the autumn of 2013, we started to find some adult salamanders with cysts at the throat level (subgular region). Following this observation, we ran a regular monitoring of the salamander population and performed multiple morphological and molecular investigations to identify the cause of this undescribed disease. The cysts surround peculiar cells, probably protists, of about 30 µm covered by numerous motile cilia/undulipodia. Despite multiple attempts with a broad spectrum of techniques, the detailed identification remains challenging as we have been unable to match its features with previously described organisms. We provide the results achieved till now to promote a rapid dissemination on this new enigmatic wildlife pathogen and to create a basis for further and deeper studies.

Abstract: New pathologies are causing dramatic declines and extinctions of multiple amphibian species. In 2013, in one fire salamander population of Northern Italy, we found individuals with undescribed cysts at the throat level, a malady whose existence has not previously been reported in amphibians. With the aim of describing this novel disease, we performed repeated field surveys to assess the frequency of affected salamanders from 2014 to 2020, and integrated morphological, histological, and molecular analyses to identify the pathogen. The novel disease affected up to 22% of salamanders of the study population and started spreading to nearby populations. Cysts are formed by mucus surrounding protist-like cells about 30 µm long, characterized by numerous cilia/undulipodia. Morphological and genetic analyses did not yield a clear match with described organisms. The existence of this pathogen calls for the implementation of biosecurity protocols and more studies on the dynamics of transmission and the impact on wild populations.

Keywords: protist; Mesomycetozoea; pathogenic; amphibians; fire salamander; *Salamandra salamandra*

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1. Introduction

Epigraph

"Но как же это так? Ведь это же чудовищно! Это чудовищно, господа", -повторил он, обращаясь к жабам в террарии, но жабы спали и ничего ему не ответили." ("But how can it be? It's monstrous! Quite monstrous, gentlemen,"

he repeated, addressing the toads in the terrarium, who were asleep and made no reply). M. Bulgakov, Роковые яйца

The emergence and spreading of novel diseases in wildlife is one of the most challenging threats to both biodiversity and human well-being [1–4]. Emerging wildlife diseases can quickly have profound broad-scale impacts, including ecological disturbance, economic and agricultural impacts, and even human losses [4,5]. The rapid detection of novel wildlife diseases is thus crucial, and often requires an interdisciplinary approach, including for instance morphological and genetic investigations, ecological assessments, and management consultations [5–7]. However, when diseases emerge in wildlife organisms, their detection is not easy and some pathogens may remain unnoticed till their spreading becomes difficult to control [8,9]. This could be particularly true for elusive or poorly studied animals, as 'non-mammals' vertebrates, and when pathologies are strongly different from the already known diseases.

Amphibians provide a clear example of the damage that the increasing spread of novel diseases may play over short periods to biodiversity at a global scale. In the last decades, recently discovered pathogens have caused dramatic declines and extinctions of populations and even species of amphibians [10,11]. The most studied pathologies threatening amphibians are chytridiomycoses caused by the fungi *Batrachochytrium salamandrivorans* and *Batrachochytrium dendrobatidis* [12–14], which are involved in the decline of >500 amphibian species and determine the greatest loss of wildlife biodiversity linked to a pathogen [13]. Amphibians are also susceptible to viruses such as *Ranavirus* sp. [15], which is the main infectious pathogen of multiple aquatic ectothermic vertebrates and is often implicated in mass die-offs of amphibians [16,17], even causing 100% mortality in tadpoles of some anuran species [18,19]. Moreover, amphibians also host understudied pathogens such as the fungal-like protists Mesomycetozoea [20–23]. Their biological features are typical of pathogens able of determining emerging infectious diseases in different amphibians as in the case of the genus *Amphibiocistydium* [24,25], but information on these organisms and their impacts remains scanty.

With this paper, we want to bring to the attention of the world's scientific community a novel disease affecting a widespread European amphibian, the fire salamander (*Salamandra salamandra* Linnaeus, 1758). In 2013, two individuals with an odd cyst at the level of the throat (Figure 1) were discovered in a small, protected area of Northern Italy. During following surveys, we observed similar cysts in multiple individuals. Therefore, we directed great effort to identify the cause of the cysts and to understand the extent of their spreading. In particular, we monitored the occurrence of the cysts during the following years and performed detailed morphological, ultrastructural and molecular analyses in order to characterize their content. We believe this information must be disseminated across scientists and managers before this phenomenon strikes other amphibian populations.

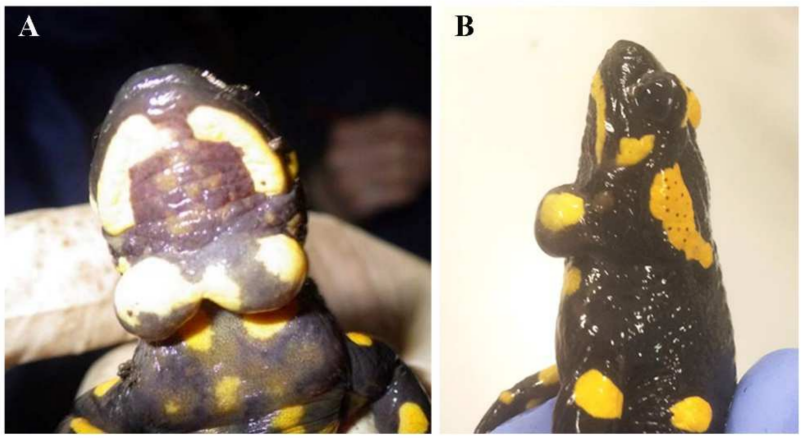

Figure 1. Salamander cysts. Ventral (**A**) and lateral (**B**) view of a male *Salamandra salamandra* with two turgid cysts occurring in a median position at the throat level.

2. Materials and Methods

2.1. The Fire Salamander: Study Area and Monitoring

The fire salamander is ovoviviparous and widespread in Europe; this species mainly inhabits hilly landscapes covered by broadleaf forests and breeds in streams and other freshwater sites [26]. When adult, fire salamanders show strong terrestrial habits, with dispersal abilities range from 200 to 1300 m (but generally up to 500 m), and nocturnal behaviour [27]. Although widespread, the species has recently suffered catastrophic declines in the northern part of its range because of the chytrid fungus *Batrachochytrium salamandrivorans* [14]. Our study focuses on the regional protected reserve named "Fontana del Guercio" located around 30 km North of the city of Milan (NW Italy) and that is recorded as a European Site of Community Interest. The area is characterized by an extended broadleaf wood crossed by a slow flowing stream and numerous springs where the fire salamander breeds. This area has undergone annual monitoring since 2010. Since 2014, after the observation of the first two individuals bearing the subgular cysts, till 2020, we established yearly surveys with three transects covering the main area of the reserve (Figure 2). From 2016, to monitor the spreading of the pathogen occurrence in the surroundings, we also sampled three additional transects in a nearby (1.6 km distant) wooded area (named "Olgelasca") occurring in a parallel valley and connected only through some wooded stretches Transects are on average ± SE 462.7 ± 95.7 m long and 18.7 ± 2.6 m wide; they follow the main paths and cover all the terrestrial habitats surrounding multiple breeding sites of the fire salamander. We performed at least three-night surveys in each autumn season (October–December) for each transect. On each survey, we collected all the salamanders we encountered; after recording the position with a GPS, we checked each individual for the occurrence of the pathogen, and then we photographed on millimetre paper and weighed it, before releasing it in the same place of collection.

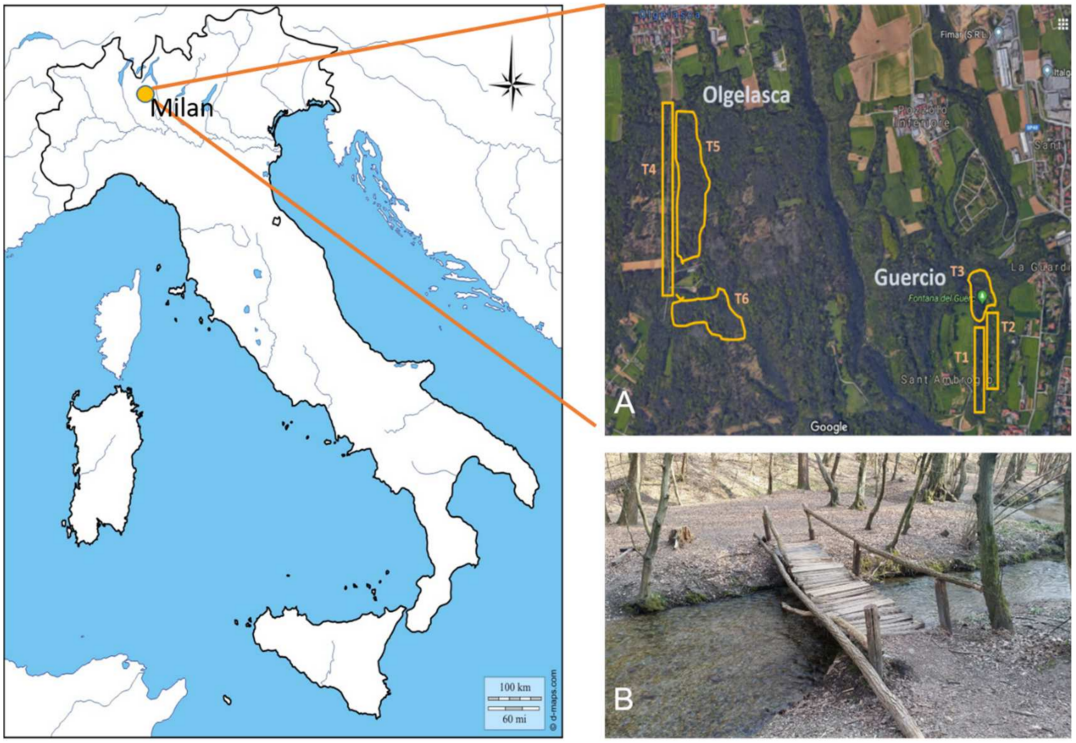

Figure 2. Location of the study area. (**A**) location and area of the transects monitored, identified by the letter T; "Guercio" identifies the main locality on the regional protected area named "Riserva del Guercio" in which the pathogen was first detected. "Olgelasca" identifies the adjacent locality in which the pathogen appeared in 2016. (**B**) picture of the protected area between transect 1 and transect 2.

The occurrence of the cyst was detected by examining the throat of the individuals. If at least one cyst was visible, we considered the salamander as affected; we considered swellings larger than 2 mm of thickness, paying attention to the fact that the throat of the fire salamanders often shows small plicas that can resemble small bumps. In cases where a second swelling occurred, we recorded it. To prevent spreading the disease during surveys we handled the salamanders with nitrile gloves that we changed for each individual; each salamander was placed for weighing and picturing on a disposable bag that was then immediately disposed of. At the end of each survey, all the material used, including shoes, was disinfected with 10% bleach.

2.2. Cyst Extraction

To understand the origin of the cyst under the skin, a few infected animals were collected during sampling and we extracted cyst samples in vivo from salamanders' throat using a non-invasive surgical protocol. Prior to surgery, the animals were anesthetized by a subcutaneous injection of tricaine/carbocaine 4 mg/mL (0.32 mL/g of weight). Loss of superficial reflexes was tested prior to the incision. The samples were removed by using a 0.3 cm, sterile, disposable biopsy punch with plugger (Bioseb lab, Vitrolles, France) and immediately processed for subsequent analyses (see below). After complete remission, usually occurring within 24 h in ozonized water, the salamanders were reintroduced in their collection site. In total we extracted samples of 15 cysts from 8 individuals from 2014 to 2018.

2.3. Cyst Cell Isolation

Under aseptic conditions, two cysts were surgically removed and immediately processed for in vitro analysis. Samples were dissected into pieces using sterile fine-tipped tweezers and disaggregated in sterile amphibian Ringer's solution. Then, cells were centrifuged at low speed (1200 rpm/9 g) and resuspended in Medium 199 with 10% foetal bovine serum and 50 mg/L gentamicin (Sigma, Igea Marina RN, Italy). Cells were cultured in petri dishes at 12 °C for 20 days. All cultures were observed daily using an inverted phase contrast microscope. Replacement of 50% of the culture medium was carried out every day.

2.4. Histology

Three cysts were processed for light microscopy analysis. Samples were immediately fixed with Bouin's solution (picric acid, formaldehyde, acetic acid, 75:25:5) for at least 24 h and rinsed several times in tap water until all the fixative solution was completely removed. Samples were then dehydrated with an ethanol series (70%, 90%, 95% and 100%), cleared in xylene and left overnight in a solution of xylene and paraffin wax 56–58 °C (1:1). Samples were then immersed in three changes of paraffin wax and finally embedded. Sections 5–7 µm thick were cut with a standard microtome and stained with Haematoxylin and Eosin (HE).

2.5. Electron Microscopy

To better describe cyst structure and the cells trapped inside, electron microscopy was also performed. Samples were pre-fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for two hours and, after overnight washing in the same buffer, post-fixed with 1% solution of OsO4 in 0.1 M cacodylate buffer. After standard dehydration in ethanol series (25%, 70%, 90%, and 100%), samples were washed in propylene oxide and embedded in Epon-Araldite 812 resin (Bio Optica, Milan, Italy). Semi-thin (about 0.9 µm) and ultra-thin (70 nm) sections were cut with a Reichert–Jung ULTRACUT E using glass knives. Semi-thin sections were stained with crystal violet and basic fuchsine, mounted with Eukitt (Bio Optica, Milan, Italy) and observed under a Leica light microscope. Ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate for electron microscopy, then observed and photographed in a Talos L120C TEM microscope.

2.6. Scanning Electron Microscopy

Cyst samples were immediately fixed in 1% glutaraldehyde and 0.4% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, 300–400 mOsm) for 30 min at 4 °C. Then, samples were washed 3 times in 0.1 M sodium cacodylate buffer over 5 min and post fixed with 1% OsO4 in 0.1 M sodium cacodylate buffer for 30 min. Cysts were washed again 3 times, dehydrated in ethanol series (50%, 70%, 80%, 90%, 96%, and absolute alcohol), and critical point dried [CPD] (Balzers CPD 030 Critical Point Dryer; Bal-Tec AG, Balzers, Liechtenstein) in carbon dioxide. Dried samples were mounted on stabs with carbon adhesive discs, then Au sputtered using a Bal-Tec SCD 050 Sputter Coater (Bal-Tec AG, Balzers, Liechtenstein), and examined with a scanning electron microscope (LEO-1430).

2.7. Genetic Analyses

Standard protocol for genomic DNA extraction with proteinase K from whole cyst samples was performed with some modifications. Briefly, overnight proteinase K digestion was preceded with triple liquid nitrogen/water bath in 100 °C for 2 min to guarantee effective genomic extraction [28]. Based on literature we selected different primers for DNA amplification. A pair of universal non-metazoan primers, 18S-EUK581-F (5′-GTGCCAGCAGCCGCG-3′) and 18S-EUK1134-R (5′-TTTAARKTTCAGCGCTTGSG-3′), were chosen to amplify a 544 bp fragment of 18S rDNA from protists without getting animal DNA [29]. Two protozoa-specific forward primers (P-SSU-342f and PR900f) in combination with a protozoa-specific reverse primer (PR900r) or an eukarya-specific reverse primer were used for targeting 18S rDNA gene. Primers sequences were: PR900f (5′-TTTCGATGGTAGATTGGAC-3′), PR900r (5′-CTTGTTACGACTTCTCCTTCC-3′) amplifying a 900 bp fragment [30,31]); P-SSU-342f (5′-CTTTCGATGGTAGTGTATTGGACTAC-3′) and Medlin B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) amplifying a 1360 bp fragment [32].

A second attempt to identify the host trapped in the salamander's cyst consisted of extracting DNA directly from the cells isolated for in vitro analysis. We selected the ciliated cells occurring in the cyst using 20 µL of TE Buffer (Tris-EDTA, 100× Solution, pH 8.0) plus 3.5 µL of liquid cystic (about 10–20 cells). We performed DNA extraction using a Proteinase K protocol. All the reagents were sterile. 2 µL of PK 20 mg/mL were added to the sample mixing the solution very well. Samples were incubated in the Thermomixer at 56 °C for two hours, then at 95 °C for 5 min and finally centrifuged at 22 °C for 10 min at 20,000× *g*. The small subunit ribosomal RNA (SSrRNA) genes were amplified with the universal eukaryotic primers forward 18S F9 [59-CTG GTT GATCCT GCC AG-39] [33] and reverse 18SR1513 Hypo [59-TGA TCC TTC (CT)GC AGG TTC-39] [34]. PCR products were purified and directly sequenced in both directions. To minimize the possibility of amplification errors, high-fidelity Taq was used. The internal primers used for sequencing were 18S R536 [5′-CTGGAATTACCGCGGCTG-3′] and 18S R1052 [5′-AACTAAGAACGGCCATGCA-3′].

Finally, we used a metabarcoding approach, trying to target the largest number of eukaryotes. The DNA extracted with proteinase K was amplified using the Euka02 primers (Forward: TTTGTCTGSTTAATTSCG; Reverse: CACAGACCTGTTATTGC) which are highly generalist primers that amplify most of eukaryotes with limited bias [35,36]. These primers amplify a short (~120 bp) region of the 18S rDNA (V7), and are thus suitable for the analysis of degraded or poor quality DNA [36,37]. DNA was amplified following the same protocol of [37] and sequenced using an Illumina Hiseq 2000 platform (see [37] for complete details on sequencing and bioinformatics processing). The detected sequences were taxonomically assigned using the Ecotag program of the OBITOOLS package, on the basis of NCBI database [38]. We analysed two DNA aliquots, each with four PCR replicates. We also ran 9 PCR controls, each replicated four times, including PCR mix but no template DNA to identify environmental contaminants [39]. To avoid the risk of false positives we only considered taxa detected in >50% of PCR replicates with >10 reads [40].

2.8. Ethics

The study design, the samplings and the surgery on the fire salamanders was approved by the ethical committee of the Lombardy Region Authority and was authorized as complying with the regional law 10/2008, permission number: T1.2016.0052349. After the removal of the cysts and recovery, each individual was released in the exact place of its collection.

3. Results

3.1. Cysts Occurrence in Fire Salamander Populations

The cyst's occurrence was first detected in 2013 in two males out of 66 salamanders observed. One of the males showed two turgid swellings occurring in a median position at the throat level, one left and one right separated by less than a millimetre (Figure 1); the cyst diameter was 10 and 8 mm respectively. The cyst size varied among individuals, while its position was always similar. We did not detect signs of external damage or injuries at the throat level, but on the cysts some small points (2 and 1 respectively) characterized by very thin skin stratus and appearing of a light black colour, were visible. The number of individuals detected per sampling in Fontana del Guercio locality, varied from five in a sampling time in 2017 to 202 during a sampling period in 2018 (Figure S1). From 2014 to 2020 the proportion of affected individuals was on average ($\pm$SE) 18.5% $\pm$ 8.1% per year. Both the proportion of individuals displaying the pathogen and the total number of adult salamanders detected along the transects varied across the years of monitoring (Figure S2); on average, in the Guercio protected area, considering the different samplings and transects, the largest proportion of affected salamanders was observed during 2015. In most cases, salamanders showed one single cyst, but 12.09 $\pm$ 6.08% of the affected individuals per year showed two of them.

In 2016, we observed for the first time, an affected adult in the transect performed in the second locality. Here, since 2016 the percentage of affected salamanders has been on average 1.18 $\pm$ 0.4%.

3.2. Histological Analysis

Macroscopically, cyst masses were spherical, located on the ventral surface of the jugular region, frequently bilateral, and, once removed, ranged between 0.2 cm and 0.4 cm in size (Figure 1A,B). They were histologically composed of a thin capsule encircling a central area of mucus material admixed with numerous granulocytes (morphologically compatible with heterophils), and a lesser number of plasma cells and lymphocytes (Figure 3D,E). Embedded in the mucus, numerous ciliated protists were detected (Figure 3F). A final diagnosis of a heterophilic cyst with intralesional microorganisms was made.

3.3. In Vitro Isolation

In cyst cell cultures, different cellular phenotypes were observed. Most of them were recognized as salamander leukocytes, mainly represented by granulocytes, among which peculiar cells were always present (Figure 3A). These latter appeared as spherical protist-like cells of about 30 µm in total length. Their cytoplast was generally heterogeneous and the cell membrane was covered by numerous motile cilia/undulipodia (Figure 3B,C).

3.4. Electron Microscopy

Electron microscopy analyses provided a detailed description of the protist-like cells in the salamander cysts. They appeared as unicellular organisms with a diameter of 10 µm, characterized by numerous undulipodia (Figure 4A,F,G). These structures protruded from the cell body (Figure 4B–D) and completely covered the cellular membrane (Figure 4H). Undulipodia were as long as the cell body, about 10 µm; together, the cell body and the undulipodia constituted what appeared as an unicellular organism of 30 µm in total length (Figure 4A,H). The cytoplasm was rich in electron dense and transparent inclusions (Figure 4A). At higher magnification, it was possible to appreciate the exogenous materials

contained in the vesicles (Figure 4B). Mitochondria with peculiar cristae were also observed (Figure 4E).

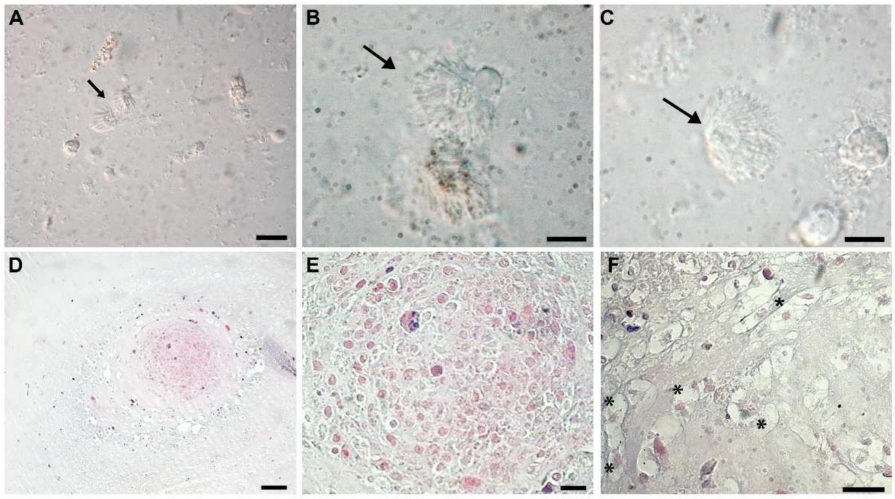

Figure 3. Morphological analyses. (**A**–**C**) Cyst cell culture in which peculiar ciliated cells are observable (arrow). These protist-like cells displayed heterogeneous cytoplast and their cell membrane was covered by numerous motile cilium-like structures (**B**,**C**). (**D**–**F**) Histological analysis revealed that the cyst masses consisted of a thin capsule of connective tissue encircling a central area of mucus material in which numerous granulocytes, plasma cells, and lymphocytes were detected (**D**,**E**). Among these, ciliated protist-like cells were present trapped in the mucus (**F**); asterisks identify trapped cells. Scale bars: (**A**) 20 μm; (**B**,**C**) 10 μm; (**D**) 250 μm; (**E**,**F**) 50 μm.

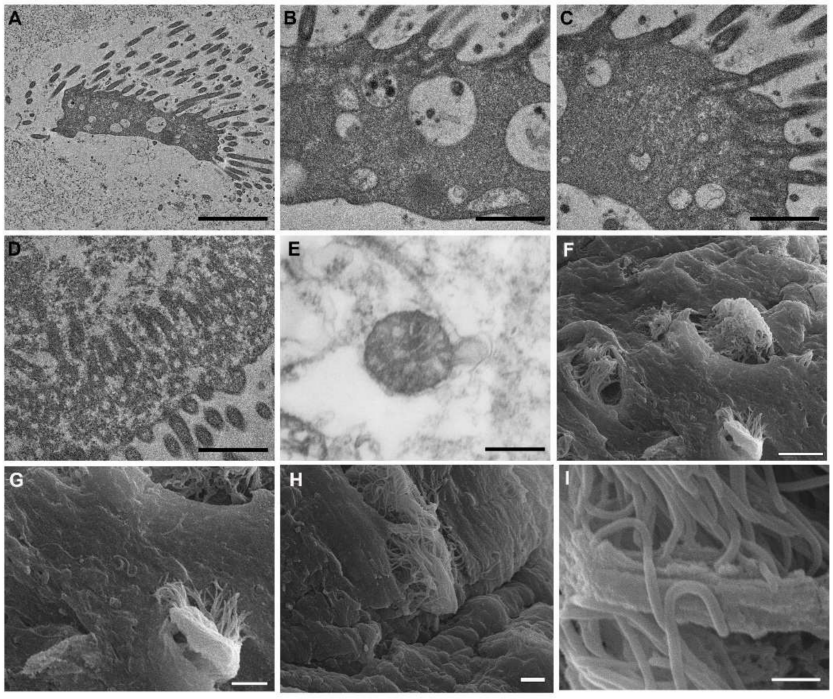

Figure 4. Electron microscopy analyses. (**A**–**E**) Transmission electron microscopy of the pathogen. In cyst mucus, numerous protist-like cells (**A**) with long undulipodia were found. At higher magnification (**B**), electron dense and transparent vacuoles were observed as well as undulipodium rootlets regularly distributed along cytoplasm periphery (**C**,**D**). Mitochondria with peculiar cristae were detected (**E**). (**F**–**I**) Scanning electron microscopy. Trapped inside mucus niches, protist-like cells were observed (**F**,**G**). Their main feature was the numerous and long cilia/undulipodia which covered the cell membrane (**H**,**I**). Scale bars: (**A**) 2 μm; (**B**–**D**) 1 μm; (**E**) 500 nm; (**F**) 10 μm; (**G**) 5 μm; (**H**) 2 μm; (**I**) 1 μm.

3.5. DNA Analyses

None of the several DNA amplifications and sequencing attempts clarified the identity of the organisms. Using genomic DNA extracted from whole cyst samples as a template, no rDNA fragment was amplified employing either protists primers [29] or protozoan-specific ones [30–32]. DNA amplification from in vitro cell isolates was also not successful. Sequencing analysis of the only amplified DNA fragment revealed the presence of fungi belonging to the genus *Cladosporium*, which was probably due to contamination. Using metabarcoding, we detected only one molecular taxonomic unit in >50% of PCR replicates (sequence: ctcaaacttccatctactaaacgtagatagtccctctaagaagccaaaaaagccaaccaaagtcgacccggctatttagcaggttaaggtctcgttcgttat). The Ecotag program assigned this sequence to the fungal genus *Meira* (Brachybasidiaceae). However, this fungus was also detected in 25% of controls, suggesting that it is a contaminant.

4. Discussion

The combination of extensive field surveys with molecular and morphological analyses allowed us to detail a new disease affecting one of the most widespread amphibians in Europe. Morphological analyses showed that the turgescence was composed mainly by mucus encapsulating numerous protist-like cells, which appeared to be embedded in small niches among the tissue. The main feature of these cells was the presence of long peculiar cilia similar to undulipodia covering the cell membrane. In the cyst, numerous leukocytes of the salamander were also recognizable, suggesting an active immune response. In vitro studies confirmed the morphology of the cells and provided more information about their cilia/undulipodia which were motile. Electron microscopy further characterized the cells. Their heterogeneous cytoplasm rich in digestive vacuoles strongly suggested phagocytosis activity. However, a definitive diagnosis remained elusive because morphological and ultrastructural data did not allow identification of the cells encapsulated by the cysts, as we were unable to match the morphology of these cells with a known pathogen.

Amphibians are affected by a large spectrum of parasites and pathologies [11,41,42] and are parasitized by multiple protists [41]. Initially we supposed a similarity between the encapsulated microorganisms and some mesomycetozoan stages [23] that parasitize fish; however, they had a different position and shape of their flagella [23]. This similarity led us to perform molecular investigations following [29], which reported for the first time the occurrence of *Amphibiocystidium* sp. in the red-spotted newts. Despite some similarities with *Amphibiocystidium*, we failed to detect its DNA and, even though we attempted multiple PCR primers and techniques, we only amplified the DNA of contaminants. The mucus of cysts could inhibit DNA extraction or amplification by reducing their efficiency [43,44], still a modification of the DNA extraction protocol according to [28] did not provide significant improvements. The absence of DNA amplification could be because, even if the cysts may attain conspicuous sizes, the encapsulated cells were quite sparse, possibly limiting the amount of DNA. Furthermore, the amplification success of primers can be highly heterogeneous across taxa [37]. Even if we employed both specific and universal primer combinations, it is possible that the potential pathogen has mismatches in the priming region that hamper amplification. In principle, the location of the cysts could also be suggestive of internal disorders linked to production of salamander cells with abnormal morphologies. Nevertheless, we can consider neoplastic formations as unlikely because the encapsulated cells are completely different from described amphibian cells and the encapsulation reaction clearly seems to underline an immune reaction toward an external agent. Ciliated amphibian cells have been recorded only in the gills of some urodeles, but they show very different shapes and features compared to the detected ones [45,46].

5. Conclusions

Despite the numerous morphological, ultrastructural, and molecular analyses, we failed to unambiguously identify the cause of the disease. The significant frequency of

salamanders showing cysts and the features of the cells encapsulated by those cysts, suggest that this disease could be caused by a slow growing and enigmatic wildlife pathogen.

Our study suggests that this disease could be caused by a pathogen that might be an unidentified protist; if dispersal by infected salamanders is likely to become a threat for the surrounding populations, the occurrence of other possible vectors should not be excluded. Waterways are considered highly suitable for the survival and spread of several pathogens [47]: a stream flows down from the Guercio protected area and connects with a complex hydrographic network in the basin of the river Lambro. Moreover, the Guercio protected area has numerous visitors each year, represented mainly by local people but also including schools and even people from abroad. Efforts will thus be necessary to make all the stakeholders of the area, from wildlife managers to visitors, aware of the presence of the phenomenon and of the caution measures to adopt to avoid its spreading. Moreover, we hope the findings of our study stimulate further studies, both on the biology of the new discovered cysts and on the occurrence of other potential animals affected, from fishes to other amphibian species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12060696/s1, Figure S1: Box-plot of the total number of adult fire salamanders recorded each year in the protected area "Fontana del Guercio". In some years, only a single survey was performed. Figure S2: Frequency of salamanders affected by cysts in the study populations through time. Error bars are Jeffrey's Bayesian 95% confidence intervals, computed using the binom package in R.

Author Contributions: R.M., G.F.F., G.U.S. and R.P. conceived the study; A.M., B.B., G.F.F., G.U.S., R.P. and R.M. performed field samplings; G.U.S. performed surgery on salamanders; G.U.S., S.M. and M.T. performed in vitro isolation and histology; G.U.S. and S.M. performed electron microscopy; G.F.F. and S.E. performed genetic analyses; A.M. and G.F.F. performed statistical analyses; R.M. wrote the first draft. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki; the study design, the samplings and the surgery of fire salamanders were approved by the ethical committee of the LOMBARDY REGION AUTHORITY and was authorized as complying with the regional law 10/2008, permission number: T1.2016.0052349. Each salamander after the removal of the cyst was released in the exact place of finding.

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Temperature and Diet Acclimation Modify the Acute Thermal Performance of the Largest Extant Amphibian

Chun-Lin Zhao [1,2,†], Tian Zhao [2,†], Jian-Yi Feng [2], Li-Ming Chang [2], Pu-Yang Zheng [2], Shi-Jian Fu [3], Xiu-Ming Li [3], Bi-Song Yue [1,*], Jian-Ping Jiang [2] and Wei Zhu [2,*]

1 Key Laboratory of Bioresources and Ecoenvironment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu 610064, China; zhaocl_1@163.com
2 CAS Key Laboratory of Mountain Ecological Restoration and Bioresource Utilization & Ecological Restoration Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chengdu 610041, China; zhaotian@cib.ac.cn (T.Z.); fengjy@cib.ac.cn (J.-Y.F.); changliming16@mails.ucas.ac.cn (L.-M.C.); zhengpy@cib.ac.cn (P.-Y.Z.); jiangjp@cib.ac.cn (J.-P.J.)
3 Laboratory of Evolutionary Physiology and Behavior, Chongqing Key Laboratory of Animal Biology, Chongqing Normal University, Chongqing 400047, China; shijianfu9@hotmail.com (S.-J.F.); xiumingli418@hotmail.com (X.-M.L.)
* Correspondence: bsyue@scu.edu.cn (B.-S.Y.); zhuwei@cib.ac.cn (W.Z.); Tel.: +86-028-82890935 (B.-S.Y.)
† These authors contributed equally to this work.

Simple Summary: The Chinese giant salamander (*Andrias davidianus*) is one of the largest extant amphibian species, and it is considered critically endangered by the IUCN Red List. Previous studies have demonstrated that future climate change could strongly affect this species. However, how to conduct the related conservation activities are still unclear. Understanding the thermal physiology of *A. davidianus* is meaningful in guiding its conservation, e.g., habitat selection and preadaptation before population translocation. In this study, the influences of temperature and diet on the metabolic capacity and thermal limits were studied for *A. davidianus* larvae based on laboratory experiments. Our results indicated prominent physiological plasticity in the thermal tolerance of *A. davidianus* in response to temperature and diet changes. This thermal plasticity likely, to some extent, buffers the effects of climate change on the Chinese giant salamander. In addition, the potential mechanisms underlying this plasticity were discussed. Our results provide insights for the formulation of conservation strategies for this species.

Abstract: The Chinese giant salamander (*Andrias davidianus*), one of the largest extant amphibian species, has dramatically declined in the wild. As an ectotherm, it may be further threatened by climate change. Therefore, understanding the thermal physiology of this species should be the priority to formulate related conservation strategies. In this study, the plasticity in metabolic rate and thermal tolerance limits of *A. davidianus* larvae were studied. Specifically, the larvae were acclimated to three temperature levels (7 °C, cold stress; 15 °C, optimum; and 25 °C, heat stress) and two diet items (red worm or fish fray) for 20 days. Our results indicated that cold-acclimated larvae showed increased metabolic capacity, while warm-acclimated larvae showed a decrease in metabolic capacity. These results suggested the existence of thermal compensation. Moreover, the thermal tolerance windows of cold-acclimated and warm-acclimated larvae shifted to cooler and hotter ranges, respectively. Metabolic capacity is not affected by diet but fish-fed larvae showed superiority in both cold and heat tolerance, potentially due to the input of greater nutrient loads. Overall, our results suggested a plastic thermal tolerance of *A. davidianus* in response to temperature and diet variations. These results are meaningful in guiding the conservation of this species.

Keywords: *Andrias davidianus*; animal conservation; metabolic compensation; physiological plasticity; respiration rate; thermal limits

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1. Introduction

Temperature is one of the most important climatic factors for ectotherms. Variations of environmental temperatures can not only impact the fitness of ectotherms directly by reducing the behavior and physiological performance [1], but they also disrupt the homeostasis of ecosystems and cause the spread of pathogens or shrink of habitat [2,3]. The critical thermal limit (T_c, including upper T_c and lower T_c) is the most widely used index representing the thermal tolerance of animals [4,5]. It is defined as the thermal point at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions in gradually heating or cooling thermal conditions [6]. Many studies suggested that the T_c are linked to species geographical distributions by matching with the local climate [7–10], making it an important index in predicting the population dynamics of animals under climate change [11,12]. However, the biological processes that determine the thermal tolerances of animals are still controversial. One of the best-known hypotheses is the "Oxygen Limitation of Thermal Tolerance" [13], which claims that a mismatch between the demand for oxygen and the capacity of oxygen supply to tissues is the critical mechanism restricting the whole animal's tolerance to thermal extremes [14]. The association between aerobic metabolism and upper T_c has been supported by a large body of evidence from fish [15] and arthropods [16]. Moreover, a higher metabolic rate is always associated with better locomotory performance under cold stress [17,18]. These results suggest that the metabolic architecture of animals can provide mechanistic insight into their thermal tolerance.

The thermal tolerances of many ectotherms are plastic with the change of the environment. These animals can remodel their cellular processes and structure to reduce the variation degree of physiological activities in response to environmental changes [19]. For example, ant foragers in late summer had higher average upper T_c compared to those in March and December [20]. More evidence is provided from laboratory studies [21,22]. This physiological plasticity is called acclimation or acclimatization capacity. From a metabolic perspective, thermal acclimation induces metabolic compensation in individuals to offset the thermodynamic variations in metabolic reactions [23]. Plasticity enabled by thermal acclimation is expected to broaden the range of temperatures at which animals are active [24] and is often highlighted as a powerful mechanism to buffer the impact of climate change [25,26]. In addition to environmental temperature, variations in other factors (e.g., oxygen level, water level, and nutrient conditions) can also affect the thermal tolerance (called the cross-talk effect) [27,28]. Therefore, it is necessary to consider the plasticity in thermal tolerance at multiple conditions to better understand species' thermal physiology.

Global decline in the amphibian populations is an urgent environmental and ecological problem [29]. The Chinese giant salamander (*Andrias davidianus*) is one of the largest extant amphibian species, and it is often referred to as a living fossil [30]. This species was once widely distributed in central and southern China [31,32]. In the past 60 years, the wild populations of *A. davidianus* have declined dramatically due to habitat degradation, pollution, and overexploitation [33,34]. Recently, this species was evaluated as critically endangered by the International Union for Conservation of Nature Red list [33] and Chinese specialists [35]. Its evolutionary and ecological significance makes it a flagship species for biodiversity conservation in China. In particular, the effect of climate change (e.g., rise of temperature) has been considered a severe challenge to its survival in the wild [34,36]. In addition to the climatic factors, empirical evidence from farmers and our laboratory indicate that the diet types (e.g., fish, red worm, and pork liver in the farming industry) and temperature can jointly affect the growth rate, a primary fitness index, of the *A. davidianus* larvae. It is interesting to know whether the diet type can shape the thermal physiology of these animals. This knowledge can be implicative in their conservation in the context of climate change.

In this study, the influences of acclimation temperature and diet on the thermal performance (i.e., metabolic capacity and thermal limits) were studied for the Chinese giant salamander larvae. Our main target was to study the plasticity in the thermal performance

of this species. This knowledge may extend our understanding of the physiology of this important species and provide useful information for the conservation of the wild *A. davidianus* population under future climate change.

2. Materials and Methods

2.1. Animals and Acclimation

Larvae of *A. davidianus* from the same clutch were purchased. They were cultured under the same environmental conditions in a farm located at Hongya, Sichuan Province, China (103°10′05″ E, 29°52′36″ N). Specifically, the average water temperature was 15 (±1.1 SD) °C, and their main food throughout the first year after hatching was red worm. Once collected from the farm, the larvae were cultured in artificial rearing tanks (length × width × height = 29 cm × 20 cm × 9.7 cm; with 3000 mL water) in laboratory conditions (light: dark = 12: 12; 15 ± 0.5 °C; dissolved oxygen level >90%) for two weeks before treatments. The larvae were fed with sufficient red worm twice a day at 09:00 a.m. and 18:00 p.m., respectively. Water was replaced daily. Animal procedures were approved by the Animal Care and Use Committee of the Chengdu Institute of Biology, Chinese Academy of Sciences.

According to laboratory studies, *A. davidianus* larvae are sensitive to temperature variations [37,38]. The optimal growth occurs in water with a temperature range of 15–21 °C [39]. Their feeding behavior was inhibited by water temperature higher than 25 °C or lower than 8 °C [40,41]. Therefore, three discrete temperature levels (7 °C/cold stress, 15 °C/optimum, and 25 °C/heat stress) were selected for thermal acclimation. The red worm (*Limnodrilus* sp.) was an empirical diet for captive giant salamanders at the first year after hatching, while wild individuals likely have more diverse prey, including fish fray, frogs, and arthropods. In this study, the red-worm and fish fray were selected to feed the experimental larvae.

Two independent acclimation programs were conducted successively to measure the respiration rate and thermal limits, respectively. For the measurement of respiration rate, 210 larvae were collected on their 60th day after hatching. After two weeks of laboratory acclimation, these larvae (1.65 ± 0.3 g, mean ± SD) were randomly divided into six groups: worm diet at 7 °C, worm diet at 15 °C, worm diet at 25 °C, fish diet at 7 °C, fish diet at 15 °C, and fish diet at 25 °C (Figure 1A). Each group included two tanks, and each tank kept 15–20 individuals. The nutrient composition of red worm and fish fray is presented in Table 1. The whole acclimation duration lasted for 20 days. The daily culture followed the conditions described above. The body weight of the larvae was measured at the nearest 0.01 g at the end of treatment, as well as before the measurement of respirate rate. To measure thermal limits, 150 larvae were collected from the farm on their 120th day after hatching. After two weeks of laboratory acclimation, these larvae (3.26 ± 0.5 g, mean ± SD) were randomly divided into five groups (30 individuals for each group): worm diet at 7 °C, worm diet at 15 °C, worm diet at 25 °C, worm diet at room temperature (15–25 °C), and fish diet at room temperature (15–25 °C) (Figure 2A). The whole acclimation duration lasted 30 days. For worm diet at 7 °C, worm diet at 15 °C, worm diet at room temperature (15–25 °C), and fish diet at room temperature (15–25 °C) acclimation groups, 15 individuals were tested for either upper or lower thermal limits. However, only 10 individuals were tested at the lower limit for worm diet in the 25 °C-acclimated group as 5 individuals died during the acclimation.

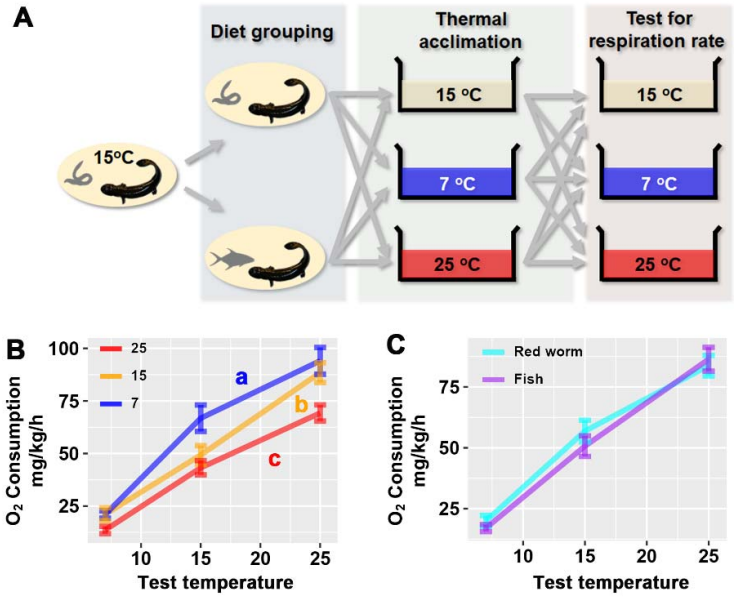

Figure 1. Plasticity in metabolic capacity after thermal and diet acclimation. (**A**) Experimental design. (**B**) Variations between thermal-acclimated groups. (**C**) Variations between diet-acclimated groups. The data were analyzed by ANCOVA (test temperature as covariant) and LSD post hoc test; the results are detailed in Table 2. Different letters denote significant differences between groups.

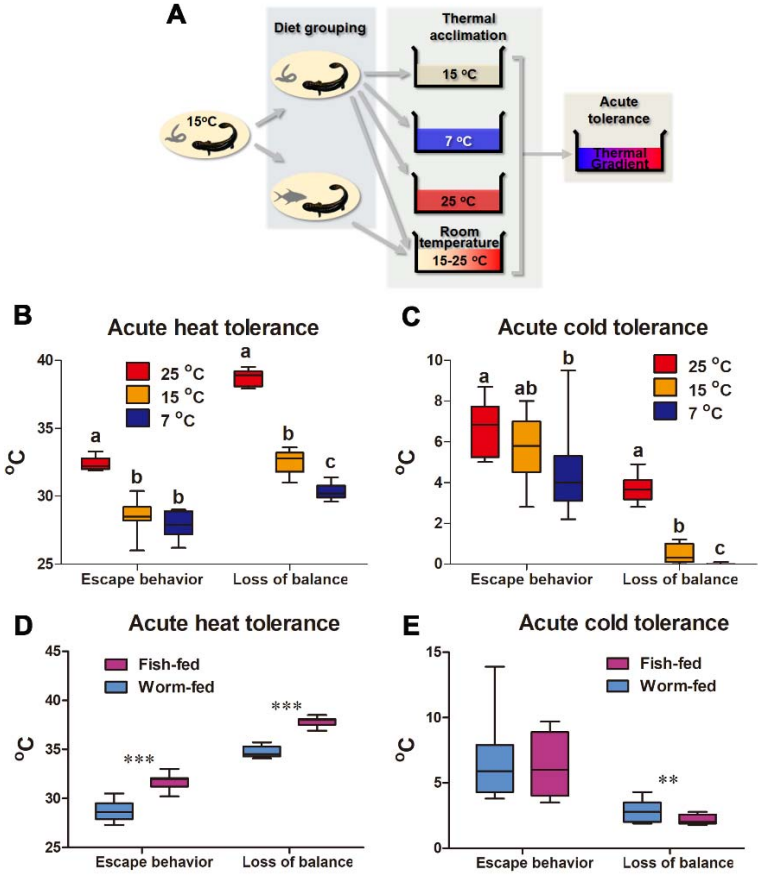

Figure 2. Plasticity in thermal limits after thermal and diet acclimation. (**A**) Experimental design. (**B**,**C**) Variations between thermal-acclimated groups. Different letters denote significant differences ($p < 0.05$) between groups; Kruskal–Wallis test. (**D**,**E**) Variations between diet-acclimated groups. The differences between worm and fish diets were analyzed by Mann–Whitney U test; **, $p < 0.01$; ***, $p < 0.001$.

Table 1. Nutrient compositions of worm and fish diet. Values are presented as mean ± SE.

Nutrients	Worm Diet (g/100 g)	Fish Diet (g/100 g)
Water	90.01 ± 0.3	69.61 ± 0.6
Lipids	1.21 ± 0.1	8.66 ± 0.8
Proteins	5.11 ± 0.2	17.70 ± 0.3
Carbohydrates	1.65 ± 0.2	0.39 ± 0.0

Table 2. Influences of temperature and diet acclimation on metabolic rate. The differences between groups were analyzed with ANCOVA, with test temperature as covariant.

Factors	Type III Sum of Square	df	Mean Square	F Value	Sig.
Acclimation temperature (AT)	1023.872	2	511.936	10.987	0.002
Test temperature (TT)	12,955.612	1	12,955.612	278.041	<0.001
Diet	21.827	1	21.827	0.468	0.508
AT × Diet	199.824	2	99.912	2.144	0.164

2.2. Respiration Rate

Oxygen consumption of *A. davidianus* individuals was measured by intermittent respirometer. For each round of tests, the respiration rate of one larva was measured. All individuals were fasted for 24 h before measurement, and the animal could swim freely in the chamber. For any acclimation groups, 11–18 individuals were randomly selected and measured at each test temperature (i.e., 7, 15, or 25 °C). For each individual, the respiration rates at 7, 15, and 25 °C were not measured continuously. Instead, these measurements were separated by recovery intervals (24 h) at the acclimation temperatures to make sure the larvae returned to their initial status. During the recovery intervals, the larvae were placed back in their respective tanks. It should be noted that the larvae from the same tank (more than one individual in each tank) could not be distinguished from each other, as there was no suitable method to label them. This meant that the same larva might be selected to measure the respiration rate at two or three different test temperatures. Thus, to avoid pseudo-replication in the statistical models, the average value of the respiration measurements, which shared the same test temperature, acclimation temperature, and diet type (n = 11–18 individuals or measurements for each condition), was treated as the only replication for each condition. See the raw data in Supplementary files.

The measurement began when the larvae became quiet. During measurement, their movements, if any, were transient and limited in small ranges as the chamber is small, and most of the time, these larvae were stationary. The respirometer consisted of one chamber (0.15 L) and one dissolved oxygen analyzer (HQ30d, HACH company, New York, USA). In addition, the intermittent respirometer was equipped with an internal circulation pump to fully mix the dissolved oxygen in the internal water. One chamber in each group without *A. davidianus* individual was used as a blank control chamber to calculate the background oxygen consumption. Then, oxygen consumption rate (VO_2) was measured one time at 1 min intervals for 30 min. The following formula was used to calculate VO_2 (mg $O_2 kg^{-1} h^{-1}$) [42]:

$$VO_2 = \Delta O_2 \times v/m$$

where ΔO_2 is the difference in the oxygen concentration level (mg O_2 L^{-1}) between the experimental and blank control chamber, v is the water flow rate in the chamber (Lh^{-1}), and m is the body weight of the *A. davidianus* individual (kg).

2.3. Measurement of Thermal Limits

The thermal limits of the larvae were measured after acclimation. The larvae were placed in a digital water bath, and a square space (height × width × depth = 23 × 11 × 18 cm) was delimited by a plastic net to avoid the direct touch between animals and the bath. The water temperature was initially set at 20 °C, and it increased or decreased by ~1 °C/min. The larvae exhibited free swimming at the beginning and then exhibited behavioral agitation (struggled to escape the confinement). The water temperature for the onset of escaping behavior was recorded, and it was defined as the escaping temperature (T_{esp}). Once the larvae exhibited escaping behavior, they were turned over by a glass hook every 1 min. The water temperature was considered to have reached the critical temperature (T_c) once the larvae exhibited a loss of righting response (five seconds). The loss of righting response was characterized by the animal's inability to correct their orientation after being flipped upside down using a glass rod, while submerged in water [43]. Note that the absolute Tc values might be overestimated, as there was likely a lag in the variation of the body temperature following the variation of the water temperature. Each individual was tested for either lower thermal limits or upper thermal limits.

2.4. Statistical Analyses

Statistical analyses were conducted on SPSS v25.0 (SPSS Inc., Chicago, IL, USA). The differences in growth rate between groups were analyzed by ANOVA, with acclimation temperature and diet as two independent factors. The variations in respiration rates were analyzed by ANCOVA and LSD post hoc test, with test temperature as a covariant. The interactive effects between the independent factors were considered in the ANOVA and ANCOVA models. The differences in upper or lower T_c and T_{esp} were analyzed by Kruskal–Wallis or Mann–Whitney U tests. Graphs were generated by Graphpad Prism 5 or ggplot2, an R package [44].

3. Results

The interaction between environmental temperature and diet affects the larvae growth rate ($F_{2,87}$ = 20.356, $p < 0.01$, two-way ANOVA; Table S1). In worm-fed groups, 7 °C larvae had decreased somatic growth compared to their 15 °C counterparts. However, no significant acceleration in growth was detected between larvae from the 7 °C group and those from the 25 °C group (simple effect analysis; Figure S1). In fish-fed groups, the growth rate tended to increase with the rise of environmental temperature, but the inter-group variations were not significant (simple effect analysis; Figure S1).

3.1. Influences of Temperature and Diet Acclimation on Larvae Metabolism

Acclimation temperature affect the larvae's metabolic rate (presented as oxygen consumption rate) independently ($F_{2,11}$ = 10.987, p = 0.002; Table 2). Cold acclimation enhanced the metabolic capacity of larvae, while warm acclimation reduced their metabolic capacity ($p < 0.05$, LSD post hoc test; Figure 1B). The larvae metabolism was not significantly affected by diet ($F_{1,11}$ = 0.468, p = 0.508; Figure 1C).

3.2. Influences of Temperature and Diet Acclimation on the Acute Thermal Tolerance Window

The influences of acclimation temperature and diet on acute thermal tolerance were studied (Figure 2A). Temperature acclimation caused a shift of the acute thermal tolerance windows of giant salamanders (Figure 2B,C). The upper T_{esp} and T_c of warm-acclimated larvae increased from 28.57 ± 1.2 °C and 32.52 ± 0.8 °C to 32.39 ± 0.5 °C and 38.72 ± 0.6 °C, respectively (mean ± SD, and similarly hereinafter). This was accompanied by an increment in their lower T_{esp} and T_c from 5.51 ± 1.5 °C and 0.48 ± 0.4 °C to 6.63 ± 1.3 °C and 3.72 ± 0.7 °C, respectively. Cold-acclimation caused opposite changes, with their lower T_{esp} and T_c decreasing to 4.64 ± 2.1 °C and 0 ± 0.02 °C, respectively, which was accompanied by a decrease in the upper T_{esp} and T_c. Fish-fed larvae exhibited a wider thermal tolerance

window than worm-fed individuals, characterized by increased upper T_{esp} and T_c and decreased lower T_c (Figure 2D,E).

4. Discussion

4.1. Temperature-Induced Shift of Thermal Limits

Our results indicated that acclimating *A. davidianus* larvae to higher and lower environmental temperatures decreased and increased their metabolic capacity, respectively, suggesting metabolic compensation and adaptive plasticity in response to thermal fluctuation. Thermal compensation enables the maintenance of physiological rates across environmental conditions. This was frequently observed in animals facing mildly cold environments [23,45,46]. This biological phenomenon is believed to partly offset the reduced physiological performance under cold and thus allows better exploitation of the environment by maintaining physiological activities such as locomotion, feeding, and development at low temperatures [47]. For animals in warming conditions, their increased metabolic activity due to thermodynamic effects resulted in accelerated resource consumption. Downregulation of metabolism can benefit their long-term survival from the perspective of resource-saving [48–50]. Given that amphibians' larval stage is devoted to somatic growth and energy storage [51], either an increased metabolic rate at a lower temperature or decreased metabolic rate at a higher temperature are aligned with their life strategy.

This study measured the thermal limits of *A. davidianus* larvae in the early developmental stage. It demonstrated their plasticity in thermal tolerance. The ability to acclimatize to changing thermal conditions is expected to be a primary factor that dictates the vulnerability of taxa to climate change. Generally speaking, the plasticity in thermal tolerance means better outcomes for *A. davidianus* larvae in response to global warming or extreme weather than expected. Currently, the existence and distribution of wild *A. davidianus* in the future has become a topic that is in great need of research and discernment. The ecological niche model raised by Zhao, et al. [34] suggested that climate change can potentially affect the distribution of these animals. To have a more reliable and refined prediction of the fate of the wild populations, physiological models considering their thermal limits and plasticity should be considered. Our results may provide a foundation for these studies. However, two factors should be still be considered, the variation of thermal tolerance with life stages [4] and the potential differences in thermal traits between different phylogenic clades of *A. davidianus* [52]. Further studies should focus on these questions, particularly in clarifying which life stage or phylogenic clades exhibit the lowest thermal tolerance and plasticity. This knowledge could be meaningful in guiding the conservation of wild *A. davidianus*, e.g., making more reliable predictions of the individual survival from different geographical populations and optimizing the trophic structure of their habitats to enhance their tolerance to extreme weather conditions.

Making clear the mechanisms underlying the thermal tolerance may guide the conservation of Chinese giant salamander in the context of climate change. Despite the complexity in the mechanisms of acute thermal intolerance, metabolic capacity is always an important contributor to the thermal tolerance of aquatic ectotherms [14,53]. This is particularly true for cold conditions, where the ectotherms have difficulty maintaining their metabolic activities. For example, the *atu* mutant *Drosophila melanogaster* has increased metabolic capacity, which improves its cold tolerance [54]. Accordingly, variations in metabolic capacity explained the improved and weakened cold tolerance in cold- and warm-acclimated individuals, respectively. For heat tolerance, as the activities of most biological reactions increase with ambient temperatures within certain thermal scopes, the metabolic capacity may no longer be a major limiting factor for heat tolerance. Instead, the overactive metabolic capacity can limit heat tolerance by overburdening the oxygen supply [13,14]. Additionally, it was reported that downregulation of metabolism could improve the acute heat tolerance of animals by reducing their requirement for oxygen [55]. Accordingly, decreased metabolic capacity after heat acclimation is likely associated with higher upper

limits and vice versa. Taken together, the metabolic rate may be an important factor for predicting the tolerance of *A. davidianus* to extreme temperature. In conservation practice, to improve the survival rate of reintroduced populations, we might screen individuals whose thermal tolerance matches the climatic characteristics of the translocation habitats, e.g., introducing cold-tolerant individuals to regions with severe winters or introducing warm-tolerant individuals to hot environments. As direct measurement of the thermal tolerance is detrimental or even lethal to animals, the metabolic rate can be an applicable indicator for their thermal-tolerance properties.

4.2. Diet-Induced Shift of Thermal Limits

Unlike the temperature acclimation, which induced a unidirectional shift of thermal limits, diet acclimation broadened the thermal-tolerance window of *A. davidianus* larvae in both directions. Diet did not change the metabolic capacity of giant salamander. It implied different mechanisms between diet and temperature acclimations in affecting thermal tolerance. Nutrition was reported to modify the critical thermal limits of ectotherms [56,57]. Despite the effects of nutrition depending on the animal size or thermal conditions [58], most studies supported that nutrient supplements (e.g., carbohydrates and amino acids) can improve thermal tolerance in animals [56,59–64]. This is reasonable, as rich nutrient storage can improve cellular metabolic maintenance and benefit the synthesis of protectants, which are necessary for survival in stressful conditions. In our study, the most prominent difference between fish and red-worm diets was that the former was richer in total energy, protein, and lipid levels (Table 1). This might be a reason for the superior of fish-fed larvae in thermal tolerance. This finding suggests that the productivity or prey abundance of the natural habitats could be a potential determinator for the thermal tolerance of wild *A. davidianus*. Introducing suitable prey with great nutrient loads to the natural or artificial habitats of *A. davidianus* may be an alternative approach to reduce the impact of climate change and extreme weather on these animals.

Some mechanisms by which the nutrients modify the thermal physiology of animals were revealed. For example, it was reported that the storage of carbohydrates, the anaerobic substrate, is associated with the tolerance of animals to extreme heat stress [16,65] when the oxygen supply cannot match the metabolic requirement [14]. Although the fish diet contains less sugar than the worm diet, the protein level in the former is much higher. Amino acids derived from proteolysis can be easily converted into carbohydrates. For cold tolerance, the abundance of lipids should be important, as these compounds can be major metabolic substrates in cold conditions [47,66]. More importantly, lipids are required for cellular membrane remodeling, which is an important mechanism underlying cold tolerance [67]. Accordingly, the rich lipid in the fish diet might contribute to the cold tolerance of fish-fed larvae. Overall, these findings shed light on the new thoughts referring to the conservation of this species under climate change. Currently, the reintroduction of captive-bred individuals to the historical natural habitats has been an important measure for the recovery of wild giant salamander populations [68]. Since the diet is a determinant of thermal performance, the prey abundance and diversity in the habitat should be considered before reintroduction. Moreover, to improve the survival rate of the released giant salamander, a preadaptation procedure should be conducted before reintroduction.

5. Conclusions

In this study, we demonstrated the plasticity in thermal physiology of *A. davidianus* larvae in response to environmental temperature and diet changes. These larvae exhibited apparent thermal compensation in metabolic capacity after thermal acclimation. Specifically, cold- or heat-acclimation improved their tolerance to more extreme thermal stress but compromised their tolerance to the opposite thermal extremes. Diet did not affect the metabolic rate; however, the fish diet, which was richer in protein, lipid, and total energy, broadened the thermal-tolerance window of *A. davidianus* larvae. This knowledge provides some implications in the conservation of this endangered animal.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12040531/s1, Table S1: Influences of temperature and diet acclimation on growth rate, Figure S1: Body weight at the end of the acclimation program.

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Data Availability Statement: The datasets presented in this study can be found in online repositories (https://figshare.com/articles/dataset/Respiration_rate_and_thermal_limits_of_acclimated_Andrias_davidianus/19188443 accessed on 17 February 2022).

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The Reproductive Success of *Triturus ivanbureschi* × *T. macedonicus* F_1 Hybrid Females (Amphibia: Salamandridae)

Tijana Vučić [1,2,*], Ana Ivanović [1], Maja Ajduković [2], Nikola Bajler [1,2] and Milena Cvijanović [2,*]

[1] Faculty of Biology, Institute of Zoology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia; ana@bio.bg.ac.rs (A.I.); nikola.bajler@outlook.com (N.B.)
[2] Department of Evolutionary Biology, Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia; maja.ajdukovic@ibiss.bg.ac.rs
* Correspondence: tijana.vucic@bio.bg.ac.rs (T.V.); milena.cvijanovic@ibiss.bg.ac.rs (M.C.)

Simple Summary: Two moderately related large-bodied newt species endemic to the Balkan Peninsula, the Balkan crested newt (*Triturus ivanbureschi*) and the Macedonian crested newt (*T. macedonicus*), coexist and hybridize in central Serbia. Many generations of mutual hybrid crossings and backcrossings with parental species shaped the genetic composition of hybrid populations. Natural populations have admixed nuclear DNA (nuDNA) of parental species and *T. ivanbureschi* mitochondrial DNA (mtDNA), which is usually maternally inherited. The mechanisms that direct gene flow and shape the first generations of hybrids could explain the formation of hybrid zones and their maintenance in nature. We followed and compared life history traits related to reproduction of the first generation of reciprocal hybrids obtained by experimental crossing. Our results suggested that possible incompatibilities between mitochondrial and nuclear genomes, which could lead to the exclusion of *T. macedonicus* mtDNA in natural populations, most likely act at later stages of development or subsequent hybrid generations. Results from this study add to the growing knowledge of *Triturus* hybrid biology and ecology, which is the baseline for conservation programs necessary to protect these highly endangered amphibians.

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Abstract: Two large-bodied newt species, *Triturus ivanbureschi* and *T. macedonicus*, hybridize in nature across the Balkan Peninsula. Consequences of hybridization upon secondary contact of two species include species displacement and asymmetrical introgression of *T. ivanbureschi* mtDNA. We set an experimental reciprocal cross of parental species and obtained two genotypes of F_1 hybrids (with *T. ivanbureschi* or *T. macedonicus* mtDNA). When hybrids attained sexual maturity, they were engaged in mutual crossings and backcrossing with parental species. We followed reproductive traits over two successive years. Our main aim was to explore the reproductive success of F_1 females carrying different parental mtDNA. Additionally, we tested for differences in reproductive success within female genotypes depending on the crossing with various male genotypes (hybrids or parental species). Both female genotypes had similar oviposition periods, number of laid eggs and hatched larvae but different body and egg sizes. Overall reproductive success (percentage of egg-laying females and viability of embryos) was similar for both genotypes. The type of crossing led to some differences in reproductive success within female genotypes. The obtained results suggest that processes that led to exclusion of *T. macedonicus* mtDNA in natural populations may be related to the survival at postembryonic stages of F_2 generation or reproductive barriers that emerged in subsequent hybrid generations.

Keywords: egg size; hybrid breakdown; life-history traits; newts; mtDNA introgression

1. Introduction

The reproduction of genetically divergent taxa is a frequent phenomenon in natural populations (e.g., [1,2]). Hybrids could be sterile or produce further generations by mutual

crossings and/or backcrossing with parental genotypes. The novel combination of genotypes in hybrids could be adaptive and beneficial for fitness, leading to hybrid speciation or these combinations could cause fitness loss [2–8]. The aforementioned outcomes of hybridization are largely dependent on the phylogenetic relatedness of parental species and/or the time of divergence from the most common ancestor. More closely related taxa, i.e., phylogenetically recent lineages, are expected to have viable hybrid offspring that could have adaptive advantages. The hybrid breakdown could be expected in the second or even in subsequent generations as a result of an accumulation of incompatibilities that produce reproductive barriers. On the other hand, hybridization between phylogenetically more divergent taxa could result in largely decreased viability or sterility of one or both sexes of F_1 hybrids [6,9–17]. One of the hybridization consequences is asymmetrical gene flow resulting in introgression of mtDNA from one species to another, which could disturb mito-nuclear compatibility and potentially lead to substantial loss of hybrid fitness (e.g., [18–20]). There are several models that describe and explain the accumulation of genetic incompatibilities over time. For mito-nuclear incompatibilities, the Dobzhansky–Muller model was proposed as the most probable model of the evolution of incompatibilities (see [18] and references therein).

Phylogenetic relations within large-bodied newts (*Triturus*, Salamandridae) and a lack of complete reproductive barriers among species, with a wide range of hybridization outcomes, provide a substantial base for evolutionary studies of hybridization consequences. This monophyletic genus consists of two major groups, marbled and crested newts, which diverged from each other around 24 mya [21]. The marbled newts are *T. marmoratus* and *T. pygmeus*, while within crested newts, four groups are recognized based on genetic data and ecomorphological characteristics: (1) *T. ivanbureschi*, *T. anatolicus*, *T. karelinii*; (2) *T. carnifex, T. macedonicus*; (3) *T. cristatus*; and (4) *T. dobrogicus* [22]. The species have mostly parapatric distribution throughout Europe and parts of adjacent Asia. In the zones of species contact, interspecific hybridization occurs [23], resulting in dynamic hybrid zones in space and time. The movement of a hybrid zone implies advantages of one species at the expense of the other, which eventually leads to species spatial displacement. Most often, this scenario includes asymmetrical introgression of mtDNA from outcompeted to colonizing species [24–31]. Hybridization between *Triturus* species was also confirmed between autochthonous species and anthropogenically introduced species in areas well outside their natural range [32–34].

Natural hybrid populations could consist mostly of F_1 hybrid generation, as in the case of two genetically well-separated species with different ecology and morphology, *T. cristatus* and *T. marmoratus,* which hybridize in western France (see [35] and references therein). These hybrids express typical heterosis; they are larger than the parental species and mostly sterile [36]. The opposite example is that of *T. ivanbureschi* × *T. macedonicus* hybrid populations on the Balkan Peninsula [27,37], which consist of an unknown generation of hybrid individuals derived from a long history of mutual hybrid crossings and backcrossing with both parental species [27,38,39]. *Triturus ivanbureschi* × *T. macedonicus* hybrids have intermediate body lengths related to parental species, and they are morphologically largely similar to both parental species [39]. In central Serbia, all populations of *T. macedonicus*, as well as *T. ivanbureschi* × *T. macedonicus* hybrids, have *T. ivanbureschi* mtDNA [25–27]. Asymmetrical mtDNA introgression is hypothesized to be a consequence of *T. macedonicus* range expansion over the range of *T. ivanbureschi* [25]. *Triturus ivanbureschi* and *T. macedonicus* are two moderately related, morphologically divergent species [22]. They diverge in body shape during postembryonic, larval development [40,41] as well as in the postmetamorphic period [42,43]. These species reproduce in similar aquatic habitats [44] but differ in reproductive traits, where *T. macedonicus* lay more eggs and have greater embryonic survival [43,45].

The mito-nuclear mismatch in *Triturus* populations in the central Balkans caused confusion in interpretations of species distribution and taxonomy (see [37] and references therein). In Serbia, many populations were considered to be *T. ivanbureschi* (syn. *T. karelinii*)

according to mtDNA analysis, or *T. macedonicus* (syn. *T. carnifex*), according to morphology, were later confirmed as hybrid populations. Therefore, in previous studies, the reproductive data of some species are actually data for hybrid populations (Pirot [46,47]; Đurđevac [47]). These hybrid populations consist of *T. ivanbureschi* × *T. macedonicus* individuals of unknown generation (F_n) with *T. ivanbureschi* mtDNA [27]. Body and egg sizes of hybrid females [46,47] were similar to those reported for parental species [43,46,48].

The first generation of *T. ivanbureschi* × *T. macedonicus* hybrids has not yet been confirmed in nature [27,38,39]. Since processes occurring in the F_1 generation are of special interest for an explanation of forming and maintenance of hybrid zones [49], we set up experimental reciprocal crossings to obtain two types of hybrid genotypes: *T. ivanbureschi*-mothered hybrids (with *T. ivanbureschi* mtDNA, confirmed in natural populations) and *T. macedonicus*-mothered hybrids (with *T. macedonicus* mtDNA, not confirmed in natural populations). Our main goal was to explore the eventual differences in reproductive success of reciprocal hybrids carrying different parental mtDNA. Since growth and reproduction in newts are negatively interconnected due to resource allocation [50,51] and the period between the first and second reproductions is characterized by considerable growth [52], we followed life history traits in the first two consecutive years of reproduction. We recorded the life history traits directly related to reproductive output: body size (length and mass), the number of egg-laying females, duration of oviposition, the number and size of eggs and the number of hatchlings. As an estimation of reproductive success, we calculated the percentage of egg-laying females and the viability of their embryos. We also tested for eventual differences in reproductive success when hybrid females were bred with the hybrid males (with the same or reciprocal mtDNA) and with the males of parental species *T. ivanbureschi* and *T. macedonicus* (backcross combinations). Overall, we expected that hybrids with *T. macedonicus* mtDNA would have at least lower reproductive success, considering that *T. macedonicus* mtDNA is not present in natural populations. Failure in their reproductive success would indicate early exclusion of *T. macedonicus* mtDNA in the first generations of crossings upon *T. ivanbureschi* and *T. macedonicus* secondary contact.

2. Materials and Methods

Individuals of *T. ivanbureschi* and *T. macedonicus* were collected from natural populations away from their contact zones: *T. ivanbureschi* from Zli Dol, Serbia (42°25 N; 22°27 E) with permission obtained from the Serbian Ministry of Energy, Development and Environmental Protection (permit No. 353-01-75/2014-08) and *T. macedonicus* in Ceklin, Montenegro (42°21 N; 18°59 E) with permission obtained from the Agency for Environmental Protection, Montenegro (permit No. UPI-328/4). Genetic data confirm that these were *T. ivanbureschi* and *T. macedonicus* populations [27]. A series of crossbreeding experiments were carried out at the Institute for Biological Research "Siniša Stanković" and approved by the Ethics Committee of the Institute (decision Nos. 03-03/16 and 01-1949). By crossing parental species, two genotypes of hybrids (HI—with *T. ivanbureschi* mtDNA and HM—*T. macedonicus* mtDNA) were obtained in two different years:

- HI in 2016: *T. ivanbureschi* ♀ (n = 8) × *T. macedonicus* ♂ (n = 5);
- HM in 2017: *T. macedonicus* ♀ (n = 4) × *T. ivanbureschi* ♂ (n = 4).

Since growth and survival, as well as reproductive traits, can be affected by external factors (e.g., temperature, density, availability of food) and their mutual interactions (e.g., [53]), we kept the experimental settings and conditions the same throughout different years of the experiment. Crossing of parental species was done outdoors in 500 L plastic vats (separate vat for each breeding crossing). Plastic strips were provided for egg deposition. When females started laying eggs, they were transferred to the laboratory in separate 10 L aquariums half-filled with dechlorinated tap water. Eggs, embryos and larvae were raised in the same controlled experimental conditions in both years. The temperature was kept constant (18–19 °C) with a natural day/night regime. Eggs and embryos were raised in Petri dishes (up to 10 eggs/embryos per dish). Dechlorinated tap water was changed every other day. All Petri dishes were checked daily to remove non-developing eggs and

arrested embryos. Larvae were raised in 2 L plastic containers (single larva per container). Containers were half-filled with dechlorinated tap water, which was changed every other day. Larvae were fed *ad libitum* with *Artemia* sp. and *Tubifex* sp. After metamorphosis, animals hibernated during winter in a cold chamber at a constant temperature (4 °C). During the first postmetamorphic year, between first and second hibernation, juveniles were kept in 200 L plastic vats placed outdoors with a similar number of animals per vat. Plastic vats were enriched with perforated bricks, providing shelter and plastic lids, which were used as floating platforms. Newts were fed *Tubifex* sp. and *Lumbricus* sp. twice a week.

When hybrids had developed secondary sexual characters during the second postmetamorphic year, they were engaged in breeding series of mutual crossings and backcrossings with parental species (Figure 1). Animals hibernated before each breeding. HI females mated in 2018 (first reproduction) and 2019 (second reproduction). HM females mated in 2019 (first reproduction) and 2020 (second reproduction). The numbers of females involved in the breeding series were 23 HI and 20 HM in the first reproductive year and 25 HI and 20 HM in the second year of reproduction. To exclude the possibility of spermatozoid retention [54], the same females were crossed with the same males in two consecutive years. The only exceptions are two HI females, which were included in the crossing with HM males. Each crossing (see Figure 1) was conducted in a separate 200 L plastic container. Containers were set in the backyard of the Institute in semi-natural conditions. After females started laying eggs, they were transferred to separate 10 L aquariums, half-filled with dechlorinated water. Eggs and embryos were raised in the same controlled experimental conditions (see above). For more details of animals' housing and experimental conditions, see [40,41,52].

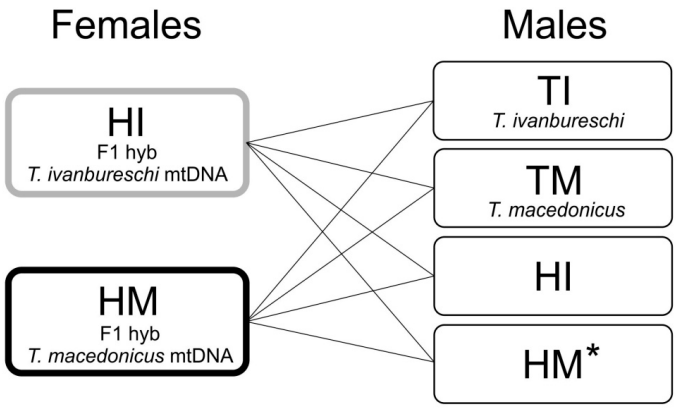

Figure 1. Schematic presentation of the experimental design. The F_1 hybrid females with *T. ivanbureschi* mtDNA (HI) were crossed and backcrossed in 2018 and 2019. The F_1 hybrid females with *T. macedonicus* mtDNA (HM) were crossed and backcrossed in 2019 and 2020. * Sexually mature HM males were available only in 2019, and therefore not involved in crossings with HI females in their first reproduction in 2018.

Data for this study were collected during two consecutive years, in 2018 and 2019 for HI and in 2019 and 2020 for HM. To obtain measures of body size, females were photographed and weighed each year right after hibernation. Imaging was performed using a camera (Nikon D7100 or Sony DSC-F828) on a fixed stand with millimeter paper as a background. Body length (snout to vent length—SVL) was measured as the distance between the tip of the snout and the level of the posterior edge of the hind legs from the dorsal view using ImageJ software v. 1.50i [55]. Body mass (BM) was weighted to the nearest 0.01g using an electronic scale (MP300, Chyo Balance Corporation, Kyoto, Japan).

The eggs of newts consist of vitellus and a protective jelly layer. Females deposit individual eggs and wrap them in submerged vegetation [56], thus providing transparent plastic strips for egg deposition enabled easy observation of newly laid eggs. To estimate the number of laid eggs and egg size, we collected eggs on a daily basis and photographed

them with a Nikon Digital Sight Fi2 camera attached to a Nikon SMZ800 stereo zoom microscope immediately after removal from the plastic strips for measurements. The number of eggs within each genotype was calculated from the first laid egg in common containers to the last laid egg in separate aquaria. Egg dimensions (maximum width of vitellus; maximum length and width of jelly) were taken using ImageJ software. Based on these measures, we calculated the volume of the vitellus and the volume of the entire egg. We calculated vitellus volume as the volume of a sphere: $Vv = 4/3 \times r^3\pi$, where r is the vitellus width/2. For the volume of egg, we measured ellipsoid volume: $Ve = 4/3 \times r_1 r_2^2 \pi$, where r_1 and r_2 are the radii length/2 and width of egg/2, respectively. The volume of the jelly was calculated as a difference between the volume of egg and the volume of vitellus: $Vj = Ve - Vv$. For statistical analysis of egg size, we used 100 eggs per genotype per year, which were taken randomly (RAND function in Microsoft Excel).

We also observed changes in other reproductive traits between the two reproductions, such as the number of egg-laying females, duration of oviposition, the total number of laid eggs and the total number of hatched larvae. Duration of oviposition was estimated as the number of days between the first and last laid egg within each genotype. To estimate reproductive success, we calculated the percentage of egg-laying females and the viability of their embryos. The percentage of egg-laying females was calculated as the ratio between the number of egg-laying females and the total number of females involved in breeding per hybrid genotype multiplied by 100. The viability of embryos was calculated as the ratio between the numbers of hatched larvae and the deposited eggs per hybrid genotype multiplied by 100.

Hybrid females of both genotypes were subdivided into four breeding groups exposed to different males (see Figure 1). As the exposure of females to different males can affect reproductive success in amphibians (e.g., [57]), we compared the percentages of egg-laying females and the viability of embryos separately within HI and HM females.

We used separated repeated measures ANOVAs to test for differences in the average body size (SVL and BM) and egg size (vitellus and jelly volumes), with female genotype as the categorical factor and year of reproduction as the within-subject (repeated measure) factor on the measured variables. Tukey's HSD post hoc test was used to determine the statistical significance of between-group differences. Other reproductive traits (duration of oviposition, total number of eggs and total number of hatched larvae) were compared by the Kruskal–Wallis test since their values did not meet the criteria for ANOVA (assumptions of normality and sphericity). Differences in reproductive success were tested using differences between two proportions. Statistical analyses were done using Statistica10 software (StatSoft Inc., Tulsa, OK, USA, 2011).

3. Results

The repeated measures ANOVA showed that reproductive year (SVL: $F_{1,38} = 317.53$, $p < 0.0001$; BM: $F_{1,38} = 109.94$, $p < 0.0001$), genotype (SVL: $F_{1,38} = 15.34$, $p = 0.004$; BM: $F_{1,38} = 10.95$, $p = 0.002$) and their interaction (SVL: $F_{1,38} = 36.99$, $p < 0.0001$; BM: $F_{1,38} = 14.57$, $p = 0.0005$) had a significant effect on the increase of body size between two reproductive years. Post-hoc tests showed a significant ontogenetic increase in body size (SVL and BM) within both genotypes. Differences in body size between genotypes were evident only in the second year of reproduction (Figure 2, Table 1).

For the volume of vitellus, repeated measures ANOVA (Figure 2) showed a significant effect of reproductive year ($F_{1,198} = 62.10$, $p < 0.0001$) and year × genotype interaction ($F_{1,198} = 27.17$, $p < 0.0001$), but non-significant effect of genotype alone ($F_{1,198} = 0.08$, $p = 0.7714$). Volume of jelly was significantly affected by both factors, reproductive year ($F_{1,198} = 34.92$, $p < 0.0001$) and genotype ($F_{1,198} = 21.41$, $p < 0.0001$), as well as their interaction ($F_{1,198} = 7.28$, $p = 0.0076$). Post-hoc tests revealed that there was no ontogenetic difference in the volume of vitellus and jelly within HI, while HM eggs were significantly larger in the second reproductive year. HI had larger eggs (volume of vitellus and jelly) than HM in the first reproductive year. In the second reproductive year, HM had a larger volume of vitellus

but a similar jelly volume to HI (Figure 2, Table 1). Kruskal–Wallis analyses showed that there were no differences in other reproductive traits (duration of oviposition, total number of laid eggs and total number of hatched larvae) between and within hybrid genotypes ($p > 0.05$ in all comparisons, Figure 2).

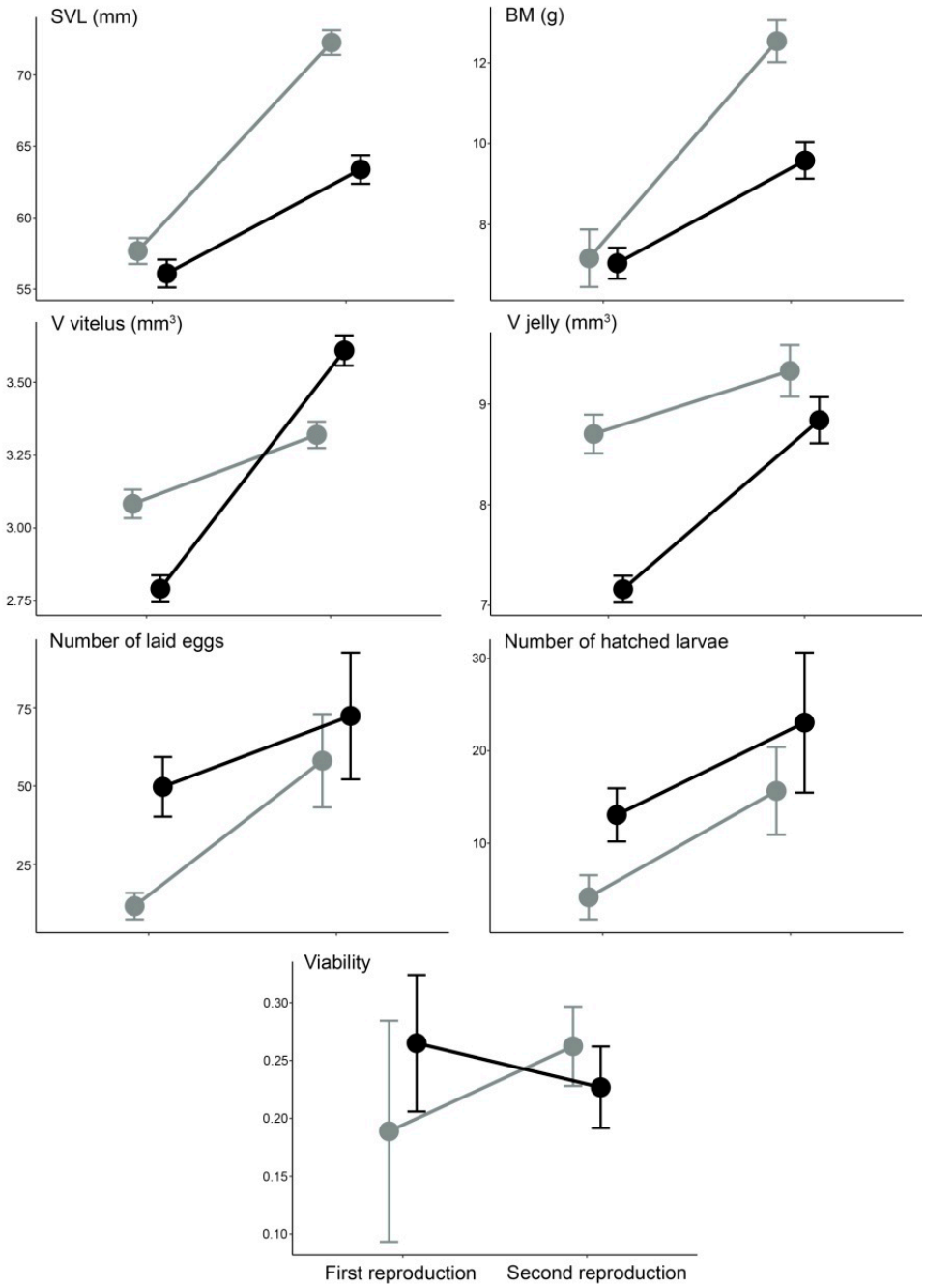

Figure 2. Ontogenetic differences in body size (length—SVL and mass—BM) and reproductive traits in the first two reproductive years of F1 hybrid females with *T. ivanbureschi* mtDNA, HI (gray) and with *T. macedonicus* mtDNA, HM (black). Values of each trait are represented as mean ± standard error per each genotype and year of reproduction.

Table 1. Differences in body size and egg volume between F1 hybrid females with *T. ivanbureschi* mtDNA (HI) and *T. macedonicus* mtDNA (HM) during the first two reproductive years. Analyzed traits: SVL—snout to vent length, BM—body mass, Vv—vitellus volume, Vj—jelly volume. Level of significances of pairwise comparisons: ns = not significant.

Comparisons	Females		Eggs	
	SVL	**BM**	**Vv**	**Vj**
within genotypes (I vs. II year)				
HI	0.0002	0.0002	ns	ns
HM	0.0002	0.0004	<0.0001	<0.0001
between genotypes (HI vs. HM)				
I year	ns	ns	0.0011	<0.0001
II year	0.0002	0.0002	0.0051	ns

As the percentage of egg-laying females and viability of embryos also did not differ between HI and HM females in both reproductive years ($p > 0.05$ in all comparisons, Figure 3), we tested for differences in reproductive success between types of crossings (male effect) within each hybrid genotype. The percentage of egg-laying females did not differ within HI in both reproductive years regardless of different types of crossings, i.e., exposure to males of various genotypes. Within the HM females, the crossing of HM ♀ × ♂ had a relatively low percentage of egg-laying females, which was significantly lower from backcrossing of HM females with males of both parental species (*T. ivanbureschi* and *T. macedonicus*) in the first year, but this difference was lost in the second reproductive year (Tables 2 and 3).

Offspring were obtained from all available breeding crossings in both reproductive years (see Figure 1). The viability of embryos obtained by different hybrid crossings and backcrossing was similar in the first reproductive year for both HI and HM females. For HI females, embryos obtained from the backcrossing of HI ♀ × *T. macedonicus* ♂ had greater viability than from the backcrossing of HI ♀ × *T. ivanbureschi* ♂. Embryos obtained from the reciprocal hybrid crossing (HI ♀ × HM ♂) had greater viability than those obtained from all other crossings of HI females and different male genotypes (HI, *T. ivanbureschi* and *T. macedonicus*) (Tables 2 and 3). For HM females, embryos obtained from the crossing of HM ♀ × ♂ in the second reproductive year had the largest viability, significantly different from the crossing of reciprocal hybrids (HM ♀ × HI ♂), as well as from the backcrossing of HM females with *T. ivanbureschi* males (Tables 2 and 3).

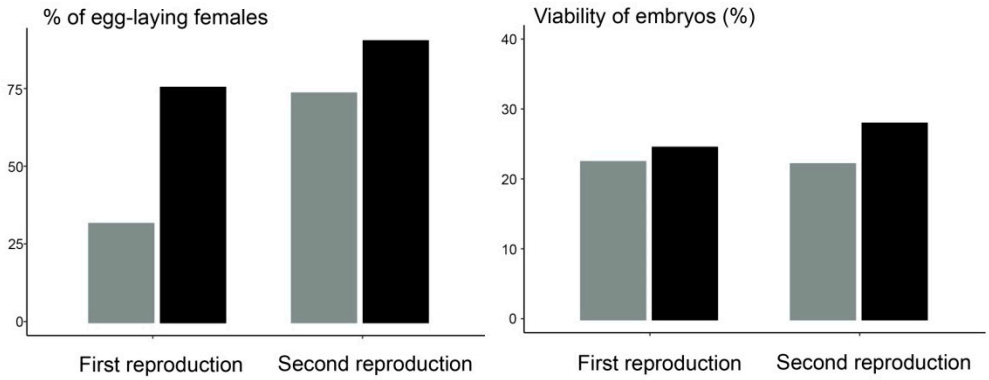

Figure 3. Differences in the percentage of egg-laying females and viability of embryos, as a measure of reproductive success during the first two reproductive years of F_1 hybrid females with *T. ivanbureschi* mtDNA, HI (gray) and with *T. macedonicus* mtDNA, HM (black).

Table 2. Reproductive success (percentages of egg-laying females and viability of embryos) of F_1 hybrid females with *T. ivanburechi* mtDNA (HI) and *T. macedonicus* mtDNA (HM) exposed to different males (see Figure 1 for various breeding crossings and abbreviations). Sexually mature HM males were not available in 2018 and therefore not involved in crossings with HI females in their first reproductive year.

Breeding Crossings (♀ × ♂)	Egg-Laying Females (%)		Viability (%)	
	I Year	II Year	I Year	II Year
HI × HI	22	43	26	20
HI × HM	/	50	/	35
HI × TI	14	100	8	18
HI × TM	57	100	33	25
HM × HM	20	100	19	35
HM × HI	80	100	20	23
HM × TI	100	100	26	26
HM × TM	100	60	23	27

Table 3. Pairwise comparison of reproductive success (percentages of egg-laying females and viability of embryos) of F_1 hybrid females with *T. ivanbureschi* mtDNA (HI) and *T. macedonicus* mtDNA (HM) exposed to different males (see Figure 1 for various breeding crossings and abbreviations). The comparisons were done within HI and HM females separately. Sexually mature HM males were not available in 2018 and therefore not involved in crossings with HI females in their first reproductive year. Level of significances of pairwise comparisons: ns = not significant.

Compared Breeding Crossings (♀ × ♂)	Egg-Laying Females (%)		Viability (%)	
	I Year	II Year	I Year	II Year
HI × HI vs. HI × TI	ns	ns	ns	ns
HI × HI vs. HI × TM	ns	ns	ns	ns
HI × TI vs. HI × TM	ns	ns	ns	0.0310
HI × HI vs. HI × HM	/	ns	/	0.0002
HI × TI vs. HI × HM	/	ns	/	<0.0001
HI × TM vs. HI × HM	/	ns	/	0.0140
HM × HM vs. HM × TI	0.0320	ns	ns	<0.0001
HM × HM vs. HM × TM	0.0320	ns	ns	ns
HM × TI vs. HM × TM	ns	ns	ns	ns
HM × HM vs. HM × HI	ns	ns	ns	0.0160
HM × TI vs. HM × HM	ns	ns	ns	ns
HI × HI vs. HM × HM	ns	ns	ns	ns

4. Discussion

In natural populations, overall reproductive output and success of F_1 hybrid generation, particularly divergence of reciprocal hybrids, have an important impact on the long-term consequence of hybridization, as they can alter the direction of gene flow [58,59]. Therefore, the mito-nuclear incompatibility could shape patterns of genetic variation in hybrid zones and impact their dynamics. *Triturus* newts are a suitable model system for studies of mechanisms that lead to asymmetrical direction of mtDNA and introgression. Hybridization of the phylogenetically distant *T. cristatus* and *T. marmoratus* resulted in mtDNA introgression asymmetry due to divergence in the reproductive success of reciprocal crossings. More than 90% of individuals in natural populations have *T. cristatus* mtDNA [60], while F_2 and further hybrid generations are rare [35,60]. Hybridization of *T. ivanbureschi* and *T. macedonicus*, two moderately related species, also resulted in an asymmetrical mtDNA introgression. *Triturus ivanbureschi* mtDNA is found across populations of *T. ivanbureschi*, *T. macedonicus* and *T. ivanbureschi* × *T. macedonicus*, in the Balkan Peninsula with the loss of *T. macedonicus* mtDNA in central Serbia [25–27].

Studies of *T. ivanbureschi* × *T. macedonicus* hybridization showed that hybrids have intermediate morphology compared to parental genotypes [39–41]. Hybridization affected physiological response resulting in raised oxidative stress parameters in hybrid larvae, which might have a negative impact on their survival [61–63]. However, fitness consequences of hybridization comprising slower growth rate, sex ratio disturbance and lower survival were not evident in F_1 hybrids compared to maternal species during the early postmetamorphic period [52]. The morphological divergences between reciprocal hybrids were not recorded in previous morphological studies of larvae and recently metamorphosed juveniles. They have similar developmental patterns and growth rates [40,41].

We found that, at the beginning of the second postmetamorphic year, both F_1 hybrids showed secondary sexual characters but did not diverge in body size. The divergence in size between reciprocal hybrids was recorded after the first reproduction, which coincides with the timing of sexual size dimorphism and divergence in size between hybrids and maternal species [52].

The other significant difference between reciprocal hybrids is related to egg size, which can have a profound impact on embryonic development and survival, as well as on larval growth and developmental rates [64]. Hybrids with *T. ivanbureschi* mtDNA laid larger eggs in the first reproduction (no differences between reciprocal hybrids in female body size), while eggs of hybrids with *T. macedonicus* mtDNA had substantially larger vitellus in the second reproduction, despite significantly smaller body size compared to hybrid females with *T. ivanbureschi* mtDNA. Additionally, reciprocal hybrids differed in the pattern of change in egg size between the first and second reproductions, where hybrids with *T. macedonicus* mtDNA showed a greater increase in vitellus size (see Figure 2). It is considered that the size of an amphibian egg is positively correlated with female body size. Thus, it is expected that older and larger females produce larger eggs, i.e., that ontogenetic changes of female body size should affect egg size [46,65,66]. Our results do not support previous notations. We suggest three mutually non-exclusive hypotheses that can be applied to explain the observed pattern of ontogenetic changes and divergences in vitellus size between reciprocal hybrids. First, the observed divergences may indicate that hybrids with *T. ivanbureschi* mtDNA invest more in somatic growth than in reproduction, while the opposite trend could be true for hybrids with *T. macedonicus* mtDNA, which could invest more in reproduction by enlarging vitellus volume. Second, our results could indicate that egg size is a heritable trait that is inherited by maternal investments through egg cytoplasm. In newts, egg size is a species-specific trait [47]. In a comparison of parental species, *T. macedonicus* has larger eggs than *T. ivanbureschi* [43]. The cytoplasm of the oocyte is full of maternal cytoplasmic components subsequently present in each cell of the new embryo [67], having an important role in the determination of egg size and possibly leading to significant divergences in egg size between two reciprocal hybrids. The third possible explanation for the divergence in egg size and female growth could be different sensitivity to external conditions of reciprocal hybrids. Previously, it was proposed that *T. macedonicus* is a thermophilic species [43,68]. Vitellogenesis occurs way before the breeding season, usually before hibernation [64], and it is dependent on different external and internal factors (e.g., [56]). It could be possible that external conditions were less suitable for F_1 hybrids with *T. macedonicus* mtDNA at the time of their preparation for the first reproduction and vitellogenesis, i.e., hybrids with *T. macedonicus* mtDNA could be more susceptible to environmental variation during these periods. Studies on the plasticity of egg size and early life-history traits under different environmental conditions could give insight into the sensitivity of different genotypes to environmental conditions, with emphasis on different mtDNA groups. Hybrid females with both *T. ivanbureschi* and *T. macedonicus* mtDNA deposited a similar number of eggs in two reproductive years during a similar oviposition period. The number of eggs that hybrid females laid was in the range reported for both parental species in the same experimental settings [43], as well as for females from *T. ivanburechi* × *T. macedonicus* natural population [46], suggesting similar reproductive potential to that of the parental species. The observed pattern is opposite to *T. cristatus* and

T. marmoratus hybridization, where hybrids have higher reproductive potential compared to parental species in experimental conditions, but low viability of eggs [69].

In our study, hybrid females were bred with four male genotypes (see Figure 1). In some amphibian taxa, females tend to choose males with whom they will engage in mating [56,57,70,71], which could affect overall reproductive success by decreasing or increasing the percentage of females that are involved in active breeding. Moreover, in hybridizing species, females' choice could be a prezygotic barrier causing unidirectional mtDNA exchange if females of one species mate with males of the other and not vice versa [71]. Considering hybrid mutual mating and mating with parental species, it has been proposed that intermediate phenotypes can be disadvantageous for hybrid males, as they could be less attractive to females [7]. This could be true for newts, as it was shown that females do prefer larger males with a prominent crest [72]. In our experimental settings, the absence of differences between reciprocal hybrids in the percentage of egg-laying females, i.e., females engaged in active reproduction, suggests that females did not refuse to breed with either male genotype and their pickiness did not represent a barrier for either mtDNA to be passed on to the next generation. In comparisons within each female genotype, slight differences in percentages of egg-laying females in the first reproduction were not recorded in the second year of reproduction. Possibly, in their first reproduction, females preferred older (i.e., larger) males with prominent crests [72], as their conspecific males were of the same age or hybrid males were less successful in their first year of reproduction.

One characteristic of *Triturus* newts is that half of embryos die during development at the tail bud stage due to balanced lethal syndrome [73]. Viable eggs that had developed to hatchlings were obtained from all types of crossings (Figure 1) with no difference in embryo mortality between females with *T. ivanbureschi* and *T. macedonicus* mtDNA. However, viability differed in the second reproduction within reciprocal hybrid females when a different type of crossing (i.e., different male genotype) was included. Differences in the viability of embryos from different crossings and backcrossings indicate a possible decrease in survival of the F_2 generation. For example, the backcrossing of hybrid females with *T. ivanbureschi* mtDNA and *T. macedonicus* was more successful than the one including *T. ivanbureschi* males. The obtained result could back up the hypothesis of postglacial species displacement and *T. macedonicus* nuclear DNA spreading [25]. This hypothesis suggests that, throughout generations, subsequent backcrossing of hybrid females with *T. ivanbuerschi* mtDNA with males of *T. macedonicus* diminished *T. ivanbureschi* nuclear DNA, rebuilt *T. macedonicus* nuclear DNA and retained *T. ivanbureschi* mtDNA [25]. The difference in the viability of F_2 generations should be confirmed and further investigated in a larger study that would include postembryonic developmental stages, as it was shown that asymmetry in the frequency of reciprocal hybrids emerged after embryonic development in *T. cristatus* × *T. marmoratus* hybridization [59].

5. Conclusions

We found that the reproductive traits and success of reciprocal *T. ivanbureschi* × *T. macedonicus* F_1 hybrids (with *T. ivanbureschi* or *T. macedonicus* mtDNA) is largely similar, expressing some differences depending on trait, year of reproduction and male genotype involved in mating. Overall, the obtained results suggest that the loss of *T. macedonicus* mtDNA emerged in F_2 or subsequent hybrid generations. Major cito-nuclear incompatibilities are often masked in the first generation and manifested in F_2 or subsequent hybrid generations due to their accumulation, the phenomenon known as hybrid breakdown (e.g., [10,16]). The viability of F_2 embryos, obtained from mutual hybrid crossings and backcrossing with both parental species, points out a possible decrease of fitness in the F_2 generation. However, these results are only preliminary and should be tested further.

Females were monitored in the first two years of reproduction, so possibly results considering success in crossings with different male genotypes could change with the age of females. The postembryonic viability of F_2 generation should be analyzed, as possible differences in survival can occur during larval development, metamorphosis or even during

the postmetamorphic period. An experiment, which would include females and males of various ages of *T. ivanbureschi*, *T. macedonicus* and hybrids with both types of mtDNA, would enable females to choose between different male genotypes. The suggested experimental design could give another insight into processes that took place a long time ago, upon the initial secondary contact between two moderately related species of *T. ivanbureschi* and *T. macedonicus.*

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