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Has the West been won? A field survey and a species distribution model of *Iberolacerta horvathi* in the Alps

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Abstract. The Horvath's rock lizard (*Iberolacerta horvathi*) is a rupicolous mountain species endemic of the eastern Alps and northern Dinaric range. The species has its known western limit of the distribution in the Veneto region of Italy. It is not known whether the species is really rare in Veneto or whether the area has been insufficiently surveyed. In addition, it is not known whether the westward distribution of the species is limited by a physiographic or by a climatic barrier. During the period 2016-2018, 118 sites were surveyed in the Veneto and Trentino-Alto Adige regions. Four new occurrences of *Iberolacerta horvathi* were discovered in Veneto that: 1) largely fill the gap between the westernmost known site and the closest site to the east; 2) extend further west the known distribution by 9 km. In addition the species was confirmed in three already known sites. A species distribution model was developed with the software MaxEnt, using 100 occurrences from Italy, Austria and Slovenia. The best model shows that the distribution is explained by the asperity of their habitat, the sedimentary bedrock, the aspect, the average temperature of the coldest quarter, the rainfall seasonality and the average summer rainfall. The last variable appears as the most likely responsible for the rarefaction of the species at its western limit. In addition, the species distribution model suggest that the Horvath's rock lizard might be present in some additional mountain groups where it has so far not been found yet.

Keywords. Horvath's rock lizard, Maxent, rainfall, temperature, roughness, aspect, Bedrock, Veneto.

INTRODUCTION

The present distribution of reptiles in Europe is much dependent on its climatic history, in particular to the Pleistocene oscillations. After the climate improvement following the last glacial maximum (18,000 BP) many species spread from southern refugia and have colonized very large areas (Joger et al., 2007). Some species were able to spread from their refugia in a more limited way due to ecological constraints or to the presence of geographical barriers like the Alps for species from the Apennines peninsula and the Pyrenees for species from the Iberian peninsula (Joger et al., 2007). The opposite happened to some cold adapted species, like the *Dinolacerta* mountain lizards of the southern Dinaric chain and the species of *Iberolacerta* of the Iberian Peninsula: they have likely reduced their range and have become restricted to high altitude isolated mountain peaks (Ortega et al., 2016). The conservation prospect of these high altitude species is likely going to worsen due to the ongoing climate change (Le Galliard et al., 2012).

A different case is the distribution of the Horvath's Rock Lizard (*Iberolacerta horvathi*), an endemic species of the northern Dinaric chain and eastern Alps (north-

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west Croatia, Slovenia and adjoining northeastern Italy and southern Austria) (Speybroeck et al., 2016), a species phylogenetically related to a group of mountain dwelling lizards of the Iberian Peninsula (Carranza et al., 2004, Crochet et al., 2004). As a matter of facts, the known presence localities in Italy and Austria are in areas that were glaciated during the last glacial maximum (Ivy-Ochs et al., 2009). So, the species should have colonized the eastern Alps from refugia in the southeastern Alps or from the Dinaric chain. Unfortunately, it is difficult to study how this colonization happened due to insufficient knowledge of the distribution of this species, as suggested by the recurrent discoveries of its presence in new mountain groups (e.g., Žagar et al., 2014). This lack of data is likely the result of the difficult accessibility of the rocky cliffs inhabited by the species and by the possible misidentification with the similarly looking (Žagar et al., 2012) and frequently syntopic common wall lizard, *Podarcis muralis* (Cabela et al., 2007). In Italy, Horvath's rock lizard is well distributed in the Friuli region (Lapini et al., 2004; Rassati, 2010; Rassati, 2012) but its presence appears to be particularly scattered west of the Piave River in the Veneto region. The westernmost site was discovered in 1993 close to the village of Listolade (Lapini and Dal Farra, 1994; Lapini et al., 2004), a site that was about 70 km away from the nearest known site. In spite of subsequent discoveries (Rassati, 2010; Rassati, 2012; Lapini, 2016) and quite extensive surveys (Tormen et al., 1998; Bonato et al., 2007; Bonato, 2011; Cassol et al., 2017) there is still a gap of around 30 km between the site of Listolade and the closest one to the east. Therefore, it is not known whether the species is present in this large gap and even west of Listolade. Moreover, it would be interesting to discover whether the species has reached its potential distribution or whether its westward post glacial expansion is still lagging behind its potential distribution, as it still happens for some plants (Svenning and Skov, 2007; Willner et al., 2009; Dullinger et al., 2012) and animals (Araújo et al 2008; Pinkert et al., 2018). In addition it is not known which factors might limit or have limited the westward spread of the species.

In order to fill these gaps in the knowledge of the species, the research has the following aims:

1) to improve the known distribution by surveying mountain groups west of the Piave River in the Trentino-Alto Adige and Veneto regions of Italy;

2) to correlate the presence of the species in Italy, Austria and Slovenia to climatic, geological, physiographic and ecological parameters in order to estimate, through a species distribution model, whether the western limit of the distribution has likely been fixed or whether new sightings further west should be expected.

MATERIAL AND METHODS

Field survey

Surveys were conducted during 94 days in the period April 2016 - September 2018 in 118 sites by one to three researchers per site. 20% (n = 24) of the sites were visited more than once. Surveyed areas were concentrated west of the Piave River but a few sites were investigated also just east of it. Due to the inaccessibility of many cliffs, investigated sites were not randomly distributed but were inevitably concentrated close to roads and mountain paths.

Species identification was rarely performed by catching the specimens with a long stick with a cotton loop. More commonly, species identification was performed by analysing pictures taken with a telephoto lens, an efficient technique also when lizards are on inaccessible rocky cliffs (De Marchi et al., 2019). Identification characters from the frequently syntopic common wall lizards are as in Corti et al. (2010).

The coordinates and altitude of the sites were obtained by hand held GPS devices. In addition, the sites where Horvath's rock lizards were found were characterized by the bedrock obtained from the geological map of Italy viewed on the Geoportale Nazionale (http://www.pcn.minambiente.it) and by the habitat classification obtained from the "Carta della Natura" of Veneto and "Carta della Natura" of Friuli-Venezia Giulia (1:50.000) (http://www.geoviewer.isprambiente.it/).

Species distribution model (SDM)

In order to elaborate the potential distribution of the species in Italy and in neighbouring Austria and Slovenia, a presence only modeling approach was chosen due to the lack of reliable absence points particularly outside our study area. A maximum entropy approach was developed with the software Maxent (Phillips et al., 2006; Merow et al., 2013; Phillips et al., 2017), which proved to be quite successful even when occurrence data were few and in presence of somehow biased samples (Elith et al., 2006). As occurrence data of Veneto are few and restricted to a too small area for statistical analyses, published data related to Italy (Lapini et al., 2004; Rassati, 2010; Rassati, 2012; Lapini, 2016; Lapini, 2017; Rassati, 2017), Austria (Tiedeman, 1992; Ortner, 2006; Cabela et al., 2002; Cabela et al., 2007) and Slovenia (Žagar, 2008a; Žagar, 2008b; Krofel, 2009; Cafuta, 2010; Žagar et al., 2011; Žagar et al., 2012; Osojnik et al., 2013; Žagar, 2016; Zauner, 2018) were collated. Such a wider area helps to reduce the risk that some local climatic conditions might wrongly highlight the importance of an environmental variable that in fact is not a relevant one (Phillips, 2006). Unfortunately, some published occurrences lacked coordinates but have only the altitude and a general description of the collecting site: only when the description was considered sufficiently detailed to be confidently associated to a single 300 m × 300 m pixel (see later) the occurrences were used by extracting the coordinated from Google-Earth. As some occurrences are aggregated due to sampling bias, some occurrence localities were manually removed so that the remaining ones are at least

one km from the closest ones. Eventually, the 100 occurrences of the Horvath's lizard were joined together with a background dataset of 10,000 points to randomly sample the analysed area (lat 45.35°N to 47.00°N, lon 11.20°E to 15.00°E).

The environmental layers (all at European scale) were as follows. 19 bioclimatic variables at 30-arc seconds (about 1km) resolution derived from the monthly temperature and rainfall values for the period 1970-2000 (http://www.worldclim.org/bioclim). Slope, Roughness (the degree of irregularity of the surface, calculated by the largest inter-cell difference of a central pixel and its surrounding cell, as suggested by Wilson et al., 2007) and Aspect (the compass direction that a slope faces with values ranging 0-360°) layers calculated with the GIS software QGIS (version 2.8.6) from a 25m digital elevation model (Layer: DEM-v1.1-E40N20) downloaded from the Copernicus Land Monitoring Service (https://land.copernicus.eu/). A 250m vegetation layer, Corine Land Cover clc_12 (version 18_5), again downloaded from the Copernicus Land Monitoring Service. A geological layer from the 1 : 5 Million International Geological Map of Europe and Adjacent Areas (IGME5000, Asch, 2003) with bedrock types reclassified in the following five categories: crystalline (all igneous and metamorphic rocks), carbonates (limestone, dolomite or carbonates as predominant bedrocks), secondary carbonates (limestone, dolomite or carbonates locally present but not as predominant bedrocks), other sedimentary rocks (like sandstone, conglomerate et clay) and undifferentiated bedrock. All environmental layers were resampled to a resolution of 300 m using the package "raster" in R (Hijmans and van Etten, 2016).

While Maxent is partly able to balance between model fitting and model simplicity, an information approach was adopted to check whether simpler models with fewer variables performed better (Warren and Seifert, 2011; Zeng et al., 2016). First, a reduced set of environmental variables was pre-selected, based on ecological or biological knowledge. Out of the 19 available bioclimatic variables, BIO1 (Annual Mean Temperature), BIO4 (Temperature Seasonality), BIO5 (Max Temperature of Warmest Month), BIO6 (Min Temperature of Coldest Month), BIO10 (Mean Temperature of Warmest Quarter), BIO11 (Mean Temperature of Coldest Quarter) BIO12 (Annual Precipitation), BIO 15 (Precipitation seasonality), BIO18 (Precipitation of Warmest Quarter) and BIO19 (Precipitation of Coldest Quarter) were selected. Then, a reiterative process of model formation and stepwise removal of the least contributing variables was utilized (Zeng et al., 2016). Unfortunately, most temperature related variables (BIO1, BIO5, BIO6, BIO10, BIO11) were highly correlated (pearson coefficient >0.7). In a similar way most rainfall related variables (BIO12, BIO18, BIO19) were highly correlated. Therefore, Maxent models were run in order to select the single most predictive temperature variable and the single most predictive rainfall variable. This was done (Table 2) by running Maxent models (regularization multiplier was set at 1 and regularization values were linear-quadratic-product) alternatively containing only one of the correlated variables for temperature (BIO1, BIO5, BIO6, BIO10, BIO11) and only one of the correlated variables for rainfall (BIO12, BIO18, BIO19). Among the alternative models the one with the lowest Akaike information criterion corrected for low sample size (AICc) calculatd with the software ENMTOOLS version 1.4.4 (Warren and Seifert, 2011; Shcheglovitova and Anderson, 2013) was selected. The AICc is an estimator of the relative quality of statistical models for a given set of data, trading-off between the simplicity of the model and the goodness of fit of the model (Warren and Seifert, 2011). Then, simpler models were constructed by stepwise removal of the variable with the lowest permutation importance (Zeng et al., 2016) and the AICc of the resulting models was calculated in order to select the final set of variables. As "feature class" and "regularization multiplier" affect the performance of the model (Morales et al., 2017) the model was fine tuned by constructing models with 7 levels of the regularization multiplier (0.05, 0.1, 0.5, 1, 2, 4, 10) and three regularization values (Linear-Quadratic, Linear-Quadratic-Product and Hinge). Finally, the best model (Regularization value = Linear-Quadratic; Regularization multiplier = 0.1) was run 100 times with bootstrap resampling and the average model was calculated. The quality of this final model was tested by calculating the area under the curve (AUC) of the receiver operated characteristic (ROC) plots in order to discriminate a species' model from a random model. Finally, the percent contribution and permutation importance of the environmental variables to the final Maxent model were measured (Phillips, 2006).

RESULTS

The Horvath's Lizard was found in four new sites (Valle di San Lucano, Val del Grisol, Casoni and Forra del Piave) and its presence was confirmed in three sites (Monte Tudaio, Val Zemola and Listolade) already known in literature (Fig. 1). Fig. 2 shows the habitat of the four new occurrences for Horvath's Lizard and Table 1 reports some characteristics of all the seven sites.

The common wall lizard was observed in 60% of the investigated sites (n = 71, Fig. 1) and was frequently syntopic with the Horvath's rock Lizard, where this last species occurred (Table 1).

Species distribution model.

The Mean Temperature of Coldest Quarter (BIO11) turned out to be a better predictor of the distribution of the Horvath's rock lizard (Table 2) compared to other highly correlated temperature variables: Annual Mean Temperature (BIO1), Max. Temperature of Warmest Month (BIO5), Min. Temperature of Coldest Month (BIO6) and Mean Temperature of Warmest Quarter (BIO10). The Precipitation of Warmest Quarter (BIO 18) turned out to be a better predictor of the distribution (Table 2) compared to other highly correlated precipitation variables: Annual Precipitation (BIO12) and Precipitation of Coldest Quarter (BIO19).

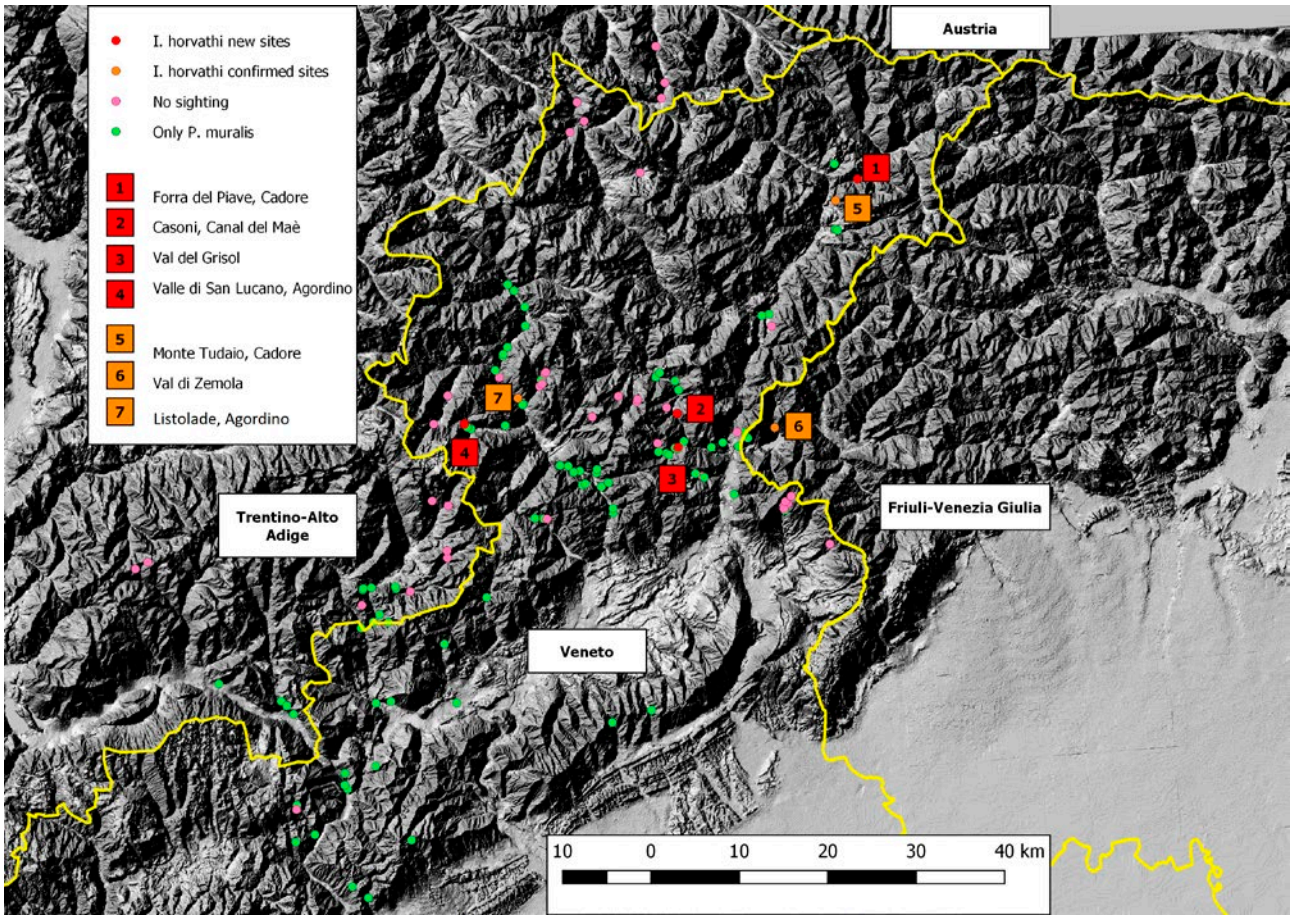

Fig. 1. Sites surveyed in 2016-2018. Red dots show the newly discovered occurrences of *Iberolacerta horvathi*, while orange dots show some confirmed occurrences.

The balancing between the simplicity of the model and goodness of fit to the known distribution resulted in a model that did not include Slope, Temperature Seasonality (BIO4) and Vegetation type (Corine Land Cover), the variables with the lowest permutation importance. As a result, the best model was explained only by Mean Temperature of Coldest Quarter (BIO11), Rainfall Seasonality (BIO15), Precipitation of Warmest Quarter (BIO18), Aspect, Roughness and Bedrocks (Table 2).

The best distribution model obtained after fine tuning (see methods and table 2) is shown in Fig. 3 (with cloglog output, which is the easiest to conceptualize as it gives an estimate between 0 and 1 of probability of presence). The model fits the distribution (average training AUC for the 100 bootstrap replicate runs is 0.922, standard deviation is 0.009) much better than a random model (AUC = 0.5). The variables that explain the distribution of the species are in order of percent contribution (Table 3): Roughness (33%), Bedrocks (26.5%), Mean Temperature of Coldest Quarter (19.5), Aspect (8.9), Rainfall of the Warmest Quarter (8.5%) and Rainfall seasonality (3.3).

Rainfall of the Warmest quarter decreases westward in the Italian Alps (Fig. 4): the 13 westernmost occurrences are in areas with a rainfall (Average±SD = 338 ± 37mm, n = 13) that is significantly lower (Welch Test = -9.67, $P < 0.001$) than the remaining occurrences (Average ± SD = 452 ± 53mm, n = 87).

DISCUSSION

Our field survey has discovered four new sites of Horvath's rock lizard in Veneto (Table 1 and Fig. 1 and 2): Forra del Piave is close to already known sites; Casoni (in the Bosconero mountain group) and Val Grisol (in the Schiara mountain group) fill the gap between the known westernmost site of Horvath's lizard and the previously known sites to the east; San Lucano Valley (in

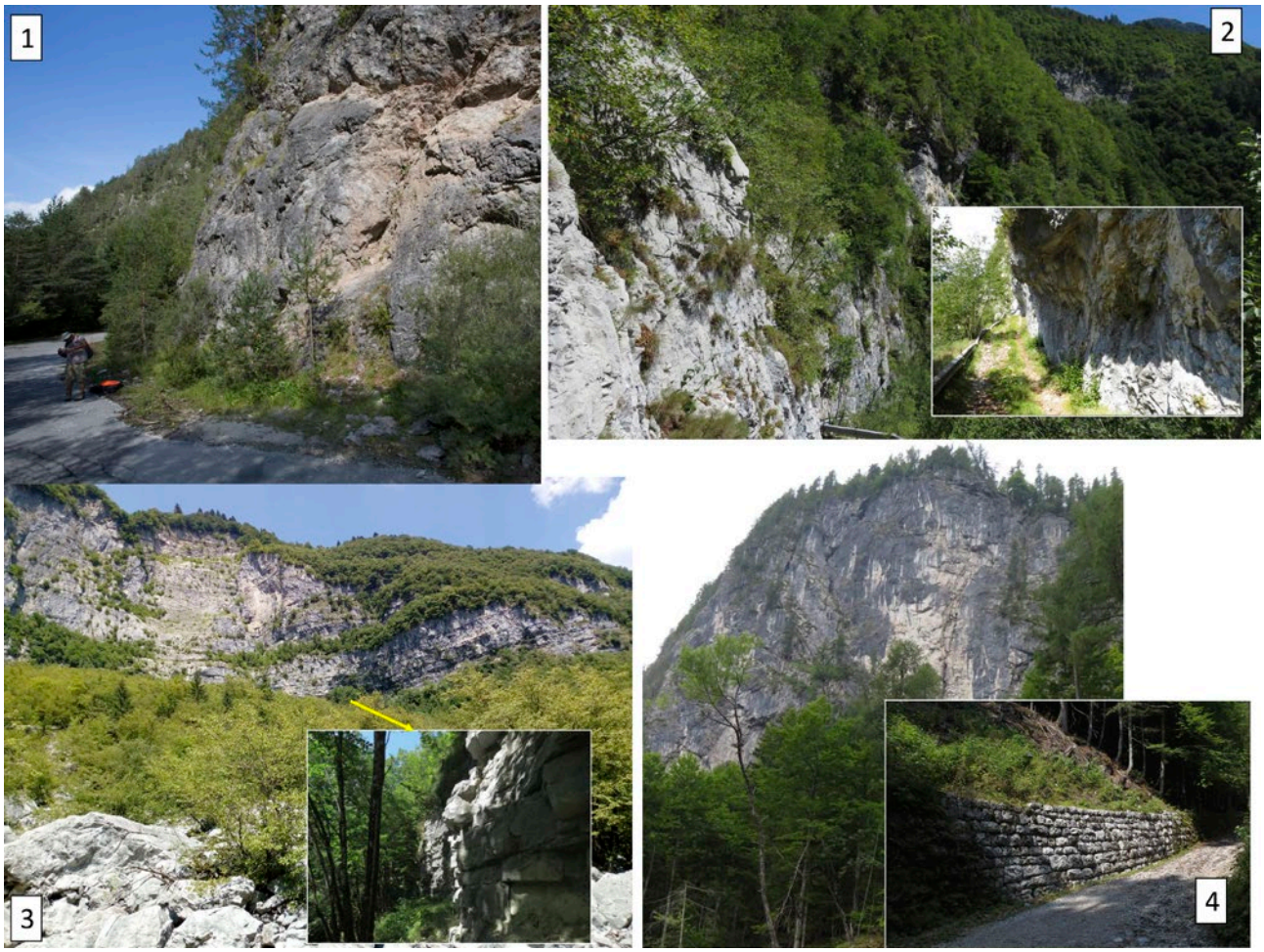

Fig. 2. Pictures showing the habitats of the newly discovered sites of Horvath's rock lizard. 1. Forra del Piave, Cadore; 2. Casoni, Canal del Maè; 3. Val del Grisol; 4. Valle di San Lucano, Agordino. In the last site, the first single individual was observed on the rock wall along the road; later, the presence of a more numerous population was documented in the neighbouring rocky cliffs (around 200 m away).

Pale di San Martino mountains) extends further west the known distribution by 9 km. Our extensive research in apparently suitable sites west of San Lucano Valley, in particular in the Vette Feltrine mountains and in the Valsugana Valley (Fig. 1 and Fig. 4), has been unable to discover additional sites. This lack of positive results suggests that the western limit of the distribution has been discovered. Interestingly, the San Lucano Valley is also the western limit in the southern Dolomite mountains of another reptile of eastern origin, the Nose-horned viper (*Vipera ammodytes*) (Sindaco et al., 2006), a species that has been found to have a close habitat similarity with Horvath's rock lizard also in another part of its distribution (Žagar et al., 2013). There is apparently no physiographic barrier to the spread of the lizards further to the west. As a matter of fact, a gravel bed mountain river like the Cordevole river (Fig. 4) should have not been a barrier to the westward spread of the species as proved by our discovery of the species just west of this river in the San Lucano Valley. In the past, gravel bed mountain rivers such as the Piave and the Cordevole (Fig. 4) should have been an even less effective barrier to the spread of the species during the phase of great sedimentation in the valleys that followed the deglaciation (Cordier et al., 2017) and continued up to 7000 BC in the Piave Valley (Carton et al., 2009): in this condition, water mostly flowed deep inside the gravel bed (Arscott et al., 2001) and could not stop the spread of lizards. Therefore, the lack of any apparent physiographic barrier lets us suggest that an environmental gradient should make the species less abundant in Veneto, where it is definitely rare, until it disappears. In general, an environmental variable might affect directly the species physiology (e.g., temperature) or might act in an indirect way by influencing

Table 1. Characteristics of the new and confirmed occurrences of Horvath's lizard surveyed in 2016-2018. The number associated to each site is the same as in Fig.1 and Fig. 2.

Site * new + confirmed	Date (day, month, year)	Coord. (°N, °E)	Altitude (m)	Aspect	Bedrock (from Geoportale nazionale italiano)	Vegetation (CNAT of Veneto and Friuli-Venezia Giulia)	Observed specimens	Syntopic Common Wall Lizard
1. Old road in Forra Piave, Santo Stefano di Cadore (BL), Veneto *	13.08.16 08.08.17	46.53516, 12.50901. 46.53258, 12.49400	825-870	S	R67: Neritic and platform dolomites (medium Triassic)	Scots pine oriental forest	Up to 3	Yes
2. Casoni, Canal del Maè, Forno di Zoldo (BL), Veneto *	25.08.17 27.08.17 01.07.18 08.07.18	46.30444, 12.22972	750-850	SO	R58: Pelagic limestones, marly limestone and marl (Jurassic)	Thermophilic calciphile beech forest of the Alps	Up to 8	Yes
3. Grisol de Entro, Val del Grisol, Longarone (BL), Veneto *	25.07.18	46.27017, 12.22878	825	SE	R58: Pelagic limestones, marly limestone and marl (Jurassic)	Ostrya carpinifolia thicket	1	Yes
4. Pont, San Lucano Valley, Taibon Agordino (BL), Veneto *	6.8.2018 9.8.2018 17.8.2018	46.30201, 11.91715. 46.30200, 11.91658. 46.30113, 11.91685.	1200-1250	NE-E	R67: Neritic and platform Dolomites (medium Triassic)	Calciphile fir forest of the Alps and central-northern Apennines	4	No
5. Monte Tudaio, Vigo di Cadore (BL), Veneto +	12.08.16	46.51413 12.47563	886	S	R67: Neritic and platform dolomites (medium Triassic)	Scots pine oriental forest	1	Yes
6. Val Zemola, Erto and Casso (PN), Friuli Venezia Giulia +	18.06.17 08.07.18	46.28607, 12.37178	1020	NE	R56: Neritic and platform limestones and sometimes dolomites (Jurassic)	Thermophilic calciphile beech forest of the Alps	Up to 2	No
7. Climbing rock, Listolade, (BL), Veneto +	22.05.16 25.09.16 13.04.17 09.09.18	46.32622, 11.99672	720	SO	R67: Neritic and platform Dolomites (medium Triassic)	Acidophile scots pine forest	Up to 11	Yes

the availability of food (e.g., rainfall) or the presence of parasites, predators or competitors (Austin, 2002). An important factor that might limit the abundance and the distribution of the Horvath's rock lizard may be the strong competition with the common wall lizard (*Podarcis muralis*) (Carranza et al., 2004). The two species have a similar overall body plan (Žagar et al., 2012) and similar ecological preferences, for example they eat similar food (Richard and Lapini, 1993). However, Horvath's rock lizards appear more adapted to rocky habitats due to their flat head and body (Corti et al., 2010), which might favour hiding in small crevices. They are more adapted to relatively cold habitats due to a higher potential metabolic activity (Žagar et al., 2015b), even if they do not exhibit the partial development of the egg inside the female body that is found in some other small mountain lizards (Ljubisavljević et al., 2012). On the contrary, common wall lizards are favoured in agonistic interactions with Horvath's rock lizards because of their larger head and consequent stronger biting force (Žagar et al., 2017), for example when competing for thermoregulation spots (Žagar et al., 2015a). Common wall lizards may be favoured as well for their higher reproductive potential (Amat, 2008). As a result, Horvath's rock lizards are confined to rocky habitats in low altitude shady gorges, at mid altitude in beech forest and to higher altitudes compared to the common wall lizards (Žagar, 2016). Vegetation type has not turned out to be important in our analysis for predicting the distribution of the Horvath's rock lizards likely because the Corine Land Cover classification that is currently available for our study area has been developed only to the "third level". This means, for example, that forests are classified only to the level of "coniferous forests" or "mixed forests", a level that is likely not enough for a proper classification.

Apart from the land cover classification, our analysis of the importance of the variables that constrain the distribution of the Horvath's lizard (Table 3) largely agrees with the previous results (Cabela et al., 2007; Žagar, 2016).

Table 2. AICc scores of different Maxent species distribution models varying in number of variables, features and regularization multipliers (see methods for details). The best model (in bold) is the one with the lowest AICc

Model #	Variables	Features	Reg.mult	AICc
1	BIO1,BIO4, BIO12, BIO15, Aspect, Asperity, Slope, Corine, Bedrock	LQP	1	2459.82
2	BIO5,BIO4, BIO12, BIO15, Aspect, Asperity, Slope, Corine, Bedrock	LQP	1	2468.09
3	BIO6,BIO4, BIO12, BIO15, Aspect, Asperity, Slope, Corine, Bedrock	LQP	1	2472.79
4	BIO10,BIO4, BIO12, BIO15, Aspect, Asperity, Slope, Corine, Bedrock	LQP	1	2465.77
5	BIO11,BIO4, BIO12, BIO15, Aspect, Asperity, Slope, Corine, Bedrock	LQP	1	2453.00
6	BIO11,BIO4, BIO18, BIO15, Aspect, Asperity, Slope, Corine, Bedrock	LQP	1	2447.56
7	BIO11,BIO4, BIO19, BIO15, Aspect, Asperity, Slope, Corine, Bedrock	LQP	1	2463.85
8	BIO11,BIO4, BIO18, BIO15, Aspect, Asperity, Corine, Bedrock	LQP	1	2452.31
9	BIO11, BIO18, BIO15, Aspect, Asperity, Corine, Bedrock	LQP	1	2447.72
10	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQP	1	2429.10
11	BIO11, BIO15, Aspect, Asperity, Bedrock	LQP	1	2430.33
12	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQP	0.05	2424.50
13	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQP	0.1	2431.21
14	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQP	0.5	2424.09
15	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQP	2	2442.47
16	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQP	4	2462.02
17	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQP	10	2529.97
18	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQ	0.05	2423.22
19	**BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock**	**LQ**	**0.1**	**2422.58**
20	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQ	0.5	2424.63
21	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQ	1	2431.17
22	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQ	2	2447.09
23	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQ	4	2459.09
24	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	LQ	10	2530.51
25	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	H	0.5	2795.60
26	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	H	1	2602.81
27	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	H	2	2529.67
28	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	H	4	2532.34
29	BIO11, BIO18, BIO15, Aspect, Asperity, Bedrock	H	10	2571.21

Table 3. Relative contributions of the environmental variables to the best Maxent model (see methods for details) for the distribution of Horvath's rock lizard.

Variable	Percent contribution (%)	Permutation importance (%)
Roughness	33.3	26.7
Bedrock	26.5	16.6
Temperature of the coldest quarter	19.5	38
Aspect	8.9	6
Rainfall of the warmest quarter	8.5	3.5
Rainfall seasonality	3.3	9.2

Roughness turns out to be important certainly due the preference of the Horvath's lizard for steep rocky areas. However, the pixel resolution of our analyses (300 m), constrained by the imprecision of the coordinates of the occurrences, is not enough to discriminate between rocky and simply steep slopes. That's why some areas that are likely unsuitable, like Visentin Mountain just south of Belluno, are predicted as suitable (Fig. 3 and 4).

The categorical variable Bedrock confirmed the importance of sedimentary rocks for the presence of the species with the highest suitability (more than 70%) in areas with the dominant bedrocks consisting of carbon-

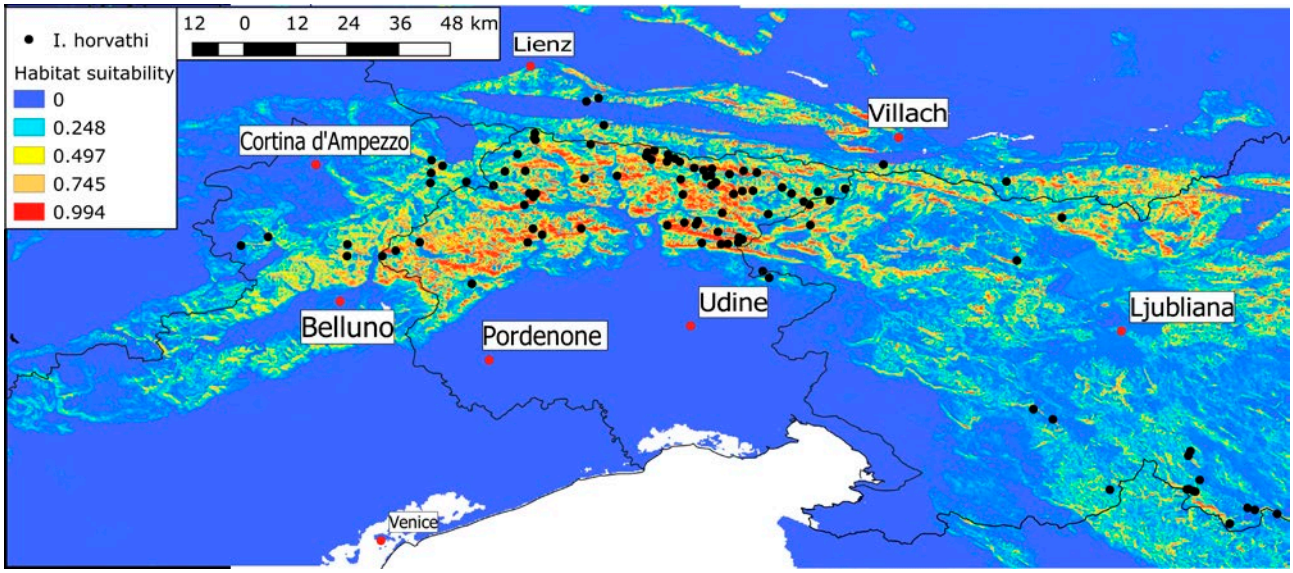

Fig. 3. The best species distribution model of Horvath's rock lizard in the Alps and neighbouring Dinaric Chain based on 100 occurrences (black dots) in Italy, Austria and Slovenia. The output is "cloglog", which gives an estimate between 0 and 1 of the probability of presence.

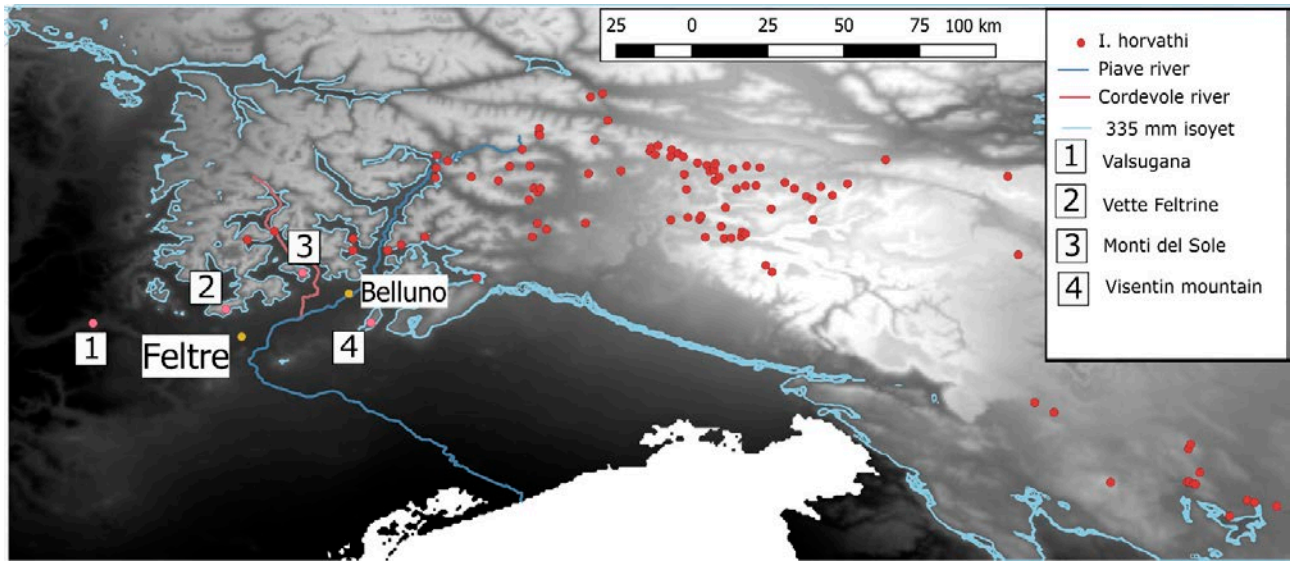

Fig. 4. Rainfall (the lighter areas the higher rainfall) during the warmest quarter. Yellow dots show the 100 occurrences of Horvath's rock lizard. The light blue line is the 335 mm isohyet. The location of some areas mentioned in the Discussion are added.

ates, dolomite and limestone, and with an intermediate suitability (40%) for areas where these three types of rocks are present, even if they are not dominant. A lower suitability (about 28%) was found for other types of sedimentary bedrocks. The remaining two types of bedrocks (metamorphic-magmatic and undifferentiated) were predicted as unsuitable. This result likely stems from the need of the lizard for fissured rocks where they can hide from predators and where rock dwelling plants (important for attracting the invertebrate food) can take root.

The inclusion of temperature as an important variable was similarly expected as the species is generally considered cold-adapted and able to live at higher altitudes and in shadier places compared to the frequently syntopic common wall lizard (Žagar, 2016). Why the temperature of the coldest quarter turned out to be the most impor-

tant temperature related variable is not clear. It might be related to the duration of the snow cover and a too brief warm season for the species to thrive. Instead, the importance of a physiological limit for survival at low temperatures would have probably resulted in a higher importance of the minimum winter temperature.

Aspect is of course important as the lizards need direct sunlight for warming up and the north aspect is unsuitable (Žagar, 2008a). So, in spite of the low resolution of the occurrences that might mask the exact aspect of the cliff, this variable is a significant percent contribution (8.9%) in explaining the best model of distribution.

Our model shows the importance of two rainfall related variables, rainfall seasonality and average rainfall during the warmest quarter. Rainfall seasonality increases westward in the Eastern Alps (data not shown) but why rainfall seasonality might be important for the ecology of the species is not clear yet. Its ability to explain the distribution is, at any rate, very low (3.3%). The higher percent contribution of summer rainfall (8.5%) might stem from its likely importance to the growth of rupicolous plants where the Horvath's rock lizards look for food. Common wall lizards are comparatively less dependent on this hunting ground as they are much more euryecious (Sindaco et al., 2006). In addition, summer rainfall might be directly important as a source of drinking water as we have observed Horvath's lizards drinking in wet, dripping parts of the cliffs. Interestingly summer rainfall decreases westwards in the eastern Alps and the 13 westernmost occurrences (almost all in Veneto) are all close to the 335 mm isohyet (Fig. 4). So, the rarity of the species west of the Piave river and its disappearance further west might effectively depend upon a reduced rainfall during the warmest quarter. Two mountain groups, the Monti del Sole and the Vette Feltrine, appear suitable (Fig. 3) and are not separated by any significant physiograghic barrier from the four westernmost occurrences (Fig. 4); they should deserve additional field surveys even if a recent research (Cassol et al., 2017) and our data have so far failed to confirm the species in those areas.

The major limitation of our study comes from the low precision of the occurrences that did not allow a deeper analysis of the roughness and the inclusion of other factors that can be important, such as solar radiation, which can change radically within a few meters in rocky areas and is certainly important in the context of the competition with the common wall lizard. Future species distribution models based on more precise occurrences (Ernst, 2017) and on finer scaled climatic variables (now at 1 km resolution) can improve our understanding of the ecological niche of the species. Hopefully, phylogeographic studies (Cocca et al., 2016) will soon shed light on the glacial refugia where the Horvath's lizards survived before colonizing the deglaciating Alps.

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First record of underwater sound produced by the Balkan crested newt (*Triturus ivanbureschi*)

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Abstract. This study presents first evidence for underwater sounds produced by adult Balkan crested newts (*Triturus ivanbureschi*). Recordings were made in spring of 2019 in controlled laboratory conditions using a commercially available omnidirectional hydrophone connected to a linear PCM recorder. A total of 27 animals (21 males, 6 females) were recorded under different conditions: (a) alone in an empty tank, (b) alone in a tank full of vegetation, and (c) a pair in an empty tank. Results indicated that both male and female newts produced a click-like sound with a mean duration of 34 ms (± 5.31 SD; range: 27-51) and mean peak frequency of 1887 Hz (± 405 SD; range: 1162-2770). Not all newts tested produced sounds and there were no statistically significant differences between males and females or recordings under different conditions in terms of click number, duration and frequency parameters, with the exception of the ratio of peak frequency/bandwidth at 50% peak amplitude, which was lower for clicks produced in the vegetated tank. Newt snout-vent length and body mass also had no effect on any of the studied parameters. The obtained results suggest that clicks could have a function in orientation and exploratory behaviour.

Keywords. Clicks, conditions, environment, interaction, orientation, phase.

INTRODUCTION

Sound production plays an important role for many taxa across the animal kingdom. For amphibians, this is mainly true for anurans – the first early attempts at more detailed studies of the functions of vocal signals in anurans date from the first half of the 20th century (Noble and Noble, 1923), and the first comprehensive work on acoustic communication in amphibians and reptiles is by Bogert (1960), which is also the first attempt to view the information of amphibian vocal signals in an evolutionary and ecological frame. In recent decades there is a growing number of publications describing the structure of anuran calls (e.g., Ziegler et al., 2011; Guerra et al, 2018; Stanescu et al., 2018; Carvalho et al., 2019), geographic variations of vocal signals in some species (e.g., Amezquita et al., 2009; Kaefer and Lima, 2012), as well as the role of the signals as phylogeographic indicators (e.g., Stöck et al., 2008; Vences et al., 2013).

In contrast, caudate amphibians have received very little attention in this regard. While they lack a tympanic middle ear, a number of studies have established that their underwater auditory abilities are sufficient for them to sense sound frequencies by other means, such as their mouths or lungs (e.g., Hetherington and Lombard, 1983; Christensen et al., 2015). Available data on their sound production is limited to just a few taxa, and even then, the information is mostly descriptive, with little effort to analyse its ecological function. In addition, most of the data is on North American species, leaving the rest of the world almost unstudied. Even though a variety of sounds made by caudate amphibians have been described as early as the mid-twentieth century (Maslin, 1950; Neil, 1952), they were assumed to be mostly non-func-

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tional and produced by unintentional expulsion of air (Bogert, 1960). On land, some caudate species are known to produce squeaking noises when stressed ("mouse-like squeaking note"; Maslin, 1950). Although a number of authors have registered underwater hisses, clicks or squeaks in various species (Maslin, 1950; Gehlbach and Walker, 1970; Wyman and Thrall, 1972; Davis and Brattstrom, 1975; Crovo et al., 2016), their exact purpose is still unknown. Maslin (1950) suggests they are unintentional, but others propose they could serve a purpose in social interactions (Gehlbach and Walker, 1970; Davis and Brattstrom, 1975; Crovo et al., 2016) or orientation (Gehlbach and Walker, 1970). A recent study by Hubáček et al. (2019) registered a high number of underwater low and mid-frequency clicks produced by *Ichthyosaura alpestris* and *Lissotriton vulgaris*, suggesting that newts are much more vocally active than demonstrated by currently available data.

This study tested the hypothesis that the Balkan crested newt *Triturus ivanbureschi* Arntzen & Wielstra, 2013 was vocally active underwater. The aim was to describe the registered sounds and to present possible explanations for the potential role of the produced clicks in the species orientation or interaction.

MATERIALS AND METHODS

Crested newts (*Triturus cristatus* superspecies) are a group of closely related species with medium size (mean adult snout-vent length [SVL] varies between 62.5-84.4 mm for different species, review in Lukanov and Tzankov, 2016) and a biphasic lifestyle (aquatic and terrestrial phase) that are distributed parapatrically in the Old World. The Balkan crested newt is distributed in both Europe and Asia – its range covers the Eastern Balkan Peninsula, including most of Bulgaria, the Eastern parts of Greece, North Macedonia and Serbia, as well as Northwestern Turkey (Speybroeck et al., 2016). In Bulgaria, the species occurs across most of the country from sea level up to 1700 m elevation, inhabiting various types of lentic water bodies (Stojanov et al., 2011). While other newt species (e.g., the Alpine newt *I. alpestris*) have been observed to produce squeaks when handled out of the water or soft gulping sounds when taking air at the water surface (Maslin, 1950), there is no literature data on the sound production of any Crested newt species.

In April 2019 a total of 27 adult newts (21 males, 6 females) were caught using funnel traps in a small natural pond overgrown with bulrush (*Typha* sp.), yellow iris (*Iris pseudacorus*) and common bladderwort (*Utricularia vulgaris*) near the city of Sofia, Bulgaria (42°35'42"N, 23°22'5"E). All animals were housed indoors in a large (100×100×60 cm) glass tank filled with water and were fed common earthworms (*Lumbricus terrestris*) ad libitum. Ambient temperature was constant at 18 °C.

Experiments were conducted in a small glass tank (40×20×60 cm) with water level at 15 cm, with the hydrophone completely submerged 5 cm below the water surface and fixed in such a position as to not touch the bottom or the sides of the tank. Three types of trials were conducted to test for sound production under different conditions – single newt in an empty tank (trial 1), single newt in a tank full of water vegetation (trial 2), and a pair of newts in an empty tank (trial 3). The pair in the third trial would consist of female/female, female/male or male/male. The trials were designed in order to assess whether any registered sounds played a role in orientation or social interaction. After sex determination, newts were placed in the tank individually for the first two trials or as a pair for the third trial, and after 5min of adaptation, newt sounds were recorded for the next 10 min. Snout-vent length (SVL) of all newts was measured with a ruler (to 0.1 cm) and body mass was weighted (to 0.01 g) using digital balance scales (Durascale D2 capacity pocket scale). Because of the crepuscular/nocturnal lifestyle of the study species, all trials were performed in complete darkness. No animals were used in the same trial twice (except for the six females, which were used once in female/female pairs and a second time in female/male pairs) or recorded more than once per day. Vegetation used in the second trial was collected from the study pond and consisted of bulrush and common bladderwort leaves (both freshly cut and old); it covered $^{2}/_{3}$ of the tank, with the hydrophone fixed in the other $^{1}/_{3}$. During the tests there was no human presence in the room and newts had approximately three days to rest between trials. All experiments complied to the international requirements for ethical treatment of animals (Lehner, 1996) and after they were completed, all newts were released at the site of capture.

Vocal activity was recorded using an Olympus LS-5 linear PCM recorder and an omnidirectional Aquarian H2a hydrophone (sensitivity: -180 dB re: 1V/µPa, useful range: 10 Hz to 100 kHz). Recordings were made in a WAV-PCM mode with sampling frequency of 44.1 kHz, 20-21.00 Hz and 24-bit resolution. The recordings were processed with the open source software Soundruler V. 0.9.6.0. (Gridi-Papp et al., 2007). The following parameters were measured: (1) number (the number of clicks produced during each recording), (2) duration (difference between the beginning and the end of a click, measured in ms from the spectrogram), (3) peak frequency (click frequency with the highest energy, measured in Hz), (4) tune 50 (the ratio of peak frequency/bandwidth at 50% peak amplitude), and (5) tune 10 (the ratio of peak frequency/bandwidth at 10% peak amplitude). Spectrograms used for the measurements of click duration had a Hanning window type and an FFT length of 512 points.

All data was tested for normality using a Shapiro-Wilk test and the null hypothesis was rejected ($P < 0.001$). A Mann-Whitney U test was used to compare clicks produced by males and females in the solitary treatment, and a Kruskal–Wallis H test was used to check for differences in the measured parameters between recordings under different conditions. A Spearman rank order correlation was used to test whether SVL and body mass were related to the studied sound parameters. A Kruskal-Wallis H test revealed no statistically significant differences between pairs in trial 3 (see Appendix) and they were treated as one sample for the statistical analyses in the comparison between the trials. The chosen level for statistical significance

was P < 0.05. All analyses were carried out using the computer program Statistica v.7.0 (StatSoft, Inc., 2004).

RESULTS

On average, newts started to produce clicks after 173 s (± 122 SD, range: 8-396) and although that latency varied widely between individuals, there were no statistically significant differences across trials (Kruskal-Wallis H test; H(2) = 0.148, P = 0.929) or sexes (Mann-Whitney U test; U = 68.50, P = 0.856). All registered clicks had a simple non-harmonic structure and their peak frequency was in the lower ranges of the spectrum (Fig. 1). Descriptive statistics for all measured parameters are presented in Table 1.

The Mann-Whitney U test revealed there were no statistically significant differences between sexes in all studied parameters of the registered clicks: number (U = 322.500, P = 0.582), duration (U = 621.500, P = 0.473), peak frequency (U = 449.500, P = 0.490), tune 50 (U = 401.500, P = 0.193) and tune 10 (U = 424.500, P = 0.313).

Results from the Kruskal–Wallis H test used for the comparison of the three trials were similar – there were no statistically significant differences among the trials in terms of number (H(2) = 0.443, P = 0.801), duration (H(2) = 5.319, P = 0.070) and peak frequency (H(2) = 3.910, P = 0.142) of the clicks, as well as in parameter tune 10 (H(2) = 2.829, P = 0.243). However, there was a significant difference between the parameter tune 50 from trial 2 and the other two trials - H(2) = 18.067, P = 0.001; values for this parameter were significantly lower in trial 2 compared to trial 1 and trial 3 (Table 1).

Normally clicks were not produced in series, but on three occasions (two in trial 1 and one in trial 2) there were more than one in quick succession. In two of the cases (one in trial 1 and trial 2) the duration of the intervals between the clicks was comparable to that of the clicks themselves at 69 ms and 34 ms, respectively; in the third case, there were three clicks in the space of around one second, with the intervals between them standing at 551 ms and 325 ms.

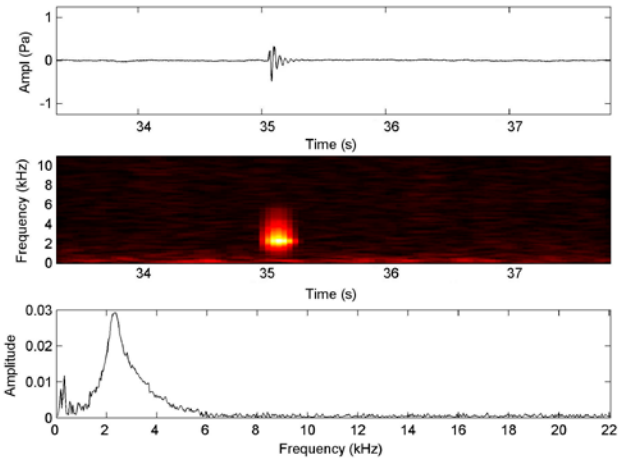

Fig. 1. From top to bottom: indicative oscillogram, spectrogram and amplitude spectrum of a click.

There was a statistically significant difference in both SVL and body mass between males and females, but there was no significant effect of SVL and body mass on any of the studied parameters (Table 2).

It has to be noted that during handling of the animals, 10 (two females, eight males) out of the 27 newts produced both clicks and squeaks – however, attempted recordings of these sounds were too faint to analyse and therefore were discarded.

Table 1. Measured parameters of the recorded clicks in *T. ivanbureschi* (number of vocalizing newts/pairs is in parenthesis). Statistically significant differences between trials are in bold; when no such differences are present, a combined value for all groups is given in the last column. Data is presented as Mean (Min-Max ± SD).

	Empty tank (trial 1), n = 27			Vegetated tank (trial 2), n = 27			Pair (trial 3) n = 13 (9)	All groups
	Male (13)	Female (4)	Combined (17)	Male (9)	Female (4)	Combined (13)		
Total clicks	26	4	30	28	11	39	17	86
Number	1.14 (0-4 ± 1.195)	1 (0-2 ± 0.632)	1.111 (0-4 ± 1.086)	1.24 (0-7 ± 2.022)	2.16 (0-8 ± 3.060)	1.444 (0-8 ± 2.258)	1.211 (0-3 ± 1.182)	1.260 (0-8 ± 1.625)
Duration	36 (26-51 ± 5.910)	33 (29-36 ± 2.655)	36 (26-51 ± 5.500)	35 (27-51 ± 6.910)	33 (27-39 ± 3.781)	34 (27-51 ± 6.050)	34 (30-40 ± 2.783)	34 (27-51 ± 5.314)
Frequency	1920 (1392-2656 ± 288)	2096 (1162-2684 ± 510)	1961 (1162-2684 ± 348)	1769 (1397-2346 ± 375)	1785 (1392-2340 ± 379)	1774 (1392-2346 ± 370)	2026 (01162-2770 ± 535)	1887 (1162-2770 ± 405)
Tune 50	9.919 (2.300-26.622 ± 6.709)	6.894 (2.232-11.214 ± 3.751)	**9.221 (2.232-26.622 ± 6.222)**	3.985 (1.599-6.779 ± 1.348)	3.628 (1.625-6.002 ± 1.5508)	**3.877 (1.599-6.779 ± 1.398)**	**8.980 (2.139-16.154 ± 5.146)**	N/A
Tune 10	1.906 (0.494-5.189 ± 1.214)	2.322 (0.527-5.378 ± 2.311)	2.002 (0.494-5.378 ± 1.490)	1.224 (0.646-3.257 ± 0.538)	1.122 (0.644-1.499 ± 0.326)	1.193 (0.644-3.257 ± 0.481)	1.861 (0.485-8.389 ± 2.082)	1.606 (0.485-8.389 ± 1.329)

Table 2. Measurements of SVL and body mass - comparison between sexes and correlations across studied parameters, with statistically significant differences in bold. Data is presented as Mean (Min-Max ± SD).

	SVL (cm)	Body mass (g)
Males	7.75 (7.20-8.70 ± 0.43)	12.01 (7.75-20.35 ± 2.79)
Females	8.3 (7.90-9.10 ± 0.46)	18.57 (12.71-21.42 ± 3.04)
Mann-Whitney U test	**U = 20.00, P = 0.012**	**U = 10.00, P = 0.002**
Spearman rank order correlation test		
Number	r = 0.074, P = 0.592	r = 0.199, P = 0.149
Duration	r = -0.218, P = 0.074	r = -0.074, P = 0.548
Frequency	r = 0.003, P = 0.980	r = -0.002, P = 0.990
Tune 50	r = -0.045, P = 0.733	r = 0.052, P = 0.698
Tune 10	r = -0.110, P = 0.408	r = -0.097, P = 0.466

DISCUSSION

The study demonstrated that the Balkan crested newt emits sounds underwater. Parameters of produced clicks highly overlapped between sexes and across the three test trials, with SVL and body mass also having no visible effect. While underwater sound production has been previously documented in four urodelan families – Ambystomatidae (Wyman and Thrall, 1972; Licht, 1973), Amphiumidae (Crovo et al., 2016), Salamandridae (Davis and Brattstrom, 1975; Hubáček et al., 2019) and Sirenidae (Gehlbach and Walker, 1970) – this is the first report for representatives of the Crested newts species group, and the second on European salamandrid species, following the recent study of Hubáček et al. (2019). Wien et al. (2011) assert that sound-producing lineages of salamanders have split from their common ancestors around 60 mya, which indicates that sound production is either a shared trait among newts, or has evolved independently in several lineages. Steinfartz et al. (2007) estimate that the last common ancestor of the *Triturus* species group lived around 64 mya, which is a very similar timeline. The latter study established that the *Triturus* assemblage included four monophyletic groups: large-bodied newts (incl. *T. ivanbureschi*, referred to as "*T. karelinii*"), small-bodied newts (incl. *L. vulgaris*, study species in Hubáček et al., 2019), *I. alpestris* (study species in Hubáček et al., 2019) and *Ommatotriton vitatus*. This early separation between large-bodied Crested newts and the smaller species of the assemblage offers at least partial explanation for the differences between the produced sounds. Hubáček et al. (2019) established two types of clicks (low frequency and mid-high frequency), but found no difference between clicks produced by *L. vulgaris* and *I. alpestris*. Their mean duration varied between 7.67 ms and 10.74 ms for the low clicks and between 10.60 ms and 12.14 ms for the mid-high clicks, which is around three to five times shorter than the clicks recorded in the present study. The low-frequency clicks had a mean peak frequency between 7.46 kHz to 7.97 kHz, which is still significantly higher than the average of 1.89 kHz established for *T. ivanbureshi*. In any case, direct comparisons should be considered with caution, as some of the differences could be due to the different equipment and settings used for the recordings.

Nevertheless, it has to be noted that clicks in *T. ivanbureschi* were more similar to the ones in the American salamandrid species *Taricha torosa*, which had duration between less than 0.1 s up to 0.4 s and frequency of 1.4-8 kHz (Davis and Brattstrom, 1975). Results of the present study also seem to be consistent with the authors' observation that clicks in *T. torosa* were produced during exploratory behaviour, when newts were placed in an unfamiliar setting; however, additional studies are needed in order to clarify this. Both species are of similar size and overall body shape (in contrast to *L. vulgaris* and *I. alpestris*, which are smaller and less robust), but at present more studies are needed in order to establish whether phenotypic similarities could result in production of similar sounds. Unlike *T. ivanbureschi*, clicks produced by *T. torosa* did have a harmonic structure, with harmonics at 3.5, 5 and 6.5 kHz (Davis and Brattstrom, 1975). While there was only one statistically significant difference between trial 2 and the other two trials (parameter tune 50), this and the relatively higher number of recorded clicks in trial 2 could implicate the role of sound in *T. ivanbureschi* orientation. For some anurans, call parameters such as call rate and pulse number have been established to have a highly directional effect (Gerhardt, 1991) and this could also be true for newts, with the dynamic ratio of frequency/amplitude being beneficial in orientation. While data for definitive conclusions is still lacking, there are studies demonstrating that acoustic information in the form of anuran calls improves orientation in other newt species (e.g., Diego-Rasilla and Luengo 2004, 2007). The only other study on sound production in caudate amphibians that also provides data for body mass is that of Hubáček et al. (2019), which also did not find any relation between weight and produced clicks, so the possible role of SVL and body mass on sound production in newts remains to be clarified. The fact that some of the newts used in this study produced clicks while being handled out of the water could indicate that clicks are some form of a distress signal, or displacement activity (involuntary response) – the latter has been demonstrated for visual signals in some anuran species (Furtado et al., 2016); however, since recordings of terres-

trial clicks could not be analysed, it is yet unclear if they are identical to the ones registered underwater.

Wyman and Thrall (1972) report two types of clicks for *Ambystoma maculatum,* with mean frequency and duration around 1.5 kHz/0.09 s and 0.5-6.5 kHz/0.04 s, respectively. The authors state that while the behavioural significance of both sounds was unknown, the lower frequency click was only recorded during the breeding season, suggesting a possible role in reproduction. Considering that April is peak breeding season for *T. ivanbureschi*, such a possibility cannot be excluded, but it is still not very likely, as none of the pairs in trial 3 produced significantly more clicks. However, juveniles or adult newts outside of the breeding season were not tested in the present study, so additional research is needed in order to establish the potential role of age and phase in sound production for this species. For the congeneric species of *A. gracile*, Licht (1973) describes sounds that are very similar to those of *A. maculatum* (peak frequency 0.4-2.5 kHz, duration 0.04-0.06 s) and suggests that they are associated with both defensive and aggressive behaviour. Again, this seems unlikely for *T. ivanbureschi*, as in trial 3 there were no signs of antagonistic behaviour.

While Crovo et al. (2016) report both low- and high-frequency clicks in *Amphiuma means* as having a role in social interaction, they admit that "the high-frequency clicks produced, however, were not associated with high-frequency hearing" – i.e., their behavioural significance remains unclear. Low-frequency clicks were associated with communication in *Siren intermedia*, but the authors also suggest that the acoustic signals may play a role in orientation when visual and olfactory cues are absent (Gehlbach and Walker, 1970). Both of the abovementioned species are exclusively aquatic, while *T. ivanbureschi* has a pronounced biphasic lifestyle, meaning that underwater sound communication could be expected to be less complex in this species.

While results for the sounds produced by *T. ivanbureschi* are not conclusive, they imply that sound plays a certain role in this species behaviour. It has to be said that sound production in caudate amphibians is likely to prove a more productive area for scientific research than previously thought - even the few existing studies so far exhibit a large variation of results and different interpretations. Crested newts in particular could be suitable study species because of their well-studied phylogeny, allowing for better inter-species comparisons.

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APPENDIX

Results of the Kruskal-Wallis H test for comparison of the studied parameters between newt pairs (female/female, female/male, male/male), n = 13.

- Number of clicks: $H(2) = 0.952$, $P = 0.621$
- Duration of clicks: $H(2) = 4.532$, $P = 0.104$
- Peak frequency: $H(2) = 0.344$, $P = 0.842$
- Tune 50: $H(2) = 0.557$, $P = 0.757$
- Tune 10: $H(2) = 0.524$, $P = 0.769$

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Identification of biologically active fractions in the dermal secretions of the genus *Bombina* (Amphibia: Anura: Bombinatoridae)

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Abstract. Amphibian skin secretions have long been considered a convenient and useful natural source of bioactive compounds, but a comprehensive study of the effects of their dermal secretions on diverse parameters of the hemostasis system has not yet been carried out. This study aimed at identifying biologically active fractions in the skin secretions of *B. bombina*, *B. variegata*, and their hybrid – *B.bombina* × *B.variegata,* and to clarify whether their components can modify certain parameters of the hemostasis system. For the skin secretion analysis, we performed ion-exchange chromatography, electrophoresis, and zymography assays. Plasma coagulation tests, chromogenic assays, and platelet aggregation assays were also conducted *in vitro*. As a result of the fractionation, a number of fractions were identified, where the proteins with miscellaneous molecular weights were revealed. The data also suggested that some fractions have proteolytic enzymes with gelatinolytic, fibrinogenolytic and collagenolytic activities. The proteins present in the fraction #5 of *B. variegata* and #5 of the hybrid secretions are characterized by the ability to prolong the clotting plug formation in the aPTT. Proteins capable of inducing platelet aggregation in the rabbit PRP are present in the fraction #3 of *B. variegata* secretions. The ability of dermal components to activate plasma proenzymes is indicative of the fact that the non-protein components of fraction #9 of *B. variegata* and fraction #7 of hybrid secretions initiated the appearance of thrombin and activated protein C in plasma.

Keywords. Amphibian skin secretions, hemostasis system, fractionation.

INTRODUCTION

Recently, a great deal of emphasis has been placed on the study of the effects of biologically active compounds derived from the sources of natural origin (Dias et al., 2012; Veeresham, 2012). These agents have found their applications in the treatment of pathological conditions, diagnosis of diseases, and have served as valuable tools in laboratory studies. Moreover, the heightened need of society for new potent medicines with strong efficiency and high safety profile, along with the increased price for drugs based on synthetic compounds, places importance on the use of natural raw material to search for various biologically active substances.

Amphibian skin secretions are enriched with complex cocktails of bioactive molecules spanning a wide spectrum of biological actions (Erspamer and Melchiorri, 1980; Daly, 1995; Conlon and Leprince, 2010). Although recently there has been a spate of interest concerning the potential therapeutic effects of the biologically active compounds derived from amphibians' dermal secretions (Mor et al., 1994; Bevins and Zasloff, 1990), most of these substances are not yet widely implemented in medical and pharmaceutical industry, as the mechanism of their action and possible side effects remain insufficiently studied (König et al., 2015).

The results of our previous study showed that the crude skin secretions of ten species of amphibians: *B.*

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bombina, *B. variegata*, *B. bufo*, *B. viridis*, *R. temporaria*, *P. ridibundus*, *P. esculentus*, *P. fuscus*, *S. Salamandra*, and the hybrid of *B. bombina* and *B. variegata*, had a pronounced protease activity with wide substrate specificity (Nikolaieva et al., 2018). In fact, it was proved that these dermal venoms can be a potential source of proteolytic enzymes. In the other research we demonstrated that the whole skin secretions affect the parameters of clotting plug formation (Udovychenko et al., 2018). It was shown that the *B. bombina*, *B. variegata* and their hybrid, *R. temporaria*, and *P. ridibundus* crude skin secretions prolonged the time of fibrin clot formation in the APTT. In contrast, the components of *B. viridis*, *P. esculentus*, *P. fuscus*, and *S. salamandra* prolonged the TT. Another research of ours showed the presence of biologically active compounds in the *P. fuscus* crude skin secretions that affect some parameters of the hemostasis system (Udovychenko et al., 2019). Our findings confirm that amphibian skin secretions are a complex material which contains a variety of biologically active substances that differ in their biological effects. So, to identify the possible mechanism of action or/and to define the components present in the whole skin secretions, fractionation of the source material is required.

Several research projects have been conducted concerning the study of the effects of skin secretion components of the toads that belong to the genus *Bombina*. A multitude of antimicrobial peptides from their dermal secretions have been discovered (Simmaco et al., 1998). For example, peptides called bombenins were isolated and found to possess antimicrobial activities, which have not been detected in other amphibian genera (Simmaco et al., 1991). In addition, bombenins are among the most studied amphibian constituents, and numerous studies have been published describing the various pharmacological activities of bombesin and its homologues (Gonzalez et al., 2008). The in vitro antitumor assay conducted by Zhou et al. (2018) showed that the bombinin-like peptide and bombinin H type peptide possessed obvious antiproliferative activity on three human hepatoma cells (Hep G2/SK-HEP-1/Huh7) at the nontoxic doses. This indicates that the peptide family of bombinins could be a potential source of drug candidates for anti-infection and anticancer therapy. A few studies also report on the antidiabetic activity of the compounds derived from the *Bombina* skin secretions: several insulin-releasing peptides have been isolated (Marenah et. al., 2004). The mechanism underlying their insulinotropic actions suggests possible involvement of a cAMP dependent G-protein insensitive pathway. Secretary glands of some representatives of genus *Bombina* also produce antimicrobial peptides called maximins. According to Lai et al. (2002), maximin 3 possesses a significant anti-HIV activity. Furthermore, maximins tend to have a potent antimicrobial activity, cytotoxicity against tumor cells and spermicidal action.

Although considerable amount of research has been conducted to study the composition of the skin secretions of the *Bombina* toads, and many biological effects of the venom constituents have been revealed, it still remains unclear whether the components of their dermal secretions affect the functioning of the hemostasis system. Moreover, considering the huge amount of research that has been conducted based on the study of the similar effects of the reptile venoms (Meier and Stocker, 1991; Markland, 1998; Manjunatha, 2006), such studies in the context of the toad skin secretions are a highly promising area for investigations. In view of these facts, the aim of the present study was to identify the biologically active fractions in the dermal secretions of the genus *Bombina* and to study their effects on some parameters of the hemostasis system. This work will provide a better understanding of the role of the proteins and enzymes present in the skin secretion of these species and might give a background for further potential medical and pharmaceutical applications of the components of amphibian skin secretions.

MATERIALS AND METHODS

Collection of amphibian skin secretions

Adult specimens of *Bombina bombina* (n = 20, females = 2), *Bombina variegata* (n = 15, females = 1), and their hybrid – *B.bombina* × *B.variegata* (n = 5, females = 0) were collected in Kyiv, Transcarpathian and Khmelnitsky regions of Ukraine, respectively. Amphibians were authenticated by the Department of Zoology and Ecology of Taras Shevchenko National University of Kyiv, Ukraine. Skin secretions were collected as follows: frogs were put into a petri dish. After mechanically stimulated with fingers for 1-2 min, the frog skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each frog with ultra-pure water. Water suspensions of skin secretions were centrifuged at 2500 g for 15 min to remove debris. The supernatants were lyophilized (Telstar LyoQuest) and kept at 4 °C till use.

Fractionation

Lyophilized skin secretions samples of *B. bombina*, *B. variegata*, and their hybrid (0.2 g) were dissolved in 1 mL 0.05 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl and centrifuged at 10,000 g for 5 min. Protein concentration in the supernatant was assayed by Bradford (1976) method, using bovine serum albumin as a standard. Chromatographic assays were performed using a system for liquid chromatography (BioRad,

USA). The samples were applied to a Superdex 200 pg (GE Healthcare, HiLOad™ 16/60) gel filtration column (flow rate 1 ml / min) equilibrated with 0.05 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl. The elution was performed with the same buffer. The appearance of peaks was monitored, and the corresponding fractions were collected in the plastic tubes. The absorbance of the eluate was monitored at 280 nm.

SDS-Polyacrylamide gel electrophoresis

Flow-through fractions were concentrated as follows: the aliquots of the fractions were mixed with 25% trichloroacetic acid (1:1) and centrifuged for 5 min at 10,000 g. The precipitate was diluted with 1 ml of acetone and centrifuged. The procedure was repeated twice. The precipitate was then incubated at 37 °C for 15 minutes. After that, the precipitate was mixed (1:1) with the standard SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% sucrose, and 0.002% bromophenol blue) without heating.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed as reported (Laemmli, 1970), using 4% (w/v) stacking gel and 15% (w/v) separating gel. SDS-PAGE was conducted using Mini-Protean Tetra System (Bio Rad, USA) at 19 mA for stacking and 36 mA for separating gels. Fraction samples were applied in 15 μL volume per well. Gels were stained with 2.5% Coomassie brilliant blue G-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15 % (v/v) isopropanol and the background of the gel was destained with 7% (v/v) acetic acid for 30 min. Apparent molecular weights of proteins were estimated using protein calibration mixture (Bio Rad, USA) containing myosin, β-galactosidase, phosphorylase b, serum albumin, ovalbumin (Mr 97; 66; 45; 31; 21; 14 kDa).

Zymography

The aliquots of the flow-through fractions were mixed with **β**-mercaptoethanol solution (9:1) and sample buffer (1:1) (0.01 M tris-HCl buffer, pH 6.8, 2% sodium dodecyl sulfate, 10% sucrose and 0.01% bromophenol blue) without heating.

Zymography was performed according to the method suggested by Ostapchenko et al. (2011). Separating gel (12% w/v) was polymerized in the presence of gelatin (1 mg/mL), fibrinogen (1 mg/mL) or collagen (1 mg/ml). Fraction samples were applied in 15 μL volume per well. After SDS-PAGE, gels were incubated for 30 min at room temperature on a rotary shaker in 2.5% Triton X-100. The gels were then washed with distilled water to remove Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.4) at room temperature for 12 hours. Gels were stained with 2.5% coomassie brilliant blue G-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15 % (v/v) isopropanol for 30 min.

Preparation of platelet-rich plasma and platelet-poor plasma

Platelet-rich plasma (PRP) and platelet-poor plasma were obtained following the standard protocol. All procedures were carried out at room temperature. Healthy adult rabbits were supplied by the vivarium of Taras Shevchenko National University of Kyiv, Ukraine. The blood was collected from the ear artery into a polyethylene tube with 3.8% sodium citrate (9:1). PRP was obtained by centrifugation of stabilized blood at 300 g for 10 min. The supernatant (PRP) was carefully separated and used further in the aggregation assay. Platelet-poor plasma was prepared by further centrifugation of the remaining stabilized blood at 1,500 g for 30 min.

Plasma coagulation tests

Activated partial thromboplastin time, thrombin time and prothrombin time were measured *in vitro* to assess the effects of the components of flow-through fractions of skin secretions on plasma clotting function. The tests were conducted using the coagulation analyzer (Rayto RT-2201C, China) and the standard set of reagents (RENAM, Russian Federation). To measure the activated partial thromboplastin time (aPTT), 45 μL of rabbit plasma was mixed with 5 μL of fraction sample and 50 μL aPTT reagent in the coagulometric cuvette. After 3 min of incubation at 37°C the 50 μL of 0.025 M $CaCl_2$ was added and the time necessary for the clotting plug formation was recorded. For the *in vitro* TT assay, the 45 μL of plasma and 5 μL of fraction sample were incubated in the coagulometric cuvette at 37°C for 2 min. Clotting time was immediately recorded after the addition of 100 μL thrombin (final activity 3 U/mL). The prothrombin time (PT) was assessed by mixing 45 μL of plasma and 5 μL of fraction sample in the coagulometric cuvette at 37°C for 2 min. Clotting time was immediately recorded after the addition of 100 μL of thromboplastin-Calcium mixture. All coagulation assays were performed in triplicate using plasma from three different rabbits. Plasma incubated with other components and equal amounts of 0.05 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl instead of fractions samples, was used as controls.

Chromogenic assays

The effects of flow-through fractions of the studied skin secretions on key hemostasis enzymes were assayed using *p*-Nitroaniline chromogenic substrates: thrombin specific substrate Phe-Pip-Arg-*p*NA (S-2238), plasmin specific substrate Val-Leu-Lys-*p*NA (S-2251), factor Xa specific substrate Ile-Glu-Gly-Arg-*p*NA (S-2222) and activated protein C specific substrate pyroGlu-Pro-Arg-*p*NA (S-2366) (RENAM, Russian Federation). The direct proteolytic activity of the components of the fractions and their ability to activate plasma proenzymes was measured *in vitro*. Assays were performed in 0.05 M Tris-HCl buffer, pH 7.4 in a total volume 250 μL. To analyze the direct activity, the fractions samples with 20 μg of total protein in 0.05 M Tris-HCl buffer were mixed with the corresponding chromogenic substrates. To study the ability to activate plasma, proenzymes 20 μL of plasma was additionally added to the incubation medium. The formation of *p*-nitroaniline was monitored at equal intervals of time at 405 nm. The amount of *p*-nitroaniline, which was hydrolyzed from the substrate, was calculated by

using molar extinction coefficient of 10,000 M^{-1} x cm^{-1} for free *p*-nitroaniline.

Platelet aggregation assay

Platelet aggregation assay was undertaken within the first 3 h after blood sampling using photo-optical aggregometer AT-02 (Medtech, Russia). Before the assessment, the number of platelets in PRP was determined (230-250 × 10^3 cells/μL). The effects of the flow-through fractions of the studied skin secretions on platelet functions were performed *in vitro*. Briefly, 380 μL PRP and 20 μL of the samples were incubated at 37°C in the aggregometer cuvette and the aggregation process was monitored for 5 min. The maximum degree of aggregation was recorded and compared with the degree of aggregation in response to one of the platelet physiological inducers – ADP (Sigma, USA) in the final concentration of 5 × 10^{-6} M.

Calculation of the results

TotalLab 2.04 program was used to analyze the resultant electrophorograms and zymograms. All experiments were performed in parallels and repeated at least three times each using the blood of different rabbits. The statistical analysis was performed using StatSoft Statistica version 7.0 for Windows. The data were analyzed for statistical significance of difference by Student's t-test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Fractionation of the whole skin secretions

The supernatants of skin secretions of *B. bombina*, *B. variegata* and their hybrid were fractionated into several fractions by Superdex 200 pg column as illustrated in Fig. 1. The purification was followed by determination of various activities for each fraction.

SDS-Polyacrylamide gel electrophoresis and zymography

SDS-PAGE analysis was applied to get information about the protein composition of the flow-through fractions of the studied skin secretions (Table 1 and Figs. S1, S2, S3, S4). The results of protein separation revealed the presence of proteins with molecular weights ranging from 3 to 128 kDa. Zymography with gelatin, fibrinogen and collagen as substrates was conducted to evaluate the proteolytic potential of the flow-through fractions of the studied skin secretions. Key results are presented in Table 1. It was revealed that some fractions have proteolytic enzymes with gelatinolytic, fibrinogenolytic, and collagenolytic activities.

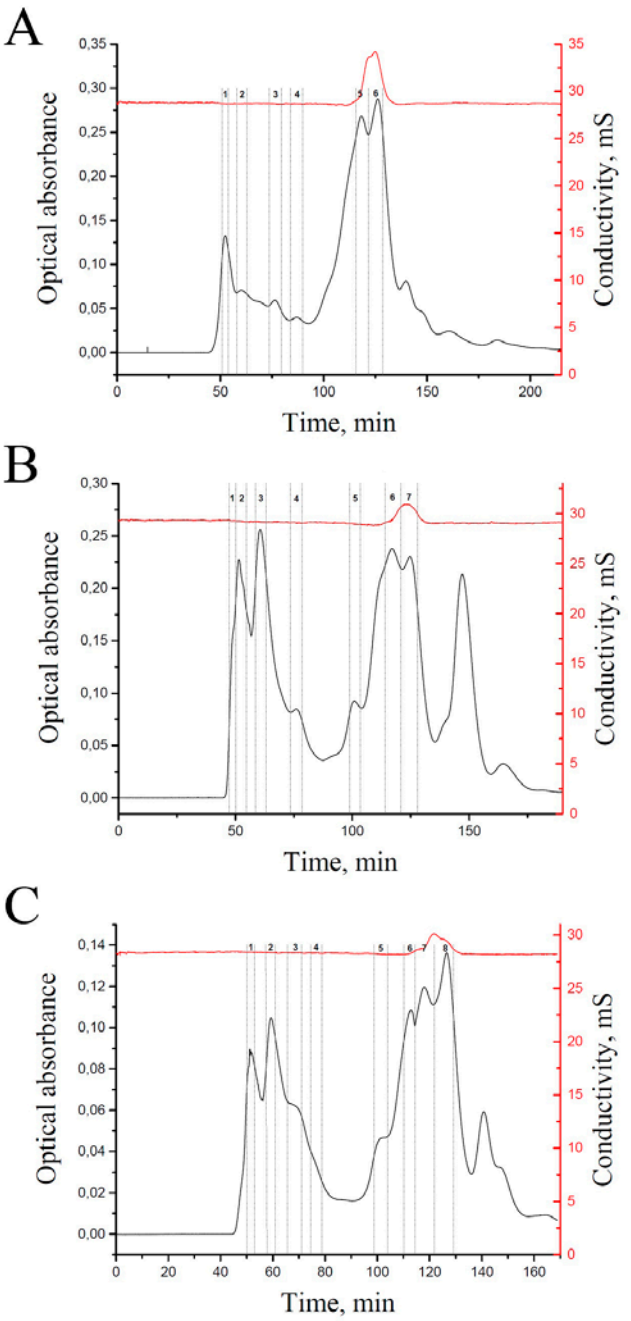

Fig. 1. Fractionation of *B. bombina* (A), *B. variegata* (B) and their hybrid (C) whole skin secretions.

aPTT, PT, and TT in vitro assays

Table 2 summarizes the effects of the flow-through fractions of the studied skin secretions on the time of clot-

Table 1. Molecular weights (MW) of proteins and the presence of proteolytic enzymes with certain substrate specificity in the flow-through fractions of skin secretions of studied amphibian species. The sign "+" - the manifestation of proteolytic activity, and the sign "-" - the absence.

	Fraction #	SDS-PAGE MW, kDa	Zymography		
			gelatin	collagen	fibrinogen
B. bombina	1	103; 78; 46; 31	+	+	+
	2	41	+	-	-
	3	74; 56; 44; 37; 33; 29; 22; 16; 13; 12; 11	+	+	-
	4	55; 48; 39; 36; 33; 27; 24; 21; 18; 12	+	+	-
	5	54	+	-	-
	6	55	+	-	-
B. variegata	1	76; 43; 39; 27; 3	+	-	-
	2	141; 129; 118; 101; 79; 57; 51; 49; 43; 39; 31; 27; 22; 17; 16; 3	+	-	+
	3	121; 109; 79; 42; 39; 27; 23; 22; 3	+	-	+
	4	124; 103; 80; 61; 48; 42; 36; 34; 29; 26; 22; 19; 17; 13; 9; 3	+	-	+
	5	16; 3	+	-	-
	6	16; 3	-	-	-
	7	3	-	-	-
Hybrid	1	100; 53; 43; 29; 22	-	-	-
	2	128; 108; 81; 53; 43; 39; 30; 27; 25; 23	+	+	+
	3	93; 65; 47; 44; 36; 32; 30; 28; 24; 23	-	+	+
	4	120; 88; 51; 42; 36; 31; 27; 24; 1	+	+	+
	5	21; 9	+	+	+
	6	8	+	-	-
	7	-	-	-	-
	8	-	-	-	-

ting plug formation, which corresponds to the plasma coagulation tests results (aPTT, TT and PT). As shown by the data, the whole skin secretions of all the studied amphibians prolonged aPTT clotting time: *B. bombina* – to 62.7 ± 0.5 s, *B. variegata* – to 73.6 ± 0.5 s, and their hybrid – to more than 90 s, compared to the control values – 21.35 ± 1.2 s. Fraction #6 of *B. variegata* skin secretions prolonged aPTT to 56.9 ± 1.1 s, and fraction #5 of hybrid skin secretions prolonged aPTT to 74.1 ± 0.8 s vs 21.35 ± 1.2 s in control. PT and TT assays showed no changes in the time of clotting plug formation while incubating with all studied whole skin secretions and flow-through fractions.

Chromogenic assays

The specific proteolytic activity of the components of flow-through fractions of the studied skin secretions was determined by the ability to hydrolyze the amide bond in the synthetic chromogenic substrates specific to thrombin (S-2238), plasmin (S-2251), factor Xa (S-2222) and protein C (S-2366). Table 3 demonstrates the results of this assay.

The ability of the studied fractions to activate plasma proenzymes was determined *in vitro*. While performing this experiment, rabbit blood plasma was additionally introduced into the incubation medium with the appropriate substrate and the skin secretion fraction sample. Table 3 indicates the emergence of activated thrombin, plasmin, and factor X in plasma while incubating with *B. bombina* whole skin secretions. Only one fraction of this amphibia – #1 – initiated the appearance of factor Xa in plasma. Fraction #9 of *B. variegata* skin secretions activated prothrombin, factor X, and protein C in plasma, whereas its whole secretions had no effects on plasma proenzymes. The hybrid *B. bombina* × *variegata* whole skin secretions and fraction 8 initiated the emergence of active prothrombin and protein C in plasma.

Platelet aggregation assay

This part of the research was aimed at investigating the potential effects of the flow-through fractions of the studied skin secretions on the process of platelet aggregation. Fraction #3 of *B. variegata* skin secretions induced platelet aggregation. As illustrated by Fig. 2, the degree of aggregation in the final concentration of 250 μg of

Table 2. The clotting time of rabbit blood plasma (sec) in the coagulation tests after incubation with the flow-through fractions of studied skin secretions.

		aPTT	PT	TT
	Control	**21.35 ± 1.2**	**6.75 ± 0.1**	**30.3 ± 2.2**
B. bombina	***Whole secretions***	***62.7 ± 0.5****	***7.1 ± 0.2***	***31.9 ± 0.4***
	1	20.3 ± 0.6	7.5 ± 0.3	32.6 ± 0.2
	2	21.3 ± 1.2	7.2 ± 0.2	32.8 ± 0.5
	3	20.7 ± 0.5	7.4 ± 0.5	32.2 ± 0.2
	4	22.5 ± 0.6	7.5 ± 0.3	32.4 ± 0.5
	5	20.8 ± 0.8	5.7 ± 1.2	33.2 ± 0.9
	6	21.7 ± 1.0	7.2 ± 0.4	32.7 ± 0.7
B. variegata	***Whole secretions***	***73.6 ± 0.5****	***6.8 ± 0.2***	***32.1 ± 0.2***
	1	23.6 ± 0.6	6.9 ± 0.4	29.7 ± 0.5
	2	19.3 ± 1.5	7.4 ± 0.3	29.0 ± 0.7
	3	25.6 ± 0.3	7.4 ± 0.2	29.8 ± 0.1
	4	28.1 ± 0.8	8.4 ± 1.2	32.2 ± 0.9
	5	56.9 ± 1.1*	6.9 ± 0.1	32.2 ± 0.7
	6	29.8 ± 0.6	7.5 ± 0.4	32.4 ± 0.7
	7	33.4 ± 0.3	6.9 ± 0.2	31.5 ± 0.2
Hybrid	***Whole secretions***	***>90****	***6.7 ± 0.3***	***31.7 ± 0.5***
	1	18.3 ± 0.6	7.5 ± 0.4	31.3 ± 0.5
	2	18.6 ± 1.2	7.4 ± 0.5	31.3 ± 0.6
	3	19.4 ± 0.3	6.8 ± 0.4	30.1 ± 0.4
	4	19.2 ± 0.4	6.8 ± 0.2	32.2 ± 0.5
	5	74.1 ± 0.8*	7.2 ± 1.1	32.2 ± 0.2
	6	17.1 ± 1.1	6.6 ± 0.2	29.7 ± 0.5
	7	18.7 ± 0.4	7.0 ± 0.6	31.0 ± 0.6
	8	18.4 ± 0.8	5.8 ± 0.5	31.6 ± 0.5

* – p ≤ 005 the difference is comparable to the control.

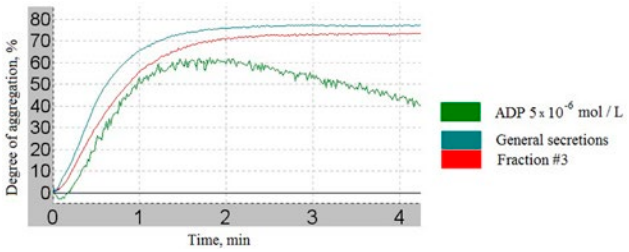

Fig. 2. Whole skin secretions and fraction #3 of *B. variegata* induce platelet aggregation in rabbit PRP.

total protein in 1 ml of PRP were 73% and 65%, respectively, which corresponds to the degree of aggregation in response to the action of $5x10^{-6}$ M ADP. Even though the components of whole skin secretions of hybrid *B. bombina* × *variegata* induced platelet aggregation in the rabbit PRP with the degree of aggregation 52%, the fraction that might have this effect was unidentified. This might be the result of the dilution of the fractions while performing the chromatography assay. Both the whole skin secretions and flow-through fractions of *B. bombina* had no effect on the process of platelet aggregation.

DISCUSSION

To adequately undertake the fractionation, a chromatographic carrier Superdex 200 pg (GE Healthcare, HiLOad™ 16/60) gel filtration column was used in our research. The prerequisite for the use of this carrier was its wide zone of separation (from 3 to 70 kDa), high stability, and inactivity to form nonspecific interactions with the fraction samples. As a result of fractionation, six protein fractions were identified in the *B. bombina* dermal secretions, seven protein fractions were determined in the *B. variegata* skin secretions, and eight protein fractions were defined in the hybrid *(B. bombina* × *B. variegata*) whole secretions. The data on SDS-PAGE assay suggests the presence of the mixture of the proteins with similar molecular weights in each identified fraction. A review of the biological effects of the fractions was conducted and compared to those of the whole skin secretions. Results of our research into the proteolytic potential indicate the presence of enzymes with gelatinase activity in all *B. bombina* (Bb) fractions, whereas the proteolytic enzymes with collagenolytic activity were determined in fractions #1, #3, and #4 of these amphibians. The fibrinogenolytic enzymes were detected only in the Bb#1 fraction. The data indicate the presence of enzymes with gelatinase activity in the fractions #1, #2, #3, #4, and #5 of *B. variegata* (Bv) skin secretions. Fractions Bv#2, Bv#3, and Bv#4 are characterized by the presence of fibrinogenolytic enzymes. No proteolytic potential was indicated in the *B. variegata* skin secretion fractions while conducting zymography assay with collagen as a substrate. The results of the study on the proteolytic potential of the hybrid fractions (Bh) indicate the presence of gelatinolytic enzymes in the fractions #2, #4, #5, #6, and #7, collagenolytic enzymes in the fractions #2, #3, #4, #5, and #7, and the presence of fibrinogenolytic enzymes in fractions #2, #3, #4, and #5. The data clarify the relationship between the emergence of specific type of activity and the release of high molecular weight proteins while performing the SDS-PAGE assay.

To assess the effects of the components of the fractions on plasma clotting function, aPTT, PT, and TT were measured. The data indicate that the *B. variegata* and hybrid whole skin secretions prolonged the clotting plug formation in the aPTT. The proteins that are responsible for the manifestation of this effect were identified in the fraction Bv#5 and Bh#5. Thus, according to the results,

Table 3. The amount of hydrolyzed *p*-NA (nmol) from the chromogenic substrates under the action of whole skin secretions and the flow-through fractions samples of studied amphibian species. S-2238 – thrombin specific substrate; S-2251 – plasmin specific substrate; S-2222 – factor Xa specific substrate; S-2366 – activated protein C specific substrate. The sign "-" - the absence of the effect.

		Proteolytic activity				Plasma enzymes activity			
		S-2238	S-2251	S-2222	S-2366	trombin	plasmin	factor Xa	protein Ca
B. bombina	***Whole secretions***	***0***	***0***	***3.73*** ± **0.07**	***23.76*** ± **0.71**	***14.48*** ± **0.72**	***3.81*** ± **0.32**	***8.61*** ± **0.45**	-
	1	9.32 ± 0.18	2.21 ± 0.04	3.62 ± 0.07	9.11 ± 0.07	-	-	1.09 ± 0.12	-
	2	13.79 ± 0.27	2.06 ± 0.04	5.98 ± 0.17	24.63 ± 0.62	-	-	-	-
	3	13.52 ± 0.07	6.53 ± 0.19	16.97 ± 0.34	25.32 ± 0.23	-	-	-	-
	4	1.03 ± 0.39	11.62 ± 0.31	17.26 ± 0.51	22.63 ± 0.43	-	-	-	-
	5	4.61 ± 0.09	-	-	4.73 ± 0.05	-	-	-	-
	6	5.85 ± 0.18	-	-	6.04 ± 0.17	-	-	-	-
B. variegata	***Whole secretions***	***3.41*** ± **0.13**	***0.73*** ± **0.02**	***1.95*** ± **0.08**	***1.47*** ± **0.07**	-	***0.35*** ± **0.02**	-	-
	1	-	-	-	-	1.52 ± 0.08	-	-	-
	2	-	-	-	-	0.78 ± 0.03	-	-	-
	3	6.01 ± 0.17	-	1.51 ± 0.07	3.57 ± 0.12	-	-	-	-
	4	9.49 ± 0.09	3.76 ± 0.12	4.42 ± 0.15	6.67 ± 0.16	-	-	-	
	5	-	-	-	-	-	-	-	1.97 ± 0.08
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	7.62 ± 0.23	-	2.91 ± 0.13	11.21 ± 0.31
Hybrid	***Whole secretions***	***3.75*** ± **0.18**	***0.75*** ± **0.05**	***1.76*** ± **0.12**	***1.60*** ± **0.09**	***6.11*** ± **0.12**	-	-	***9.71*** ± **0.35**
	1	2.37 ± 0.12	-	-	2.31 ± 0.04	-	-	-	-
	2	2.31 ± 0.09	-	-	-	-	-	-	-
	3	2.37 ± 0.11	-	-	2.27 ± 0.07	-	-	-	-
	4	6.82 ± 0.34	-	1.78 ± 0.15	3.78 ± 0.25	-	-	-	-
	5	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	6.34 ± 0,15	-	-	10.89 ± 0,43

the Bv#5 fraction were characterized by the presence of two proteins with the molecular weight 16 kDa and 3 kDa and had a gelatinolytic activity. Fraction Bh#5 was characterized by the presence of proteins with molecular weight 21 kDa and 9 kDa and had a pronounced protease activity with wide substrate specificity. These results may be attributed to the presence of the inhibitors of certain factors of coagulation hemostasis that are present in the fractions of skin secretions, or, to the degradation of the factors of coagulation hemostasis by the active components of crude skin secretions. Another test – PT – is used to evaluate the functioning of the coagulation factors V, VII, and X and the time necessary to generate fibrin after activation of factor VII in the extrinsic coagulation pathway (Azevedo et al., 2007). TT measures the time required for fibrinogen to form fibrin strands in the presence of thrombin. This test only reveals disturbances in the final stages of coagulation (Koch and Biber, 2007). Although the data shows no potential effect of the components of the flow through fractions on these coagulation tests, mention should be made of the specificity of action.

The data on the dermal components ability to activate plasma proenzymes indicate that the whole secretions of the hybrid initiated the appearance of thrombin and activated protein C in plasma. This effect may be attributed to the action of the components of fraction Bh#7. Although the fraction Bv#9 initiated the appearance of thrombin and activated protein C in plasma, no effect was observed under the action of the whole secretions of this amphibian. According to the SDS-PAGE assay, the components responsible for the manifestation of these effects have a low molecular weight, e.g., fraction Bv#9 – 3 kDa, or, are non-protein nature and have little interest in the context of studying of the effects of the amphibian skin secretions. These results have failed to support our hypothesis that the appearance of thrombin and activated protein C may be due to the presence of active forms of proteolytic enzymes in the crude skin

secretions that can directly activate the cleavage of the corresponding proenzymes of thrombin and protein C or affect the cofactors which can promote a cascade of reactions that result in the formation of zymogens, as the data of SDS-PAGE shows no proteolytic activity in the active fractions Bv#7 and Bv#9. The data indicate that the whole skin secretions of *B. bombina* initiated the appearance of thrombin, plasmin, and factor Xa in plasma, whereas the results have failed to define the fractions that were responsible for these effects. We can assume that such effects of the whole *B. bombina* skin secretions might be the result of the sequential activity of the proteins, when two proteins from different fractions initiated the appearance of thrombin. Or this may also be due to the action of non-protein components or might be related to the high dilution of the fractions as a result of gel filtration chromatography.

The results of the study on the platelet aggregation capability suggest that the *B. variegata* whole skin secretions induced platelet aggregation in the rabbit PRP. The proteins that were responsible for this effect are concentrated in the fraction Bv#3. Mention should be made of the fact that the effect of the whole secretion and the protein fraction corresponds to the effect of ADP, which is known as one of the physiological inducers of platelet aggregation. Previous results of SDS-PAGE assay indicate the presence of several proteins in the fraction Bv#3 with various molecular weights: 121 kDa, 109 kDa, 79 kDa, 42 kDa, 39 kDa, 27 kDa, 23 kDa, 22 kDa, and 3 kDa. Moreover, presence was also indicated of proteolytic enzymes in this fraction with specificity to gelatin and fibrinogen. The proteins present in fraction Bv#3 could be a prominent source of inducers of platelet aggregation. Although inducers that can modulate platelets function are not relevant in the treatment of hemostasis system disorders, they could be a useful tool for studying platelets functions and their signaling pathways. Despite the activity of the components of the hybrid *B. bombina* × *variegata* whole skin secretions to induce platelet aggregation (data is not shown), the results have failed to identify the fractions that were responsible for this effect. This may be due to the significant dilution of the fractions resulting from gel filtration chromatography.

In conclusion, the results of our study demonstrate that the studied amphibian skin secretions of the genus *Bombina* are a potential source of bioactive constituents that can affect different stages of the hemostasis system. It was defined that certain flow-through fractions have active molecules that demonstrate some specific effects. It was established that these molecules are proteins by nature and some of them have pronounced proteolytic activities. Furthermore, the data showed that the proteins with the ability to prolong the clotting plug formation in the aPTT *in vitro* assay are present in the fraction #5 of *B. variegata* and #5 of the hybrid skin secretions. The ability of dermal components to activate plasma proenzymes indicates that the non-protein components of fraction Bv#9 and fraction Bh#7 skin secretions initiated the appearance of thrombin and activated protein C in plasma. The proteins capable of inducing platelet aggregation in the rabbit PRP are present in the fraction #3 of *B. variegata* skin secretions.

Our research has made a contribution into the study of the genus *Bombina* dermal secretions, in particular, the effect of the venom constituents on the hemostasis system and the nature of the active components; however, the mechanism of their action still requires additional research. Such findings may be used by biomedical researchers as a source of potential novel drug leads or pharmacological agents with a direct therapeutic effect and can rapidly provide a basis for related scientific studies such as those involved in systematic or the evolution of species.

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All animal procedures followed the European Directive 2010/63/EU (EC, 2010) on protecting animals used for experimental and other scientific purposes. All manipulations were approved by the Ethical Committee of Educational and Scientific Center Institute of Biology and Medicine, Taras Shevchenko National University of Kyiv, Ukraine. All animal experiments were approved by the Animal Care Committee of Taras Shevchenko National University of Kyiv, Ukraine.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> manuscript number 7858

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Don't tread on me: an examination of the anti-predatory behavior of Eastern Copperheads (*Agkistrodon contortrix*)

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Abstract. Venomous snake species across the globe have been historically categorized as aggressive and dangerous, leading to widespread persecution and killings. Despite the conservation importance of educating the public about the docile nature of these species, few studies have attempted to quantify the response of viperid species to human interactions. Here we report the responses of free-ranging copperheads to a potential human encounter using a set of hierarchical behavioral trials. Out of a total of 69 snakes, only two individuals feigned striking and only two attempted to bite (3% of all individuals). Our results support the findings of previous studies documenting the docile nature of other viperid species and can hopefully be used to change the public perception of venomous snakes. Convincing the public and policy makers that viperid species are docile is critical to long-term conservation of these species in the U.S. and around the globe.

Keywords. Human-wildlife conflict, optimality theory, venomous species, Viper, Viperidae.

INTRODUCTION

As human populations continue to grow and encroach further into uninhabited or sparsely populated areas, there is a subsequent increase in the prevalence of human-wildlife conflict (Woodroffe et al., 2005; Skogen et al., 2008; Dickman, 2010). The outcomes of human-wildlife conflict are rarely more pronounced and potentially lethal to both parties than the interaction between humans and venomous snakes. Venomous snakes have long been a source of great fear for the general public, and have been historically (and currently) mischaracterized as being aggressive and dangerous (Blythe, 1979; Seigel and Mullin, 2009; Burghardt et al., 2009; Pandey, 2016). Even scientific medical publications describing envenomations as late as 2002 (Juckett and Hancox, 2002) continued to perpetuate the myth that viperid species like the cottonmouth (*Agkistrodon piscivorus*) are aggressive and readily attack humans. Misinformation and negative public perception have led to the wholesale slaughter of venomous pit vipers across North America and Europe. Events such as Rattlesnake and copperhead roundups in the US (Adams et al., 1994; Fitch, 1998; Burghardt et al., 2009), and the killing of individual snakes, such as the meadow viper (*Vipera ursinii*), the Cyperian Blunt-nosed Viper (*Macrovipera lebetina lebetina*), and the Northern adder (*Vipera berus*) when they are encountered in Europe (Edgar and Bird, 2006; Stumpel et al. 2015, Julian and Hodges, 2019) are examples of direct persecution against viperid species. This widespread persecution continues to take place in both areas, and is a serious conservation concern for many viperid species, despite these species causing very low numbers of fatalities across these two continents (Chip-

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paux, 2012). It is imperative that any conservation action plan seeking to protect these species will need to incorporate some type of public outreach to reduce direct persecution of these species (Seigel and Mullin, 2009).

Despite the largely negative public perception surrounding pit-vipers, papers have been published in the last two decades that clearly demonstrate the passive and even cowardly nature of other viperid species (Shine et al., 2000; Gibbons and Dorcas, 2002; Glaudas et al., 2005). If snakes are confronted by a large potential predator, the decision to no longer rely on passive defensive behaviors and strike could have a host of potentially short and long-term negative consequences for the snake (Gibbons and Dorcas, 2002; Broom and Ruxton, 2005). For cryptic species, optimality theory predicts that the most efficient strategy to both reduce energy waste and avoid potential mortality is to remain in hiding if possible and to flee immediately if detected by the predator (Ydenberg and Dill, 1986; Broom and Ruxton, 2005; McKnight and Howell, 2015). Like other cryptic species responding to large predators, cryptic viperid species should rely primarily on crypsis or fleeing as primary sources of predator evasion, followed only after these two tactics have failed, by striking and envenomation. When confronted and detected by a potential predator, a snake should first attempt to escape, then employ a suite of passive deterrents (e.g., musking, tail vibrating, mouth gaping), and finally commit to active defenses (biting or striking; Roth and Johnson, 2004).

In addition to the decision-making process driven by optimality theory (see Ydenberg and Dill, 1986), there are a host of intrinsic and extrinsic factors that may act to mediate the chance that a snake will strike (Cooper and Vitt, 2002; Roth and Johnson, 2004). Intrinsic factors such as size (Hailey and Davies, 1986; Whitaker and Shine, 1999), body temperature (Layne and Ford, 1984; Goode and Duvall, 1989), sex (Scudder and Burghardt, 1983), time since feeding (Herzog and Bailey, 1987), prior predator exposure (Glaudus, 2004), and gestation may all play a role in the likelihood of striking (Glaudas et al., 2005). However, studies have found contradictory results regarding the role that each of these factors may play, suggesting that the exact influence of these factors are likely species specific (Roth and Johnson 2004). Extrinsic factors like the severity of the threat and the relative location of the snake may also impact strike likelihood (Gibbons and Dorcas, 2002; Shine et al., 2002; Glaudas et al., 2005).

The Eastern copperhead (*Agkistrodon contortrix*) is perhaps the most commonly persecuted snake species in the Eastern US. This wide-ranging species can be found throughout the eastern United States from Massachusetts to Florida, west into Texas and across a wide variety of habitat types (Ernst and Ernst, 2003). Copperheads are an ideal species for a study examining the defensive behavior of a Viperid species to human presence and subsequent interaction, because they are widespread across the heavily populated areas of the Mid-Atlantic and Southeastern US, are responsible for a large proportion (49.2%) of the reported envenomations in the US (Gummin et al., 2017), and are both widely feared and heavily persecuted when located by the general public. While there were 2,048 reported copperhead envenomations in the US in 2016 (Gummin et al., 2017), the overwhelming majority of these envenomations (94%) were either of a moderate or lower health-risk and there were zero reported fatalities (Gummin et al., 2017).

With the continued and rapid expansion of urbanized areas across the copperhead's range, especially in the Southeastern US, where copperheads are still abundant, the number of copperhead-human interactions is likely to increase in the future. Therefore, an understanding of the anti-predatory behavior of copperheads may be used to dispel misinformation, inform the public about the behavior of this common and widespread venomous species, and potentially serve to mitigate the negative consequences of future human-snake encounters.

The aim of the present research is to examine the anti-predatory behavior of the Eastern copperhead when contacted by a potential human predator. Based on optimality theory, we predict that copperheads will rely on crypsis to avoid predation and will very rarely resort to defensive anti-predatory tactics.

MATERIALS AND METHODS

Our study areas (n = 10) were dispersed throughout the state of Maryland, a small state located in the mid-Atlantic region of the US, and included a variety of habitats within each of the state's physiographic provinces. Maryland is comprised of six physiographic provinces, the Atlantic Continental Shelf Province, the Coastal Plain Province, the Piedmont Plateau Province, the Blue Ridge Province, the Ridge and Valley Province, and the Appalachian Plateau Province (Reger and Cleaves, 2002). Copperheads are widely distributed across Maryland and occupy different habitats within these physiographic provinces (e.g., bottomland swamps, rocky stream banks, south facing slopes) across the state. The location of each site was non-random, with study sites chosen based on prior distribution records collected through the Maryland Amphibian and Reptile Atlas (Cunningham and Nadrowicz, 2018) or historical localities gathered from Harris (1975). All encounters occurred within the state of Maryland. Searches were conducted during the copperhead's active season, from 01 May 2017 to 01 November 2017, and again from 01 May 2018 to 01 November

2018. The snakes were located by visually searching each study site by foot (Karns, 1986; Gibbons and Dorcas, 2002). In general, snakes were found in areas adjacent to wintering den sites with a large amount of adjacent cover in the form of rock crevices and piles. Once a snake was visually located, the body position of each individual, either coiled or extended, was recorded prior to any further approach (Shine et al., 2000; Glaudas et al., 2005). Since body surface temperature can play a role in defensive behavior (Arnold and Bennett, 1984), we used a Ryobi Tek4 non-contact infrared digital thermometer (Ryobi, Chicago, IL, USA) to record body temperature at three different locations on the body (head, mid-section, and cloaca) from a distance of ~2 m and then averaged these values together (Garrick, 2008). Ambient environmental temperature was recorded by extracting local weather data from the nearest weather station using the Weather Underground mobile application software (v5.11.9, TWC Product and Technology, Atlanta, GA) from the National Weather Service, operating under the National Oceanic and Atmospheric Administration (2018).

Once located, snakes had 1) an apparatus with a boot attached placed directly adjacent to the snake to simulate a possible human interaction while actively hiking (Gibbons and Dorcas, 2002; Shine et al., 2002). After the initial approach and first trial, the snake then had 2) the apparatus placed gently on top of it (to simulate accidental contact with a hiker; Gibbons and Dorcas, 2002). Finally, the snake was 3) grabbed and picked up using a pair of snake tongs covered with a leather glove to simulate a human hand (Gibbons and Dorcas, 2002; Glaudas, 2004; Glaudas et al., 2005; Maritz 2012). A previous study showed that a human hand elicits a strong anti-predatory response, suggesting that faux gloved hand might elicit a similar anti-predatory response (Herzog et al., 1989). Each stage of the test (1-3) was carried out for 20 seconds and was videotaped using a digital video camera (to allow post hoc analysis of the defensive response).

During each phase, the observers recorded the defensive behavior of the snake from the anterior end. Behaviors were categorized into four separate categories during each stage of the experiment (fleeing, tail vibrating, feigning a strike, and striking; Gibbons and Dorcas 2002) to represent escalating levels of anti-predatory responses. A feigned strike was classified as a lunge forward without any discernible opening of the mouth. To test the effect of human activity, environmental temperature, snake's body temperature, and the snake's initial posture on anti-predatory behavior, we categorized each snake's response across trials into one ordered value based on their most defensive response to any of the trials (no-response [0], fleeing [1], benign anti-predatory response [2; tail vibrating], or defensive anti-predatory response [3; feigning a strike or striking]). To test for associations between ambient and body temperatures and behavior we used an ANOVA. Both environmental temperature and the snake's body temperature were normally distributed (Shapiro-Wilk W Test, W = 0.94 and 0.97 respectively). To test for associations between initial body condition and anti-predatory behavior we used a Mann-Whitney U-Test. All statistical analyses were conducted in JMP Pro (v14, SAS Institute Inc., Cary, NC).

We did not collect and individually mark snakes since it would have been impossible to collect many of the snakes that rapidly fled into adjacent cover (e.g., deep rock crevices, den sites, heavy vegetation) in a manner that did not harm the snakes or lead to the potential envenomation of the researchers. Additionally, since contact prior to the trials would have biased behavior, marking could not have been performed prior to the initiation of the trials. To help prevent "double-testing" of the same snake, integument patterns (specifically the darker "hourglass" bands that may have been thin or wide, uneven, broken on the dorsal side, etc.) were used as a basis for individual recognition and were supplemented by recordings of scale abnormalities (Carlstrom and Edelstam, 1946; Shine et al., 1988; Moon, et al., 2004). Post-hoc visual photo comparison between each individual snake was conducted to remove any duplicate trials. In total, we removed one snake trial from all analysis after post-hoc comparison confirmed that it had been tested during a prior sampling period.

RESULTS

In total, we recorded encounters with 69 snakes across all 10 sites (Fig. 1). Of these 69 snakes, 15 escaped immediately upon discovery without performing any other anti-predatory behavior and were not available for any further trials. During the initial approach, one snake performed tail vibrating before fleeing, one snake performed tail vibrating and a feigned strike before fleeing, and one snake attempted a strike. After accounting for these 18 snakes, 52 snakes remained for further trials. For a summary of the responses to each of the individual trials (stepped next to (N = 52), stepped on (n = 33), and picked up (n = 14)), see Fig. 2. In total across all trials, five snakes displayed tail-vibrating behavior and one exhibited a feigned strike followed by fleeing (Fig. 2). Across all trials, we recorded only two instances of striking (3% of all snakes).

There was no relationship between snake anti-predatory behavior and either ambient temperature (ANOVA: $F_{3,65}$, P = 0.92) or snake body temperature (ANOVA: $F_{3,62}$, P = 0.45). Similarly, there was no difference in anti-predatory behavior between snakes that were initially coiled or extended (U = 255, P = 0.496). Thus, across all conditions snakes showed similarly low percentages of anti-predatory behavior.

DISCUSSION

Overall, our results provide evidence to support the hypothesis that copperheads respond to potential predators in a manner consistent with their cryptic patterning. Specifically, copperheads are more likely to either remain in crypsis or flee in the presence of a human rather than display defensive behavior. Across the various trials of

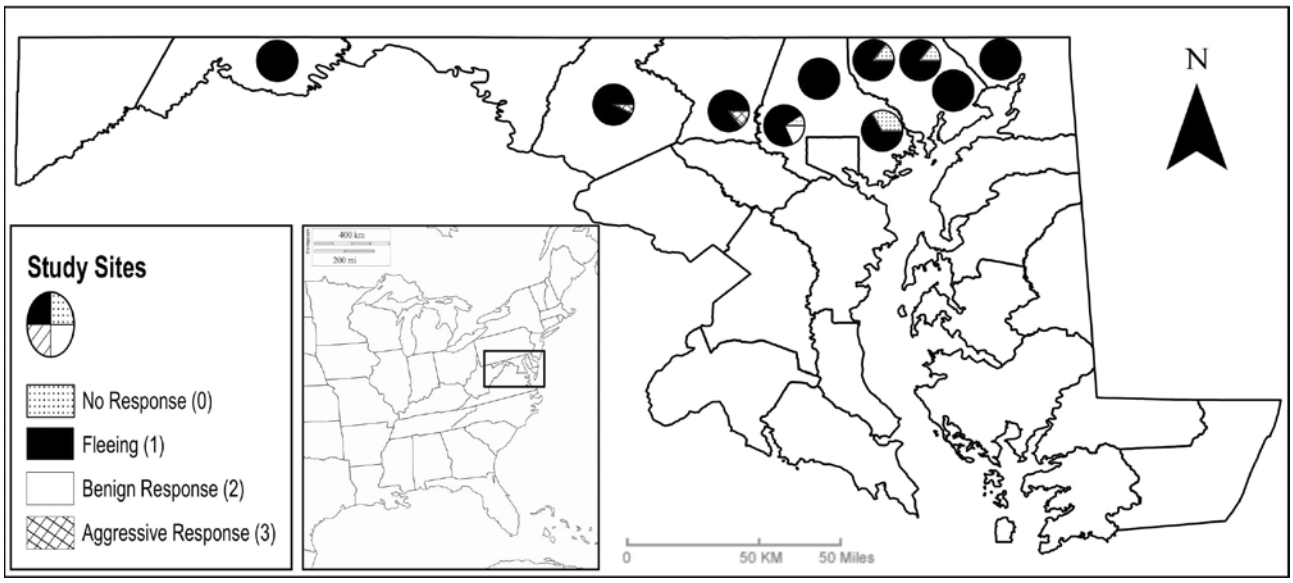

Fig. 1. Geographic distribution of the study sites across the state of Maryland. Each study site is represented by a pie chart with the binned behavioral responses from all individuals at that site.

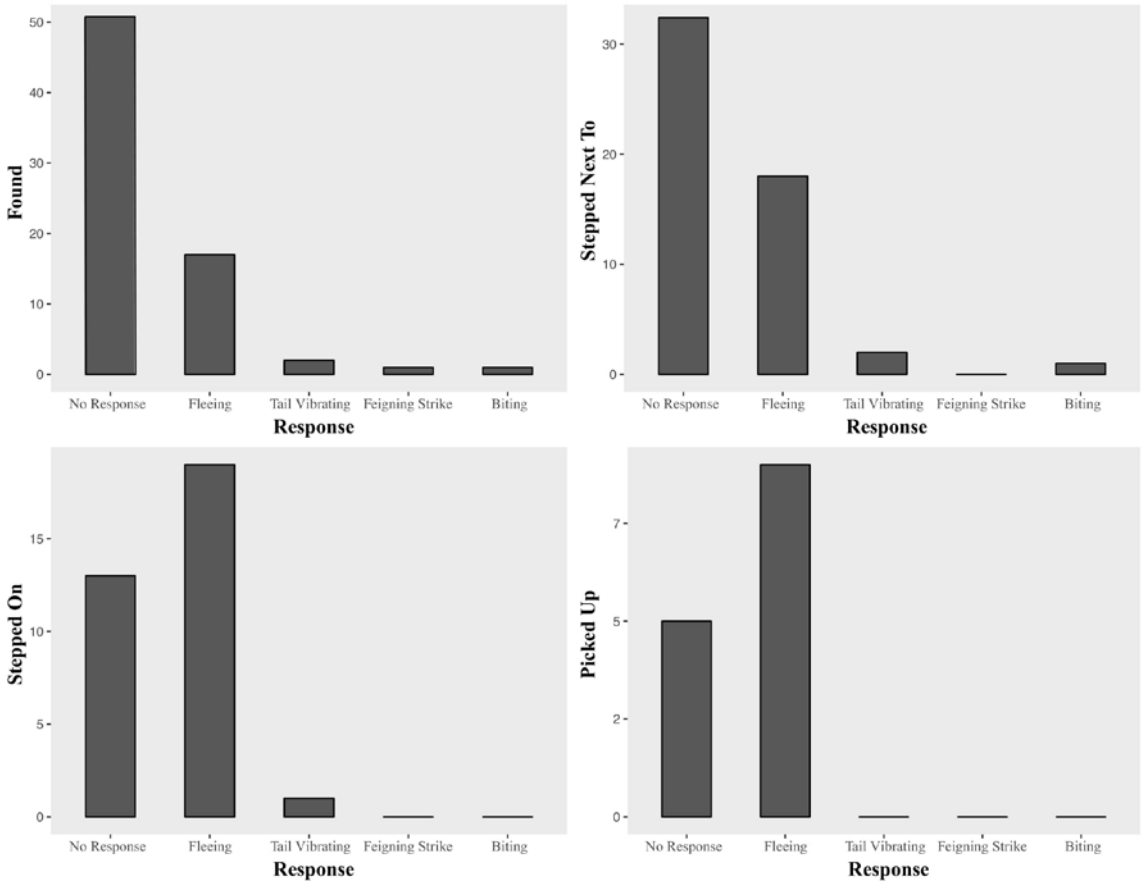

Fig. 2. Responses of copperheads to four increasing threat levels in a hierarchical anti-predatory trial (Found, Stepped Next To, Stepped On, Picked Up).

the study, 93% of the snakes fled when we approached or made physical contact with them. Furthermore, a higher proportion of snakes (6%, n = 4) displayed no response to any of our interactions (including being picked up) than those snakes that struck during one of the trials (3%, n = 2). While the proportion of strikes was low, these results mirror the findings of other studies examining pit-vipers' responses to humans and consistently demonstrate that despite the public's perception of these species as being dangerous and aggressive, that Pope (1958) was correct when claiming that snakes are "first cowards, then bluffers, and last of all, warriors."

To examine how different intrinsic and extrinsic factors influenced anti-predatory response, we analyzed the effect of the initial body posture and body temperature of each individual, and ambient environmental temperature on anti-predatory responses. While we did not collect individuals to gather morphometric data, the extremely low prevalence of snake strikes makes it highly unlikely that any effect of sex or size class on a snake's anti-predatory response would have been detected. However, with a larger sample size, differences in anti-predatory behavior based on various intrinsic or extrinsic factors may be detected. The published literature on the anti-predatory behavior of snakes is full of conflicting results regarding the role of intrinsic and extrinsic factors on anti-predatory behaviors both between and among species (Shine et al., 2000; Roth and Johnson, 2004). Unfortunately, our work does little to elucidate the differences between these conflicting studies, except perhaps to further emphasize that differences in evolutionary history may be prohibitive when attempting to produce general models of anti-predatory behavior in snakes.

While for obvious safety reasons we were unable to approach the snakes with an exposed hand or forearm, other studies have also used a gloved apparatus to simulate the human hand (Gibbons and Dorcas, 2002; Glaudas 2004; Glaudas et al., 2005). While it is possible that the difference in temperature between the gloved apparatus and a human hand may have modified the anti-predatory behavior of the copperheads due to their heat-sensing capabilities, no study has yet assessed the importance of thermal cues in the modification of anti-predatory behavior in free-ranging pit-vipers. Since no studies have examined the effect of predator temperature on anti-predatory behavior, examining the literature on the influence of temperature on predatory behavior may be instructive. However, in the only field studies conducted to this point examining the importance of thermal cues on predatory behavior, Shine and Sun (2003) found that while adult snakes were more likely to strike at warmer objects, temperature was not a predictor of juvenile strikes, and Schraft et al. (2018) found that absolute temperature was not an important predictor of predation attempts.

The public perception of venomous snakes as aggressive and dangerous leads to a suite of problems for the conservation of viperid species (see Seigel and Mullin, 2009 for an overview). Most notable among these issues are the large organized round-ups that may lead to localized extirpation of rattlesnakes (Adams et al., 1994; Fitch, 1998; Burghardt et al., 2009) and the lack of resources that are made available for habitat protection or management of these species (Seigel and Mullin, 2009). While some studies provide cautionary tales about the potential backfiring of educational material (Hoff and Maple, 1982), it is clear that increasing the public's positive perception of snakes will be a necessary component of any long-term conservation plan (Seigel and Mullin, 2009). More recent studies have shown that well designed education programs focusing on biodiversity conservation, the ecological role or snakes, or the use of antivenom for medicinal use can improve feelings and attitudes about snakes (Murphy and Xanten, 2007; Markwell and Cushing, 2009). As part of this public outreach and education, providing examples demonstrating the docile nature of most venomous species may convince some individuals to support legislation to prevent the organized killings that persist to this day.

The results of this research provide previously unavailable information to inform the public of the docile nature of copperheads and potentially assuage fears surrounding the perceived aggressive nature of viperid species. These striking results should prove useful in convincing the proportion of the public that is still impressionable of the copperheads' benign nature and may result in an increase in positive public perception. Future conservation of imperiled viperid species may hinge on the ability of scientists to persuade policy makers and the public of the importance and docile nature of these species (Seigel and Mullin, 2009). This study provides further evidence that common venomous species are not aggressive and rely on striking only as a last resort. As Charas (1677) noted over 300 years ago, "The viper is taken by many for an image of malice and cruelty; but in reality, she is guilty of no such thing".

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An overview of research regarding reservoirs, vectors and predators of the chytrid fungus *Batrachochytrium dendrobatidis*

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Abstract. This review presents an overview of research from 1998-2018 regarding interactions of *Batrachochytrium dendrobatidis* with both potential hosts and predators. To this end, 23 different studies collected from the Web of Science database along with two external journals were utilized, encompassing numerous taxonomic groups. Numerous groups of animals were identified as potential vectors for the fungus, with crayfish and reptiles standing as the most prominent and consistent non-amphibian hosts warranting their inclusion in any future broadscale distribution surveys. an important area for future research. Additionally, *Daphnia* were noted to serve as predators of the zoospores when exposed in mesocosm scenarios, reducing infection levels in corresponding tadpoles. Caecilians have also been observed to be carriers of *Bd*, though the level as to which the chytrid impacts these organisms needs to be further researched. In total, this review indicates that future research needs to begin including freshwater crustaceans, caecilians and reptiles in field studies for presence/absence, while a broader range of taxa need to be tested to see whether they serve as vectors or hosts in natural scenarios.

Keywords. *Batrachochytrium dendrobatidis*, crayfish, reptiles, *Daphnia*, fish, caecilians, alternate hosts, review.

INTRODUCTION

Since its identification in the late 1990s, amphibian declines have been heavily attributed to a parasitic fungus called *Batrachochytrium dendrobatidis* (Longcore et al., 1999) as it spreads around the globe (Longcore et al., 1999). A member of the fungal division Chytridiomycota, the zoospores encyst in an amphibian's skin, often resulting in deleterious effects, posing especially difficult to eradicate to its ability to persist in areas for years despite an apparent lack in hosts and easy spread via multiple vectors. While anthropogenic actions and naturally resistant amphibian species have long been considered potential vectors for *Batrachochytrium dendrobatidis* (*Bd*), interest has steadily grown over the past decade in whether non-amphibians could serve as either a vector for the disease or potentially as alternative hosts from which the fungus could complete its lifecycle (McMahon et al., 2013). As the zoospores are specifically attracted to highly keratinized cells, such as those in the frog's skin, early studies proposed and investigated whether the fungus would be attracted to keratin from non-amphibious sources such as snakeskin and crayfish with varying results (Longcore et al., 1999; Piotrowski et al., 2004; Rowley et al., 2006). Since then, research into the topic has been few and far between, with the subjects ranging from traditional aquatic species to those who may serve as unconventional vectors such as reptiles.

MATERIAL AND METHODS

In this review, we sought to provide an overview regarding research into the roles that taxa outside of the clade Batrachia

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(frogs and salamanders) play as potential hosts or predators of *Bd*. To select studies for inclusion in this review, we combined the Web of Science database along with the journals *Herpetological Review* and *Herpetological Conservation & Biology* to find most of the studies/reports. When searching the database, each major taxa group investigated, combined with the chytrid, were used as keywords, with new taxa being included every time they were first eluded to in a previous study. While it is possible that some studies could have been overlooked due to differences in keywords or not being present in the database, this search method covered a broad range of taxonomic groups and serves as a solid base for this research.

RESULTS

These studies have been conducted in a range of fashions in both the lab and the field on animals ranging from crayfish, to reptiles and even nematodes, with several such individuals found capable of carrying the fungus. All investigated species were selected due to the presence of keratin either on surface structures or internally (such as the gastrointestinal tract), while also being associated with freshwater environments where they could easily contract the pathogen. While macroinvertebrates were the initial focus of this form of study, reptiles have become increasingly more researched.

Potential Reservoirs

When it comes to invertebrates, crustaceans have been the most prominently studied for a potential relationship with *Bd*, with two separate approaches for two distinct groups. Decapods (crayfish and shrimp) were first investigated as carriers for *Bd* in 2006 in a study on both *Caridina zebra* (a shrimp) and *Cherax quadricarinatus* (Queensland Red Claw) in field studies and in lab infection studies that initially reported the presence of the chytrid in both species before being retracted a year later with false positives being attributed to an error in the transcription of raw data (Rowley et al., 2006; 2007). Despite this early controversy, a later study by McMahon et al. (2013) confirmed the presence of zoospores on both the carapace and in the gastro-intestinal tract of three crayfish species; *Orconectes virilis* (Blue Crayfish), *Procambarus alleni* (Blue Crayfish) and *Procambarus clarkii* (Louisiana Crayfish) both in captivity and in the wild. This model study presents a few notable findings, most importantly that tadpoles exposed to infected crayfish were highly susceptible to having the pathogen transmitted to them in captivity (McMahon et al., 2013). Discharge tubules being observed in the gastrointestinal tract of both captive and wild-caught crayfish also indicates that they can serve as reservoir hosts for *Bd* (McMahon et al., 2013). Interestingly, these crayfish were found to be both carriers and victims of *Bd*, with gill recession occurring in individuals who were exposed to either *Bd* filtrates or the zoospores themselves (McMahon et al., 2013). The finding that in the natural systems where crayfish tested positive for *Bd*, all the amphibians were negative, also helps to indicate that the crayfish may act as true alternate hosts in a broader cycle (McMahon et al., 2013).

Some of these findings were corroborated by another study, that identified *Bd* in both wild and farmed populations at 3.31% and 6% prevalence respectively across various *Procambarus* species in Louisiana (Brannelly et al., 2015). Unfortunately, this study was not conducted with a concurrent amphibian survey to assess a potential relationship between infection rates of the crayfish and the amphibians, limiting the ecological importance of this study. Additionally, the close quarters of the farmed populations would easily result in cross-contamination that might have inflated the number of positives found in these populations. Inflated or not, the finding of *Bd*-positive (*Bd*+) individuals pose an interesting potential for disease spread as fishermen will often purchase live crayfish as bait, potentially introducing contaminated individuals to *Bd*-negative (*Bd*-) sites. This potential vector needs to be further explored on a larger scale, as the various methods of transmission must be understood if we are to curb the spread of this disease. These findings of crayfish positivity were not universal, however, as the study by Betancourt-Roman et al. was performed in a similar fashion but found no evidence of gill recession in *Procambarus alleni* and a fairly low prevalence rate (5%) on sampled carapaces (Betancourt-Roman et al., 2016). However, this study was only performed with nine exposed individuals in the lab (small compared to the previous two studies) and the restriction of sampling to the carapace, excluding the GI tract, could have potential resulted in false negatives (Betancourt-Roman et al., 2016).

Research involving crustaceans was not limited to crayfish, as a study by Paulraj et al. (2016) investigated the possibility of *Macrobrachium rosenbergii* (Giant Freshwater Prawn) serving as a vector for *Bd*, due to the prominent commercial species being heavily impacted by fungal infections. A field survey was conducted across 15 culture ponds in South India over the course of four years, with infection being determined through visual inspection and subsequent swabbing (Paulraj et al., 2016). Physical signs of infection noted by the researchers included: dullness of color, abnormal protrusion of scales, tufts of fungal growth, lethargic movement and an overall spongy appearance (Paulraj et al., 2016). This

study mirrored previous research on crustaceans with a *Bd* prevalence of 17.4-38.8% being detected across the sampled farming ponds with prevalence noted to be lowest in the months of April, May, June, and highest in October, November and December (Paulraj et al., 2016). These findings continue to support the idea that the infection may transfer from amphibians in the spring to crustaceans in the fall, with this specific scenario needing further study. Another study investigating the presence of *Bd* on the island of Jamaica included two Sesarmid land crabs and 12 crayfish (unidentified) in their survey, with one of each being *Bd*+ (Holmes et al., 2014). Neither of these studies investigated presence within the gastrointestinal tract (which was shown by previous studies to have higher levels in crayfish compared to carapace swabs) and did not directly observe discharge tubules, but the potential that they mirror other decapods means that these species should be investigated in a manner similar to McMahon et al. (2013).

Certain fish have also been shown to function as reservoirs in the laboratory, with samples taken from *Danio rerio* displaying various stages of development, including discharged mature zoosporangia, indicating the ability to reproduce off the larvae (Liew et al., 2017). Infected individuals were found to suffer from fin erosion at the 72-hour mark and later, along with skin blisters, the disruption of muscle striations, and an increased level of degeneration with these same symptoms occurring in larvae exposed to only filtered supernatants (Liew et al., 2017). This damage seems to be similar in nature to the negative impacts of *Bd* on crayfish, with the same symptoms in filtered treatments indicating the presence of some enzyme causing the damage rather than just encystation. The ability for the chytrid to clearly encyst and replicate successfully in *D. rerio* larvae further illustrates why the view of potential hosts for *Bd* needs to be expanded and indicates that this is a knowledge gap that should be focused on more extensively.

Potential Vectors

Reptiles ranging from squamates to those in the clade Aves have been investigated the most, with the first study being undertaken in 2012 (Garmyn et al., 2012). Waterfowl have been of interest due to their consistent exposure to water systems such as ponds and wetlands where they would be likely to encounter *Bd*+ environments and serve as vectors between various sites. All these studies focused on sampling the toe scales of various individuals either through swabs alone or through toe clippings. All living species that have been sampled across two studies (Garmyn et al., 2012; Hanlon et al., 2017) were found to have individuals positive for the chytrid, with 14.9% of *Branta canadensis*, 19.5% of *Anser anser domesticus*, 45% of *Anas strepera*, 50% *Anas carolinensis* and 62% of *Anas platyrhynchos* being *Bd*+. In the laboratory it was also found that spores would adhere and encyst to toe scales after 24-hours while desiccation tests found that spores remained viable after 30 minutes, indicating that the range of transmission would be based off an animal's flight speed over thirty minutes (Garmyn et al., 2012). Furthermore, they were also able to have zoospores produce zoosporangia from Day 4-14 after initial exposure (Garmyn et al., 2012). Notably, in the study by Garmin et al (2012) the feet of sampled waterfowl were rinsed to remove transient spores differently from the study by Hanlon et al (2017) and this could have been the reason for increased levels of positive individuals. The fungus has also been detected from museum specimens of waterfowl, but this appears to be more variable depending on the sampling technique. When comparing specimens from both the La Paz Museum and Seville Museum, 13 individuals were found to be *Bd*+ when performing tissue sampling while only one of those same individuals was positive from swabs (Burrowes and De la Riva, 2017). This study also found that the oldest positive sample dated back to 1982 from an *Anas flavirostris* (Yellow-billed Teal) and a *Plegadis ridgwayi* (Puna Ibis) collected from Ulla Dam, Bolivia (Burrows and De la Riva, 2017). Other species positive for *Bd* include *Anas georgica* (Yellow-billed Pintail), *Rollandia rolland* (White-tufted Grebe), *Fulica ardesiaca* (Andean Coot), *Fulica gigantea* (Giant Coot), *Lophonetta specularioides* (Crested Duck) and *Spatula puna* (Puna Teal), having been collected from various sites across Bolivia (Burrows and De la Riva, 2017).

Squamate reptiles have also been investigated as potential vectors by testing for the fungus on the ventral surface of multiple species and comparing its presence to nearby amphibian populations. *Anolis lionotus* and A. *humilis* were found to be positive for *Bd*, with a prevalence of 9% and 32% respectively among sampled individuals and an overall prevalence of 16% in survey sites in Panama (Kilburn et al., 2011). Three snake species were also positive for the chytrid: *Imantodes cenchoa* (Blunthead Tree Snake), *Nothopsis rugosus* (Rough Coffee Snake) and *Pliocercus euryzonus* (Cope's False Coral Snake), with one individual of each species being tested and a total prevalence of 38% among all sampled snakes (Kilburn et al., 2011). Habitat may have had an influence on which species tested positive for the fungus with the three species of snakes as *A. humilis* routinely encountering wet leaf litter that could retain spores, while *A. lionotus* is specifically noted as being semi-aquatic. It is possi-

ble that the snakes may have acquired zoospores through predation upon infected amphibians, while *Bd*+ reptiles may serve as vectors for the spread of this disease by direct contact or indirect environmental spreading. This study is markedly contrasted by a similar study conducted in Australia, which investigated whether *Physignathus lesueurii* (Eastern Water Dragon) juveniles were positive for *Bd* by conducting opportunistic sampling in Murray Upper National Park (Phillott et al., 2009). While all of the *P. leseuerii* were found to be negative, concurrently sampled amphibians in the area were found to be positive for *Bd* showing that the fungus is present in the region (Phillott et al., 2009). The reason infection was not able to set in on these agamids may be due to prolonged periods of desiccation or to the natural cutaneous microfauna present, but neither of these factors were specifically investigated.

The potential for fish to be vectors has also been investigated in two studies in a laboratory setting, including *Poecilia reticulata* (Ornamental Guppies) and *Gambusia holbrooki* (Eastern Mosquitofish) and *Danio rerio* (McMahon et al., 2013; Liew et al., 2017). While both *D. rerio* and *P. reticulata* had high levels of *Bd* shortly after initial exposure, the guppies showed a consistent decrease in the genomic equivalents of *Bd* DNA and the mosquitofish showed no evidence of infection (Liew et al., 2017). The previously mentioned colonization of *D. rerio* in combination with these findings shows that *Bd*'s relationship to fish can vary extensively across taxa, with fish serving as both potential hosts and/or vectors with more extensive research being required to better shape how fish relate to *Bd* ecology. Bacteria were also shown to have a significant effect in mitigating infection of the larvae in exposed zebrafish coinciding with research regarding amphibian microbial communities having an impact on susceptibilities (Liew et al., 2017). Forming a profile of the three fish species' bacterial communities would allow comparison between susceptible and resistant amphibians and may explain why infection was able to persist in *D. rerio* larvae and produce mature zoosporangia while infection waned in the *P. reticulata* and failed in *G. hoolbrooki*. Another important note is that if the mosquitofish reacted to *Bd* in a similar manner to the guppies, the difference in time before sampling between these two studies could be responsible for the differences in susceptibility through false negative readings.

Abiotic factors may also be potential vectors of *Bd* zoospores as explored in a study based in Honduras that investigated zoospore presence in raindrops. Out of the 20 rainfall events that Kolby et al. (2015) sampled in this study, one was found to have a *Bd*+ sample with an extremely low density As the study ensured collection sheets were emplaced in clearings to prevent throughfall of contaminated water from the forest canopy, these zoospores were at least from drops that were windblown off contaminated amphibians and may have even been directly carried by the precipitation (Kolby et al., 2015). This study proposes a potentially game-changing method of transportation if the zoospores can truly be transported through precipitation and definitely requires follow on research to verify these findings and expand upon their possible implications.

Predators

Taxa of the Order Cladocera (Water Fleas) and various other micro-invertebrates have been investigated as predators of the fungus and whether they might be effective as biological controls. Individuals of the genus *Daphnia* have been shown to have a clearly negative impact on zoospore concentrations, reducing genomic equivalents to nearly undetectable levels in a mesocosm experiment combining infected tadpoles with more than ten *Daphnia* (Hamilton et al., 2012). Infected *Rana aurora* (Northern Red-legged Frog) tadpoles were used, with *Daphnia* being noted to have an indirect positive effect on tadpole biomass alongside a negative impact on overall tadpole survivability (Hamilton et al., 2012). To verify that zoospore presence in *Daphnia* intestines was due to predation, another study exposed starved individuals to the zoospores and compared treatments with living and dead individuals, finding significantly higher concentrations in the intestines of living *Daphnia* (Buck et al., 2011). While these studies show evidence that members of *Daphnia* would be beneficial in fighting the chytrid, the reduced scope of these makes it hard to draw any major generalizations, especially regarding natural systems.

Outside of *Daphnia*, microfauna presence in general has been observed to be negatively correlated with zoospore prevalence in natural systems with lab experiments indicating that a portion of this impact comes from the microfauna predating upon the zoospores (Schmeller et al., 2014). One study noted that higher prevalence of microfauna was a significant differentiator between low and high prevalence sites (Schmeller et al., 2014), while another noted the presence of a more diverse rotifer and micro-arthropod community in bromeliads had a more negative impact on *Bd* prevalence than the protist diversity found in the surrounding streams (Blooi et al., 2017). This information coupled with the studies on *Daphnia* illustrate why it is important to consider *Bd*'s role in the ecological community outside of simply being a parasite if we desire to truly understand its distribution pattern along with what may help curb its spread.

Caecilians

Despite being amphibians, the finding that *B. dendrobatidis* can infect caecilians is fairly recent and can potentially provide information regarding the ecology of the fungus that has been long overlooked. While their secluded nature and primarily subterranean lifestyle make them unlikely to serve as vectors, it is interesting to note that this has not precluded them from encountering this fungus. Furthermore, it also continues to demonstrate how broadly ranging this pathogen's potential hosts are, though the full impact of chytridiomycosis on members of order Gymnophiona are hard to determine due to the difficulties of studying members of this taxa. The first study to investigate caecilians for *Bd* specifically sampled both African and South American species, including nearly 200 individuals upon initial capture and an additional 31 being tested after being kept in captivity for two years (Gower et al., 2013). While small in terms of geographic coverage, this study was quite broad regarding taxa diversity, including representatives from 10% of known species and at least one species from 12/34 known genera and 8/10 known families (Gower et al., 2013). While none of the South American species were positive for the chytrid, all African genera tested had at least two positive individuals except *Schistometopum*, showing a broad range of infectivity (Gower et al., 2013). Unfortunately, however, improper sampling techniques initially including handling without gloves could have led to false positives and inflated the present findings. Another recent study also performed in Africa confirmed the prevalence of *Bd* in four species not reviewed in the Gower study, providing confirmation of the pathogen's presence in African species of caecilians (Thorpe et al., 2018). However, this study had small sample sizes making it difficult to extrapolate the range of this disease across the broader population. A third study was also conducted in Africa as a broad survey of caecilians and anurans for the fungus and found it to be present on various caecilians but gave no specific identifications regarding species sampled and which were positive for *Bd* (Hydeman et al., 2017). Overall, members of Gymnophiona should be investigated more thoroughly to determine the presence of *Bd* across the order worldwide, though this may be difficult as a result of their isolated and subterranean nature.

Unclear Relationships

Investigation of invertebrates was not limited to crustaceans, with the nematode species *Caenorhabditis elegans* also being the subject of investigation in two separate studies due to their habitat overlap with amphibians/crayfish and the similarity of function between their tissues. Both studies utilized heat-killed *Bd* as controls and determined mortality by counting both the living and dead individuals present in each treatment (Shapard et al., 2012; Betancourt-Roman et al., 2016). These two studies had contradictory results, however, as Shapard et al. noted an increase in mortality from control to infected treatments, while Betancourt-Roman et al. saw no significant differences between the two groups. While these separate experiments were run as similarly as possible, the Betancourt-Roman study noted that differing lab conditions made the study hard to replicate and that double the concentration of zoospores was used. In the Shapard study, the use of staining dye to mark the zoospores displayed an apparent attachment of *Bd* to the external cuticle of the nematodes, with the cuticle frequently becoming breached afterward (Shapard et al., 2012). Due to the effects of zoospores on the cuticle, it is probable that the zoospores release proteolytic enzymes that serve to degrade the collagen forming the cuticle, however this enzyme was not isolated. This interaction between nematodes and *Bd* warrants further examination, due both to the contradictory findings of these two studies and the potential for use as either vectors or hosts (as indicated by observed encystation in the laboratory).

The potential of other parasites to influence the prevalence of *Bd* in amphibians has also been investigated across many field sites and multiple anuran species. A negative correlation with both parasite abundance and diversity was noted in *Bd* infected *Rana aurora* tadpoles (Nieto et al., 2007). *Echinostoma* sp. specifically were negatively correlated with *Bd* presence across various amphibians while *Ribeiroia ondatrae* was positively correlated with *Bd* presence at the site level (Stutz et al., 2018). This aspect of disease interaction has been largely underresearched and warrants further study to better understand the overall ecology of the chytrid fungus.

DISCUSSION

When looking at this research it seems clear that non-amphibian hosts pose a potentially important role in both transmission and ecology of *Batrachochytrium dendrobatidis* across numerous taxonomic groups. The presence of *Bd* zoospores on the toe scales of waterfowl could allow for the fungus to be spread across a wide range, while the potential for zoospores to be dispersed via rainfall poses an even wider avenue for distribution. While birds are potentially capable of transporting *Bd* the longest distance, the detected presence

on some squamate and fish species raises the potential these taxa could serve to distribute among large, interconnected water systems and wetlands. The presence of zoospores in and on crayfish could also allow for a wider spread of the fungus through anthropogenic methods, especially as *Bd* has been observed successfully replicating within their gastro-intestinal tract. Fishing especially could allow for the fungus to be spread easily and much more rapidly than it would naturally, as infected crayfish can be transported long distances to potentially isolated water systems where the zoospores can then become introduced via fecal matter or upon predation of the crayfish. The finding that crayfish can be positive for the disease while amphibians in the same system are negative could also help explain how *Bd* has managed to persist in systems after the initial extirpation of frogs. Predation of the zoospores by various microfauna such as *Daphnia* presents an alternate end of the spectrum by potentially helping to explain how and why certain areas have been more impacted by the presence of *Bd*. Repeatedly, lab studies would also observe that an enzyme produced by the zoospores had a negative impact on non-amphibian species, with gill recession in crayfish, fin erosion in fish and cuticle breaching in nematodes being tied to this enzyme. While none of these studies identified the enzyme, the fact that filtrate has the same deleterious effects as zoospore presence could mean that negative impacts experienced by non-amphibian hosts/vectors are caused primarily by the enzyme, but future research on the subject is needed.

CONCLUSION

Based on these findings, it is clear that an important concern for *Bd* field surveys is the incorporation of other known hosts outside of amphibians. The most likely taxa to be carrying the fungus besides anurans and caudatans seems to be crustaceans such as crayfish species within the genus *Procambarus* which reside in largely the same habitats as anuran species and have been observed to have full lifecycles being conducted through the chytrid. Birds could be a potentially important factor when trying to model the chytrid's spread across broad areas, and should definitely be investigated further to observe whether they are truly viable vectors for the zoospores. This is especially true for waterfowls due to their ability to fly long distances in the thirty minutes required for the zoospores to become susceptible to desiccation, posing a major potential threat to the prevention of this disease's spread. Transportation via rainfall poses a similar potential problem and should continue to be investigated to better determine the prevalence of this phenomenon and how it may impact distribution. Similarly, predation of zoospores by microfauna needs further research, potentially utilizing a mesocosm approach, to test what taxa have the largest impacts and how this interaction relates to how *Bd* fits into the community at a larger scale. Finally, it is important to continue investigating other taxa to help broaden the list of potential hosts, with a special focus on other crustaceans/macroinvertebrates and semi-aquatic reptiles along with caecilian taxa when performing studies in South America or Africa.

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Genetic characteristics of an introduced population of *Bombina bombina* (Linnaeus, 1761) (Amphibia: Bombinatoridae) in Moselle, France

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Abstract. The fire-bellied toad *Bombina bombina* has recently been introduced in Moselle, north-eastern France, in an area where the yellow-bellied toad *Bombina variegata* occurs naturally. Both species hybridize in a wide area throughout Europe where their distribution overlaps. Therefore, there is a risk of introgression regarding the *Bombina variegata* population in north-eastern France. In order to assess the status of the introduced population of *Bombina bombina* and its origin, we investigated its genetic characteristics and structure using both mitochondrial (cytochrome b) and nuclear DNA (microsatellites markers). The results demonstrated a lack of introgression in the *Bombina variegata* population. Though experiencing a bottleneck effect, the introduced *Bombina bombina* population displays a high genetic diversity. If a propensity for expansion is found within the introduced population of *Bombina bombina*, it could be considered as a potential invasive species in France, and thus threaten the native species.

Keywords. Invasive species, population genetics, conservation, cytochrome *b*, microsatellites.

INTRODUCTION

Introduction of allochthonous species in natural habitats represents one of the aggravating factors of the current loss of biodiversity (Kats and Ferrer, 2003). Such introductions may induce numerous effects on native species, such as ecological competition, over predation, transmission and dispersal of pathogens, and genetic introgression (Mooney and Cleland, 2001; Strayer et al., 2006). In the context of conservation biology, it is important to understand the factors which enabled introduced species to adapt to their new environment, in order to define prevention, monitoring, and management plans for these species (Strayer et al., 2006).

Amphibians are currently in the focus for conservation biologists as they represent the most endangered group of vertebrates worldwide (Stuart et al., 2004; Stuart et al., 2008). Invasive alien species are a major threat to amphibians (Kats and Ferrer, 2003). Among them, other amphibian species can have an impact on native ones. For example, they can be an important source of dispersal of the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), a pathogen which affects many amphibian species around the globe and causes their decline (Ficetola et al., 2008; Fisher and Garner, 2007; Garner et al., 2006). Another possible threat linked with introduced species is genetic introgression, which can, in some cases, lead to local extinctions (Arntzen and Thorpe, 1999; Dufresnes et al., 2016; Rhymer and Simberloff, 1996).

In France, at least six species of amphibians have been introduced: *Triturus carnifex*, *Discoglossus pictus*, *Lithobates catesbeianus*, *Xenopus laevis*, *Pelophylax*

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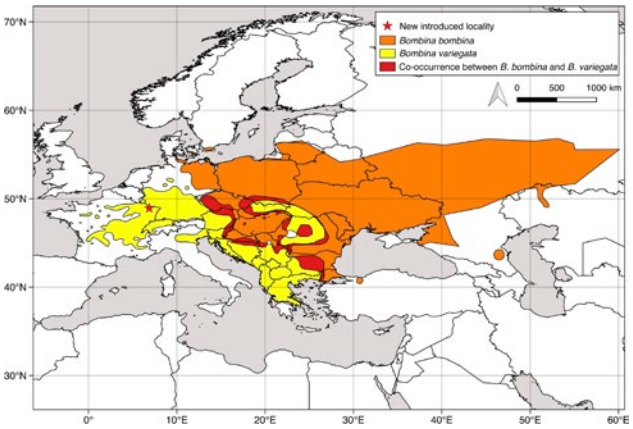

Fig. 1. Global distribution of *Bombina bombina* and *B. variegata* and location of the newly introduced population in France. Map sources: Naturalearth/IUCN.

ridibundus, and *P. bedriagae* (Duguet and Melki, 2003; Lescure and de Massary, 2012). In 2009, an introduced population of the fire-bellied toad *Bombina bombina* has been discovered in the eastern part of the Moselle department, close to the Sarre valley (Vacher and Pichenot, 2012) (Fig. 1). In this region, the yellow-bellied toad *Bombina variegata* occurs naturally (Lescure et al., 2011; Lescure and de Massary, 2012; Thiriet and Vacher, 2010). This last species is considered as endangered in France and listed as 'Vulnerable' on the national red list published by the French Committee of the IUCN (UICN France et al., 2015). The occurrence of introduced *Bombina bombina* close to natural populations of *B. variegata* raises conservation concern as *B. bombina* could lead to the decline of *B. variegata* through introgression. Indeed, hybridization has already been demonstrated where both species are in contact in their native range (Gollmann et al., 1988; Szymura, 1976; Yanchukov et al., 2006). In order to characterize the status of this introduced population, and its possible interaction with the native *Bombina variegata*, we assessed if hybridization already occurred between the native and the introduced species using genetic markers as well as the putative origin of the introduced individuals.

MATERIALS AND METHODS

Sampling design and laboratory methods

We collected 61 DNA samples from individuals morphologically assigned to *Bombina bombina* from three localities in Moselle (6.87°E, 48.92°N) and 64 samples from individuals morphologically assigned to *Bombina variegata* from four neighbouring localities in Moselle and Bas-Rhin in 2011 and 2012. DNA samples were collected through buccal swabbing (Beebee, 2008; Pidancier et al., 2003). The two westernmost *Bombina bombina* localities were 1 km distant from each other, and the third was 5 km south-east from the others.

DNA was extracted from the buccal swabs using the QIAGEN DNeasy Blood & Tissue kit (QIAGEN®). As we suspected that all *Bombina bombina* individuals would originate from the same locality, we amplified by PCR a fragment of 1200 bp of the mitochondrial cytochrome *b* (*cytb*) using L16245 and H17444 primers (Hofman and Szymura, 2007) from only five out of the 61 samples. Amplifications were performed following Hofman and Szymura (2007) and sequencing were performed by Macrogen (Amsterdam, the Netherlands). The new sequences were deposited in GenBank (Table 1).

Ten microsatellite loci specifically developed for the fire-bellied toad (BobomF2, Bobom5F, Bobom9H, Bobom1A, BobomF22, Bobom10F, Bobom8A, BobomD2, BobomB13 and Bobom11D) were amplified by PCR for all 125 samples, following the PCR conditions suggested by Hauswaldt et al. (2007) and Stuckas and Tiedemann (2006). Forward dyed primers were used in order to analyse them with an automatic sequencer (AB3130xl Applied Biosystem). Allele lengths were then read with the software PEAK SCANNER v.1.0 (Applied Biosystem).

Data analysis

We used *cytb* sequences from GenBank to confirm taxonomic assignation of our samples. The *cytb* sequences obtained were first aligned automatically using the software MAFFT (Katoh and Standley, 2013), and then the alignment was checked in MEGA (Tamura et al., 2011). We subsequently grouped our sequences with other *Bombina bombina cytb* sequences published in a previous study on the phylogeography of the species (Fijarczyk et al., 2011). After inferring the best sequence evolutionary model in PartitionFinder v.1.1.1 (Lanfear et al., 2012), using a BIC approach, we constructed a phylogenetic tree with a Maximum Likelihood method in RAxML v.8 (Stamatakis, 2014) under the GTR+G model. We used one sequence of *Bombina variegata* as an outgroup to root our tree. After visualizing the position of our samples in the tree, we subsequently selected the sequences that were the closest to the samples from Moselle, and constructed a haplotype network using the software Hapview (Salzburger et al., 2011).

Each microsatellite locus was first examined for null allele occurrence with MICRO-CHECKER v.2.2.3 (Van Oosterhout et al., 2004) for each population. Loci showing a high probability ($P > 0.05$) of null alleles were discarded from the dataset. For each retained locus, we estimated allele frequency, allelic richness (A_R), observed and expected heterozygosity (H_O, H_E), and intrapopulation structuration (F_{IS}) with the packages *adegenet* (Jombart, 2008) and *hierfstat* (Goudet, 2005) implemented in R (R Development Core Team, 2016). Moreover, Hardy-Weinberg equilibrium was tested for each locus with allele randomizations (1000 permutations per test) with the package *pegas* (Paradis, 2010) implemented in R. In addition, we evaluated the number of genetic clusters (K) using a Bayesian clustering approach implemented in the software STRUCTURE v.2.3.3 (Pritchard et

al., 2000). First, we conducted the analysis for the three putative populations of *B. bombina*, and then for *B. bombina* and *B. variegata* populations together. We performed ten independent runs for each K, and tested between 1 and 3 for *B. bombina* alone, and up to seven clusters for *B. bombina* and *B. variegata* grouped together, according to the number of localities we sampled. Each replicate was run for 400,000 iterations following a burn-in period of 200,000. As the three sampled populations of *B. bombina* were supposedly closely related, we used the admixture model with allele frequencies that were correlated among populations (Falush et al., 2003). In order to identify the most likely value of K, the logarithmic probability of the data [Ln P(D)] was estimated for each simulation. Additionally, the value of ΔK, representing the second order of change, was estimated (Evanno et al., 2005). Finally, we tested if a bottleneck effect (significant heterozygosity excess) was detected within the population of *Bombina bombina* with the software BOTTLENECK v.1.2.02 (Cornuet and Luikart, 1996; Piry et al., 1999), using a Wilcoxon signed-rank test. Such an effect is expected after a strong reduction in population size (Hedrick et al., 1986), such as in recent introduced populations.

RESULTS

BLAST and haplotypes

The BLAST results showed that the five samples matched with *Bombina bombina cytb* sequences deposited in GenBank. Maximum Likelihood inference suggests that the samples from Lorraine are nested within a clade that originates from Austria and Czech Republic (Fig. 2). The haplotypes found in Moselle cluster within haplogroup B3-1 (Fijarczyk et al., 2011), that contains specimens from southern Europe. More precisely, one individual of Moselle is identical to haplotype B14 (Fig. 3), that includes specimens from Czech Republic, Slovakia, Austria, Croatia, Serbia, Hungary, and Ukraine (Fijarczyk et al., 2011), and the four other haplotypes retrieved from the specimens from Moselle only differ from B14 by three to eight substitutions (Fig. 3).

Genetic variation and diversity

We detected an excess of homozygosity, thus the probable presence of null alleles for BobomF2, BobomF22, and BobomD2 within *B. bombina* populations only. Therefore, these markers were discarded from the subsequent analyses for *B. bombina*, which were consequently conducted with seven microsatellites markers (Bobom5F, Bobom9H, Bobom1A, Bobom10F, Bobom8A, BobomB13, and Bobom11D).

The number of alleles in *B. bombina* of Moselle varies from three (Bobom5F and Bobom1A) to ten (Bobom9H)

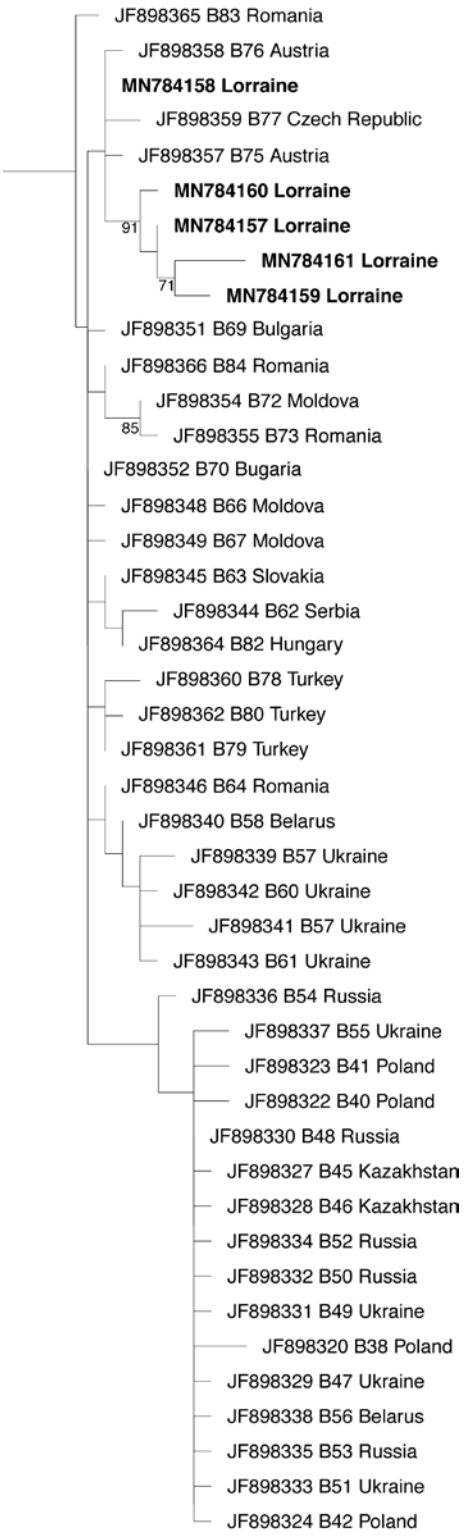

Fig. 2. Best Maximum Likelihood inference obtained from RAxML using ~1000 bp of *cytb* mtDNA of *Bombina bombina*. Bootstrap values above 70 are given at each nodes. The tree is rooted on *Bombina variegata* (not shown). The GenBank accession numbers are provided, new sequences are indicated in bold.

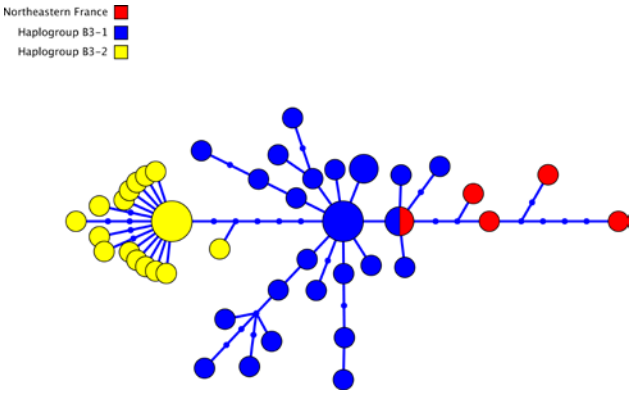

Fig. 3. Haplotype network based on a fragment of the mitochondrial *cytb* in *Bombina bombina*. The haplogroups were defined in Fijarczyk et al. (2011). The five new haplotypes from the introduced specimens in Moselle (this study) are represented in red. Each line represents a single mutation, and the size of the circles represents the frequency of a haplotype.

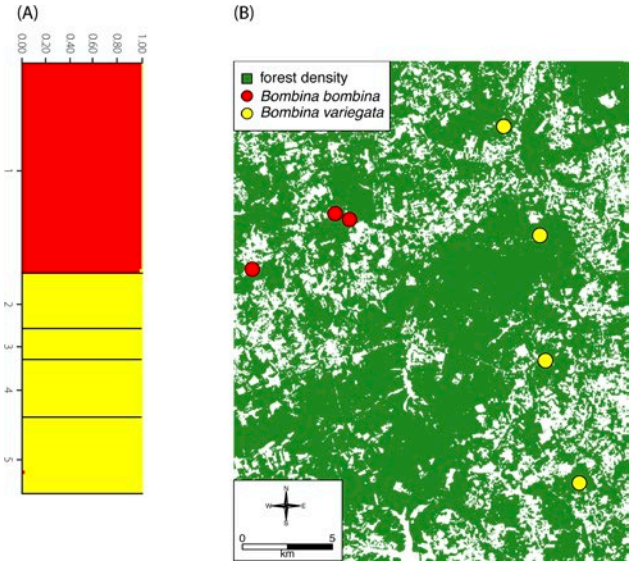

Fig. 4. (A) Clusters from retrieved in the STRUCTURE analysis from seven microsatellite markers; (B) Distribution in space of the two nuclear DNA clusters. The geographic coordinates are not provided on purpose. Map source: Global Forest Watch.

Table 1. GenBank accession numbers and additional information of the locality of the newly introduced *Bombina bombina* in France (in bold) and the samples from GenBank used in the genetic analysis.

No	Location	Accession GenBank	Reference
1	**Moselle, France**	**MN784157**	**This study**
2	**Moselle, France**	**MN784158**	**This study**
3	**Moselle, France**	**MN784159**	**This study**
4	**Moselle, France**	**MN784160**	**This study**
5	**Moselle, France**	**MN784161**	**This study**
6	Hungary	JF898363	Fijarczyk et al., 2011
7	Austria	JF898357	Fijarczyk et al., 2011
8	Romania	JF898366	Fijarczyk et al., 2011
9	Bulgaria	JF898352	Fijarczyk et al., 2011
10	Hungary	JF898356	Fijarczyk et al., 2011
11	Romania	JF898353	Fijarczyk et al., 2011
12	Ukraine	JF898347	Fijarczyk et al., 2011
13	Bulgaria	JF898351	Fijarczyk et al., 2011
14	Bulgaria	JF898350	Fijarczyk et al., 2011
15	Moldova	JF898348	Fijarczyk et al., 2011
16	Hungary	JF898364	Fijarczyk et al., 2011
17	Slovakia	JF898345	Fijarczyk et al., 2011
18	Romania	JF898346	Fijarczyk et al., 2011
19	Belarus	JF898340	Fijarczyk et al., 2011
20	Turkey	JF898362	Fijarczyk et al., 2011
21	Turkey	JF898361	Fijarczyk et al., 2011
22	Ukraine	JF898343	Fijarczyk et al. , 2011
23	Ukraine	JF898342	Fijarczyk et al., 2011
24	Turkey	JF898360	Fijarczyk et al., 2011
25	Moldova	JF898349	Fijarczyk et al., 2011
26	Austria	JF898358	Fijarczyk et al., 2011
27	Serbia	JF898344	Fijarczyk et al., 2011
28	Romania	JF898355	Fijarczyk et al., 2011
29	Moldova	JF898354	Fijarczyk et al., 2011
30	Ukraine	JF898339	Fijarczyk et al., 2011
31	Romania	JF898365	Fijarczyk et al., 2011
32	Czech Republic	JF898359	Fijarczyk et al., 2011
33	Ukraine	JF898341	Fijarczyk et al., 2011
34	Belarus	JF898338	Fijarczyk et al., 2011
35	Russia	JF898330	Fijarczyk et al., 2011
36	Poland	JF898321	Fijarczyk et al., 2011
37	Poland	JF898326	Fijarczyk et al., 2011
38	Slovakia	JF898325	Fijarczyk et al., 2011
39	Russia	JF898334	Fijarczyk et al., 2011
40	Ukraine	JF898333	Fijarczyk et al., 2011
41	Russia	JF898332	Fijarczyk et al., 2011
42	Ukraine	JF898329	Fijarczyk et al., 2011
43	Kazakhstan	JF898328	Fijarczyk et al., 2011
44	Poland	JF898324	Fijarczyk et al., 2011
45	Kazakhstan	JF898327	Fijarczyk et al., 2011
46	Ukraine	JF898337	Fijarczyk et al., 2011
47	Russia	JF898335	Fijarczyk et al., 2011
48	Ukraine	JF898331	Fijarczyk et al., 2011
49	Poland	JF898322	Fijarczyk et al., 2011
50	Russia	JF898336	Fijarczyk et al., 2011
51	Poland	JF898320	Fijarczyk et al., 2011
52	Poland	JF898323	Fijarczyk et al., 2011

Table 2. Microsatellite loci used for the genetic analyses of 61 individuals of *Bombina bombina* introduced in Moselle, north-eastern France. The estimations were conducted with the *adegenet* and *hierfstat* packages implemented in R. bp: base pairs; A_R: allelic richness; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: intrapopulation structure index.

Microsatellite	Length (bp)	Alleles number	A_R	H_O	H_E	F_{IS}
Bobom5F	126-146	3	3	0.54	0.64	0.09
Bobom9H	115-203	10	7.6	0.83	0.86	0.00
Bobom1A	341-353	3	2.95	0.57	0.54	-0.04
Bobom10F	207-223	4	3.97	0.75	0.73	-0.02
Bobom8A	275-315	5	3.77	0.61	0.63	-0.01
BobomB13	117-147	4	3.21	0.63	0.66	0.12
Bobom 11D	268-296	6	5.12	0.82	0.78	-0.05

(Table 1). The mean A_R was 4.23 (calculated from 61 diploid individuals). The mean H_E value was 0.65, and the mean H_O value was 0.66. There was no significant difference between the overall values of H_O and H_E (Bartlett's K-squared = 0.035, df = 1, P = 0.8). The overall F_{IS} value was 0.01 and ranged from -0.05 for Bobom 11D to 0.09 for Bobom 5F (Table 1).

Genetic structure of populations

The analysis conducted with STRUCTURE did not reveal any population differentiation between the three sampling sites of *Bombina bombina* in Moselle. In the analysis conducted with all the samples of *B. bombina* and *B. variegata*, both species formed two well-differentiated clusters, indicating a complete lack of introgression (Fig. 4).

Bottleneck

The BOTTLENECK analysis revealed a bottleneck effect within the *Bombina bombina* population of Moselle. Indeed, the Wilcoxon test showed an excess of heterozygotes compared to the expected equilibrium heterozygosity under both the SMM and TPM models (Wilcoxon test: P = 0.007).

DISCUSSION

Globally, the genetic diversity observed in this introduced population of *B. bombina* is rather high, since the mean H_E value of 0.65 for seven loci is similar to the ones found in 11 natural populations of *Bombina bombina* that occur in the core of the range of the species in Germany and that averages 0.70 [0.59-0.78] for six loci (Dolgener et al., 2012). This could be explained by the introduction of numerous individuals, maybe through different episodes. As the biggest population was observed in a series of lakes that are used for fish farming, it is highly probable that the presence of *Bombina bombina* in this area resulted from the transport of tadpoles caught together with young fishes from one or several close localities in Central Europe. Such cases of translocations have been observed in Brandenburg and in Saxony (Berger, 1996; Dolgener et al., 2012), so it is very likely that the occurrence of *B. bombina* in Moselle might result from a similar event. As tadpoles are small organisms, it is possible that hundreds, or maybe thousands of them have been introduced, therefore maintaining a high genetic diversity. Still it was expected to detect a bottleneck effect within this population as it is the case with recently introduced populations (Lee, 2002; Puillandre et al., 2008). In comparison with other amphibian species, the fire-bellied toad does not have a high fertility rate, with around 300-400 eggs per female per year (Gollmann et al., 2011). Therefore, the high diversity and the high number of alleles observed in some markers indicate that introduction of only a few founder individuals, as observed in other introduction of amphibians in France such as the bullfrog *Lithobates catesbeianus* (Ficetola et al., 2008), is unlikely. We could think that even though the primary source of the *B. bombina* population in Moselle resulted from numerous tadpoles, they still represent a small fraction of a broader population located in the core area of distribution and that though a bottleneck effect could be detected, it was not sufficient enough to affect the genetic diversity of this population.

The discovery of *Bombina bombina* in Lorraine is recent, certainly dating back to 2009 (Vacher and Pichenot, 2012). Right now, its distribution is geographically restricted and does not directly overlap with that of *B. variegata* (Fig. 4). As it can hybridize with *B. variegata* in the wild, a monitoring of *B. bombina* in the area should be set up to track the population dynamics, its dispersal behaviour, the evolution of its distribution in the area, and to determine possible concurrence with *B. variegata*. However, both species seem to display contrasted ecological preferences in their native habitat: *B. bombina* is known to prefer ponds or swamps for reproduction compared to *B. variegata* that favours puddles (Barandun and Reyer, 1997; Gollmann B. et al., 2011; Gollmann G. et al., 2011; Kruuk and Gilchrist, 1997). Consequently, we might expect that ecological competition should be limited. Moreover, *B. bombina* shows

higher site fidelity, suggesting lower dispersal capacities (Gollmann et al., 2011). Still, *B. bombina* seems to display a broader ecological tolerance by colonizing puddles in areas where ponds are scarce and where both species occur and hybridize (MacCallum et al., 1998). Therefore, a close attention to habitat components in the landscape (i.e., density of ponds and small lakes) should be integrated in a monitoring protocol to track the dynamics of this introduced population. The removal of a species at an early stage is normally the best method to avoid future competition with native species. We can consequently recommend to monitor the competition between both species of *Bombina* in the area, and perhaps also conduct some actions to reduce or remove this introduced population. Additionally, it would be necessary to scan for *Bd* and maybe other pathogens within this introduced population, as they can represent a further threat on native amphibian populations that occur in the area such as the European tree frog *Hyla arborea* or the common frog *Rana temporaria* (Ohst et al., 2013).

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Adaptive significance of the transparent body in the tadpoles of ornamented pygmy frog, *Microhyla ornata* (Anura, Amphibia)

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Abstract. In Southern India, during the Southwest monsoon phase, the newly formed ephemeral water bodies harbour several species of tadpoles as well as some of their predators. Tadpoles of *Microhyla ornata* dwell in surface or column zones of water. They face predation threat from aquatic insect predators and carnivorous tadpoles of other anurans though they are invisible due to their transparent body form. We tested whether transparent body form of *M. ornata* tadpoles is a useful attribute against predation by exposing tail fin stained (with the Nile blue) subjects to a naturally occurring predator (*Hoplobatrachus tigerinus* tadpoles that detect prey using both visual and chemical cues). The study shows that susceptibility of stained *M. ornata* tadpoles to predation increased significantly compared to the unstained transparent individuals. We conclude that the transparent body form is of great significance in escaping predation during the larval phase of life in *M. ornata*.

Keywords. *Hoplobatrachus tigerinus*, *Microhyla ornata*, predation, tadpoles, transparent body

Most anuran amphibians are opportunistic breeders; in South India, they often breed communally in rain-filled puddles soon after the onset of southwest monsoon. Therefore, tadpoles of several species coexist during their aquatic larval phase (Saidapur et al., 2009). In such ephemeral ponds, the tadpoles commonly experience desiccation threat, competition for resources from homospecific and heterospecific members (Mogali et al., 2011a, b, 2015, 2017). In addition, predation threat from aquatic insects and their larvae are routinely encountered by the herbivorous tadpoles in such ephemeral ponds (Skelly, 1997). Furthermore, the tadpoles of several species are carnivorous or omnivorous (Saidapur et al., 2009). In response to coexisting predators, the prey tadpoles have evolved defence strategies that often involve utilization of refuge shelters when available, reduced movements, high burst speed when movement is necessary, species-specific habitat choices (Hiragond and Saidapur, 2001; Saidapur et al., 2009; Hossie and Murray, 2010; Mogali, 2018; Mogali et al., 2019) and so on. Within the community of anuran larvae found in the ephemeral water bodies of Southern India, tadpoles of *Hoplobatrachus tigerinus* are notorious predators and hunt actively (Saidapur, 2001; Saidapur et al., 2009).

Of the several species of anuran larvae found in Southern India, the tadpoles of *Microhyla ornata* are unique in many aspects; they are predominantly found in the surface or column zone of the pond, devoid of teeth, feed on micro-plankton, delicate, slow movers, remain still for longer time and more importantly, their body is transparent (Hiragond and Saidapur, 2001) and hence not easily visible. Despite a highly vulnerable nature, *M. ornata* is highly successful and show wide distribution throughout Asia and the Indian subcontinent. Evidently, the success of any anuran species with aquatic larval phase depends on the survival rate of

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their larvae, emergence on the land with optimum size and, subsequent reproduction. We hypothesized that transparent body of *M. ornata* tadpoles is a key feature in ensuring their survival and escape from predation. Therefore, we conducted experiments on *M. ornata* tadpoles whose tail fins were stained with Nile blue or left unstained and then tested for their efficiency to escape from predation using highly predacious sympatric tadpoles of *H. tigerinus*.

Six egg clutches of *M. ornata* were collected from rain-filled puddles/ ponds located on the Karnatak University Campus, Dharwad (latitude 15.440407°N, longitude 74.985246°E) in June 2008. Each egg clutch was placed separately in plastic tubs (42 cm diameter and 16 cm deep) containing 3 L of pond water. Eggs from all clutches hatched almost synchronously at Gosner stage 19 (Gosner, 1960) a day after their collection. Soon after hatching, the tadpoles of different clutches were mixed and reared in 3 separate glass aquariums (75 cm L × 45 cm W × 15 cm H) each with ~ 600 tadpoles in 15 L of pond water to provide plankton material for feeding. The tadpoles in the Gosner stage 25-26 with comparable body size (length 12.65 ± 0.42 mm, Mean ± SE) were used in trials. The tadpoles of *H. tigerinus* (Gosner stage 27-28; ~ n = 70) were also collected from the rain-filled ponds located on the University Campus. They were reared individually to avoid cannibalism in plastic bowls (16 cm diameter and 7 cm deep) in 0.5 L of aged tap water. *Hoplobatrachus tigerinus* tadpoles of Gosner stage 29-30 having comparable body size (length 32.15 ± 0.49 mm Mean ± SE) were used in the trials.

The experiment was made with two sets of tadpoles of *M. ornata* (n = 25 per set) chosen randomly. In the first set of experiment, tadpoles of *M. ornata* (n = 25) with a natural transparent body, were released into a rectangular glass tank (40 cm L × 25 cm W × 30 cm H) with 10 L of aged tap water that created a column height of 10 cm. After acclimatization for 15 min, a single *H. tigerinus* tadpole (starved for 24 h) was introduced into the tank and left there for 24 h to estimate the quantum of predation. After the trial period, the number of surviving prey tadpoles was recorded to compute the number of tadpoles lost due to predation. In the second set of experiment, the tail fins of prey tadpoles were applied Nile blue (1 mg/mL water and filtered by using Whatman filter paper no. 40) using a fine brush before the trials. The Nile blue coloured tadpoles (n = 25) were left with one starved *H. tigerinus* tadpole for 24 h as in the first set of experiment. After completion of the trial duration, the number of tadpoles consumed by the predator was recorded. Twenty-five trials were carried out for both sets of experiments. The data on the number of tadpoles consumed by the predator between the two experiments were analyzed by the Mann-Whitney U test.

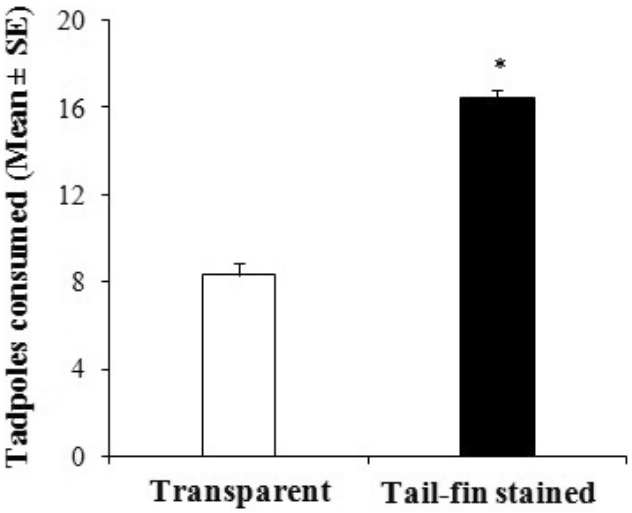

Fig. 1. Number of *Microhyla ornata* (transparent or tail-fin stained) tadpoles consumed by the predator, *Hoplobatrachus tigerinus* tadpoles in trials of 24 h. Data represent Mean ± SE and analyzed by Mann-Whitney U test; n = 25 trials per group with 25 tadpoles per trial. An asterisk indicates a significant difference between the groups ($P < 0.001$).

The results show that the predator consumed a significantly greater number of prey tadpoles that had blue tinge on their tail fins ($U = 15.500$, $P < 0.001$, Fig. 1) compared to those that did not have any colour on the tail fins. Thus, the insatiable predatory tadpoles of *H. tigerinus* that are active chase hunters predated a significantly smaller number of prey subjects that were transparent compared to those whose tail fins showed blue tinge due to Nile blue.

Most previous studies on prey-predator interactions have dealt with the tadpoles residing in the substrate or benthic zones (Relyea, 2001; Saidapur et al., 2009; Jara and Perotti, 2010; Mogali et al., 2011a, 2012). It may be noted that the aquatic insects and their larvae that prey on tadpoles are generally found along with anuran larvae at the substratum and these are mostly 'sit and wait' predators; they wait for the prey to come near before attacking them (Luttbeg et al., 2008; Miller et al., 2014). *Microhyla ornata* tadpoles are principally surface/ column zone dwellers (Hiragond and Saidapur, 2001). Therefore, one can expect the least predation of *M. ornata* tadpoles by the aquatic insects and their larvae. On the other hand, predatory *H. tigerinus* tadpoles are generally substrate dwellers but also capable of moving all over. Besides they are active chase hunters which make use of both visual as well as chemical cues of the prey subjects in devouring them (Hiragond and Saidapur, 2001; Saidapur et al.,

2009). The predation threat from such predators is enormous even to surface dwellers like the *M. ornata* tadpoles. The present findings clearly show that *M. ornata* tadpoles have a greater capacity to escape predation from the active and chase hunting *H. tigerinus* tadpoles. However, a mere blue tinge of tail fins following application of the vital dye Nile blue made them highly susceptible to predation.

Nile blue used in this study was in micro quantities. Hence, in such large quantity of water, chemical cues arising from the dye, if any, would be presumably very weak. However, the study shows that *H. tigerinus* tadpoles perceive even slightly coloured blue tinged *M. ornata* tadpoles more efficiently than that of the transparent subjects. Thus, it appears that less vulnerability of this tadpole species to predation is largely due to its unique transparent body form. The present study, however, does not convey us about the perception of the type of visual cues arising after application of Nile blue to prey tadpoles by *H. tigerinus* tadpoles. Therefore, further additional and differently designed studies are needed to decipher the impact of different colours/ wavelengths to know what exactly is perceived by the predator. In conclusion, the transparent body form of *M. ornata* tadpoles appears to be a key feature ensuring larval survival and escape from predators.

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Comparison of complement system activity amongst wild and domestic animals

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Abstract. Multiple mechanisms have evolved for the defensive recognition of foreign components, such as microorganisms. The majority of immunological studies with vertebrates have been focused on endothermic species, and relatively little attention has been directed toward ectothermic vertebrates. We employed a colorimetric assay designed to compare plasma hemolytic activities based on the serum complement system (CS) activities amongst some representative reptiles, wild and domestic birds, and mammals. Results obtained from the hemolytic assays conducted with plasma derived from all of the animal species used showed that broad-snouted caiman had the highest activity, and no differences were observed in the hemolytic activities of plasma from birds or the other reptile species. In contrast, the CS activity obtained with mammalian plasma was markedly lower than that from the other taxa. This assay has many advantages, such as the requirement of small sample volume, reproducible results, and low cost. In addition, unsensitized sheep red blood cell hemolysis can be successfully used for the evaluation of innate immune system activities in non-mammalian species; however, for mammals, it should be combined with other immunological determinates to evaluate integral innate immunocompetence.

Keywords. Innate immunity, immunocompetence, complement system, wildlife, domestic animals.

Organisms are continually exposed to a multitude of pathogens through contact, ingestion, and inhalation. Multiple mechanisms have evolved for the recognition of foreign antigens such as microorganisms. These strategies are the result of multiple cascade events that converge in the release of molecular signals that stimulate the recognition of foreign antigens. Those mechanisms are strategically divided in two distinct, but related, systems: acquired immunity and innate immunity. The innate immune system also plays a critical role in priming and stimulating the adaptive immune response (Medzhitov and Janeway, 1997). The concept of innate immunity refers to the first line host defense that serves to restrain infection in the early hours after exposure to microorganisms (Hoffmann et al., 1999). In turn, the adaptive immune system, more complex in nature, activates innate effector mechanisms in an antigen-specific manner as a second line of defense. The connections between the various immune components are not fully understood, but recent progress has brought us closer to an integrated view of the immune system and its function in host defense.

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The majority of studies on vertebrate immunity have focused on endothermic species, and relatively little attention has been focused on ectothermic species (reviewed in Juul-Madsen et al., 2008; Hamon and Quintin, 2016). These species are not commonly studied because the features that control their growth, reproduction and general physiology are largely unknown. However, some studies have shown that the complement system, as part of the innate mechanism of fish and other poikilothermic vertebrates, is more diverse than that of higher vertebrates, and thus a broader range of antigens can be recognized (Sunyer et al., 1998).

Crocodilians exhibit well-characterized social behaviors and responses to stressors that can trigger serious disputes between co-specific species, predators, and even conflicts with human activities. As a result, they sometimes exhibit physical trauma, serious injuries and even the loss of entire limbs. Frequently, these animals live in environments, either natural or captive, containing a high concentration of potentially pathogenic microorganisms. In most cases, crocodilians tolerate these circumstances without showing signs of infection (Siroski et al., 2010).

The colorimetric assay used in this study detects and characterizes the serum complement system (CS, Merchant et al., 2006). It is based on the disruption of sheep red blood cells (SRBC) by immunological proteins circulating in plasma. These proteins recognize SRBCs as foreign antigens and initiate activation of the CS cascade, which culminates in the formation of a protein complex that generates a pore (membrane attack complex, MAC) in the SRBC membrane and its subsequent lysis. Upon lysis, released hemoglobin is quantified using a spectrophotometer and considered proportional to the CS activity. This assay is routinely used in clinical laboratories to assess CS activity (Nagaki et al., 1980).

To compare plasma hemolytic activity amongst different species of vertebrates, we collected blood samples from reptiles, wild and domestic birds, and mammals. Blood samples from juvenile broad-snouted caiman (*Caiman latirostris*; n = 8) were obtained from the spinal vein; from tegu lizard (*Salvator merianae*; n = 6), lagoon turtle (*Phrynops hilarii*; n = 5) and painted turtle (*Trachemys dorbigni*; n = 6) from the caudal vein. Blood samples were obtained from wild pochard ducks (*Netta peposaca*; n = 5), domestic ducks (*Anas domesticus*; n = 5), and swan geese (*Anser anser domesticus*; n = 6) from the brachial vein. Blood of following mammals were obtained from the jugular vein: maned wolves (*Chrysocyon brachyurus*; n = 4), spider monkeys (*Ateles chamek*; n = 5), and horses (*Equus caballus*; n = 5). All blood samples were collected using heparinized syringes. All animals appeared healthy, and none were undergoing antimicrobial treatment. Plasma was separated within 1 h of collection by centrifugation at 1500x*g* for 30 min, and stored at -18°C until analysis.

The hemolytic assay was adapted and performed to evaluate the hemolytic properties of serum CS activity from different animals. As mentioned above, the SRBC hemolysis assay is based on the hemolytic disruption of SRBCs by means of serum immunological proteins (Merchant et al., 2005; Merchant and Britton, 2006; Merchant et al., 2009; Siroski et al., 2010). Fresh SRBCs were obtained from heparinized whole blood collected from the jugular vein of Merino sheep (*Ovis aries*). Whole blood from sheep was washed several times with phosphate-buffered saline (PBS, pH 7.4) until the supernatant was clear, and then a 2% SRBC (v/v) solution was prepared (Siroski et al., 2010).

To make a comparison between the hemolytic properties of plasma caused by the serum CS from different animals against SRBC, each sample was treated independently. Validation assays were performed with the plasma of each species with and without ethylenediaminetetraacetic acid (EDTA) and mild heat treatment (56°C for 30 min), both considered as classical inhibitors of the CS (Morgan, 2008). The tests were conducted at laboratory ambient temperature (25°C ± 2°C) by placing 0.5 ml of plasma from each animal together with 0.5 ml SRBC (2% v/v in isotonic saline). After 30 min incubation, the mixture was centrifuged and 300 µl of supernatant was transferred to a well of a microtiter plate for analysis on microplate reader at 540 nm.

A positive control for hemolysis was obtained by 1% SRBCs solution and 0.1% (v/v) Triton X-100. This mix was aggressively injected and ejected several times through a tuberculin syringe until complete hemolysis was confirmed with an optical microscope (Olympus BH-2, Tokyo, Japan) at 400×. Optical density of the supernatant was measured in a microplate reader at 540 nm, and was considered to be maximum hemolysis. The samples were performed in quadruplicate to obtain valid statistical evaluations and results were expressed as the percentage of maximum hemolysis (MH%; mean ± standard error). Statistical analysis was performed using SPSS 16.0 software (SPSS for Windows 2007). Data were tested for normality with the Kolmogorov-Smirnov test, and homogeneity of variances between groups was verified by the Levene test. One-way analysis of variance (ANOVA) and Tukey's test were used to test for differences among groups, and a P value of 0.05 was considered statistically significant.

The hemolytic method based on the rupture of sheep red blood cells was very effective at estimating complement activity in every species tested. Plasma samples

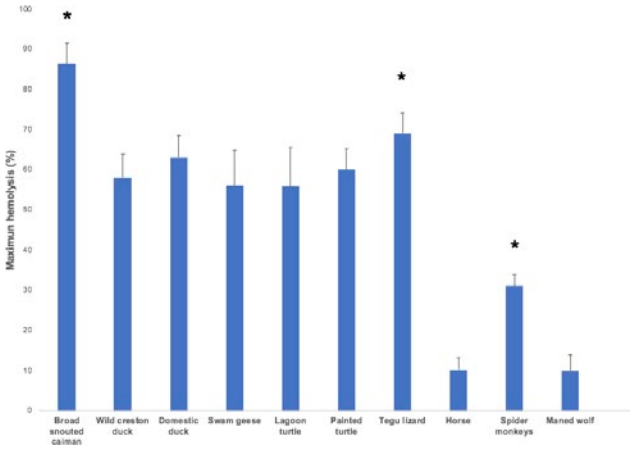

Fig. 1. Results obtained from the evaluation of the complement system activity from reptiles, wild and domestic birds and mammals. * Indicates significant differences.

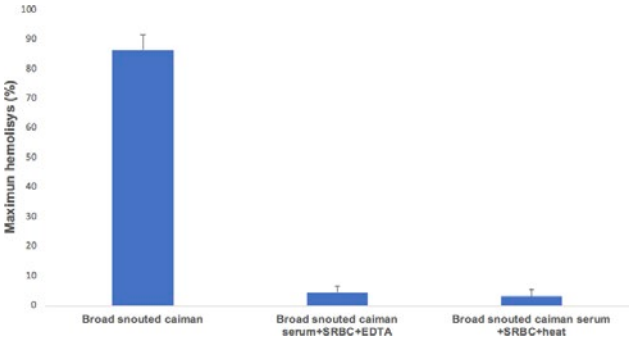

Fig. 2. Results obtained from the evaluation of the complement system activity from Broad-snouted caiman serum+SRBC with EDTA and heat.

from all species responded to the exposure to SRBC solution (2%). Results obtained from the hemolytic assays of plasma derived from ten species of vertebrates showed that *C. latirostris* plasma (86.38 ± 5.1) had the highest activity ($P < 0.05$; Fig. 1). No differences were observed in the %MH generated with plasma from birds and reptiles other than the caiman. However, the tegu lizard had the second highest %MH (69 ± 5.2) after caiman. There were no significant differences between %MH of domestic duck, swam geese and wild pochard ducks (63 ± 5.6, 56 ± 8.9 and 58 ± 5.9, respectively). Conversely, the %MH obtained with mammalian plasma was markedly lower than those from birds and reptiles. While the spider monkey exhibited 31 ± 2.9 activity, the maned wolf and horse had very low values of %MH (10 ± 3.9 and 10.2 ± 3, respectively).

In order to eliminate the possibility that the observed hemolysis was mediated by other hemolytic mechanisms, the CS assay included 2 classical inactivators of the serum complement, mild heat treatment of the serum and ethylenediaminetetraacetic acid (EDTA) (Morgan, 2008). Untreated serum, preheated serum (56°C for 30 min), and serum treated with 50 mM EDTA were exposed to 2% (v/v) SRBCs. Data demonstrated the ability of serum to rupture SRBC membranes (Fig. 2). In this case, %MH values of SRBCs with EDTA (4.4 ± 2.11) and heat (3.2 ± 2.17) were compared. The absorbance recorded indicated that there were significant reductions in the maximum hemolysis of SRBCs when complement-system inhibitors were added ($P < 0.001$; Fig. 2).

The hemolytic activities found for *C. latirostris* in this work were similar and comparable to that values previously reported from other crocodilian species, such as *Alligator mississippiensis* (Merchant et al., 2005), *Crocodylus porosus* and *Crocrodylus johnstoni* (Merchant and Britton, 2006) and *Crocodylus acutus* (Merchant et al., 2010). These data provide evidence that the mechanisms of complement activities for diverse crocodilian species are similar. The differences between the %MH values of each species is a consequence of different habitat where they live and therefore exposure to the large variety of pathogens.

The greater plasma hemolytic capacity of *C. latirostris* compared with that of turtle plasma had already been indicated by Ferronato et al. (2009) for *Phrynops geoffroanus*, who suggested that the higher activity detected in crocodilian species might be due to evolutionary pressure selection on this group because their environment is rich in potentially pathogenic microorganisms. The same reasoning might be extended to the species *S. merianae*, which showed high values, similar to those of turtles, but lower than that obtained for *C. latirostris*. In addition, it is interesting the fact that the values observed in these other reptilian species are lower than that observed for crocodilian species. The high complement activity of *S. merianae* could be associated with the aggressive behaviors displayed toward member of their own species. These conspecific aggressions may have been compensated by the evolutionary development of potent innate immunity (Merchant et al., 2010).

Birds have been shown to have a potent nonspecific immune mechanism based on the alternative pathway of CS activity. Mekchay et al. (1997) reported a significant hemolytic capacity in the plasma of birds and reported higher complement activities in wild birds than in captive birds raised under intensive commercial breeding conditions. These results are consistent with those previously detected by Skeeles et al. (1988) that chickens reared for human consumption exhibited lower CS activities compared to wild turkeys. Differences could be related to the

genetic composition of these birds or reflect a difference in the environment in which the chickens were raised. However, the results from our study revealed no differences between species of domestic and wild birds. The antimicrobial activity of one species of commercial poultry (*Gallus gallus*) was high, similar to that of reptiles, possibly resulting from a common evolutionary origin and developed in response to periodic exposure to a significant amount of pathogens (Siroski et al., 2010).

The highest hemolytic capacity among the plasma samples derived from mammals was detected in spider monkeys but was still much lower than that of non-mammalian species. Similarly, in a study conducted to determine levels of serum hemolysis in the plasma of nine primate species against sensitized and desensitized SRBC, the results were varied, but the plasma from primates mostly caused only a slight hemolysis of unsensitized SRBC (Ellingsworth et al., 1983). Other determinations were performed with camel (*Camelus dromedarius*) serum where lytic capacity was evaluated against desensitized erythrocytes from different species. They found that the erythrocytes in homologous species (goats, sheep, rat and bovine) were resistant to lysis, while the effect on heterologous erythrocytes was attributed to the presence of the alternative pathway (Olaho-Mukani et al., 1995). Similar findings were previously reported by Arya and Goel (1992) in buffalo (*Bubalus bubalis*) serum. In a study focused on the complement-mediated disruption of erythrocytes from 18 species of mammals and birds, it was observed that erythrocytes were not lysed by homologous complement, with the exception of guinea pig complement, which weakly lysed homologous erythrocytes, but with only 37% lysis maximum (Ish et al., 1993).

The low hemolysis values of erythrocytes exhibited by mammalian serum suggests that this restriction might be involved in the regulatory principle of non-specific and role-specific factors (Van Dijk et al., 1983). From these studies we can assume that plasma of some mammals did not hemolyze the SRBCs because they were not recognized as foreign and thus the mammalian CS was considered to recognize more limited range of antigens to trigger activation.

Although the main objective was to evaluate the differences between the activities of CS in the plasma of the various species studied, it is important to note that the assessment of immune function in wildlife has become an important tool for the investigation of ecological and evolutionary processes. The variety of tests that can be used in wild animals are often limited by the difficulty of capture and handling, and also difficulties of recovery or recapture, lack of specialized specific reagents, and small sample sizes of the study species (Matson et al., 2005). In this case, the assay employed had many advantages, such as the requirement of small sample volume, reproducible results, and low cost for the assessment of immunocompetence. Hemolysis of SRBCs has been used to assess the serum complement activity of crocodilians (Merchant et al., 2005; Merchant and Britton, 2006; Merchant et al., 2010; Siroski et al., 2010; Merchant et al., 2013a, 2013b), varanids (Merchant et al., 2012), snakes (Baker and Merchant, 2018), turtles (Ferronato et al., 2009; Baker at al., 2019), and amphibians (Major et al., 2011). The results showed that the test of unsensitized SRBC hemolysis can be used successfully for the evaluation of the innate immune system in a wide variety of species of reptiles and birds, but for mammals it should be utilized with other immunological determinations. These findings may reflect underlying differences in the biology and life history of each species.

Our study confirmed that the hemolytic method was very effective and advantageous at estimating complement activity in every species tested because small plasma samples from all species responded to the exposure to SRBC solution (Merchant et al., 2006). Furthermore, this assay allows to be routinely used in clinical laboratories to assess CS activity. Through the use of this hemolytic assay, we demonstrated a greater plasma hemolytic capacity of *C. latirostris* compared with other reptiles, birds and mammals. This means that the higher activity detected in crocodilian species might be due to evolutionary pressure selection on this group because their environment is rich in potentially pathogenic microorganisms and they exhibit aggressive territorial defense behaviors. Due to the close phylogenetic linkage with the reptiles, we built similar deductions with the group of birds.

Finally, it is important to highlight that the use of hemolytic method in the current study offered a contribution to the knowledge of comparative immunology. Also, it could be an appropriate tool to evaluate the role of complement in the immune function for some other wildlife species other than reptiles and for the investigation of ecological and evolutionary processes.

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tina). All animals were handled according to the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals (CONICET, 2005). Samples were collected from captivity wild and domestic animals, which were maintained in a registered zoo by a National Resolution.

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Effects of temperature and food level on plasticity of metamorphic traits in *Bufo gargarizans gargarizans* larvae

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Abstract. Many environmental factors such as temperature or food level may influence growth and mortality risks of ectothermic vertebrates in both aquatic and terrestrial habitats. In this study, plasticity in growth rates, survival, larval period, and size at metamorphosis were examined in *Bufo gargarizans gargarizans* larvae under different combinations of temperature and food level. Our results showed that larvae metamorphosed at an older age when reared at 17.3°C. A significant interaction between food level and temperature revealed that the food level has obviously affected length of larval period when tadpoles raised at 17.3 °C, but not at 27.3 or 31.3 °C. Also, we found clear evidence that growth rates are influenced by both temperature and food level. Interestingly, tadpoles reared at 17.3 °C had larger size at metamorphosis than those reared at other temperatures, suggesting that *B. g. gargarizans* larvae reared at cold temperatures have a longer developmental period but they are also larger as metamorphs than conspecifics reared at warmer temperatures. Therefore, the global climate change or local manipulations of the environment may promote growth and development of *B. g. gargarizans* larvae, but not large size at metamorphosis.

Keywords. Amphibia, Anura, Bufonidae, *Bufo gargarizans gargarizans*, larval period, metamorphic size, phenotypic plasticity, China.

In animals with complex life cycles, such as amphibians, metamorphic size and timing are important fitness components (Arnold and Wassersug, 1978; Wilbur, 1980). Many environmental factors such as temperature or food availability may influence size and age at metamorphosis (reviewed by Álvarez and Nicieza, 2002). Generally, low temperatures resulted in a longer developmental periods, but larger metamorphic size than conspecifics reared at warmer temperatures because of differential effects on growth and differentiation (Smith-Gill and Berven, 1979; Álvarez and Nicieza, 2002). However, previous studies indicated that the temperature variation of the reaction norms among species and phylogenetic groups was a puzzling problem, suggesting an interplay between phylogeny and adaptation to specific habitats (aquatic and terrestrial) (e.g., Blouin, 1992; Morand et al., 1997; Joly et al., 2005).

A steady food supply generally elicits a younger age and larger size at metamorphosis (Werner, 1986). This also correlates with younger age and larger size at first reproduction, and thus potentially higher fecundity (Harris, 1999; Werner, 1986; Semlitsch et al., 1988). This variation in metamorphic traits may have strong effects on later fitness as early metamorphosis, and large size at metamorphosis are favoured because of their positive effects on juvenile survival and adult fecundity (Wells, 2007).

Temperature and food availability have been studied in numerous anurans (see review by Angilletta and Dunham, 2003), but little is known about how the interaction between temperature and food availability can affect tadpole growth and development (but see: Laugen et al., 2005; Castano et al., 2010; Courtney Jones et al., 2015; Yu et al., 2015). In this study, we examined the potential

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interactive effects of food level, and rearing temperature on the plasticity of metamorphic traits of *Bufo gargarizans gargarizans*, including the length of larval period, survival, the size at metamorphosis, and growth rate. As model species we used *Bufo gargarizans gargarizans*, a widely distributed toad, breeding in different aquatic habitats (Yu and Guo, 2013), which may offer a large variation in both temperature and resource availability during larval development.

Bufo gargarizans gargarizans is sexually dimorphic and is widely distributed in East Asia. Female toads are the larger sex, and clutch size is positively correlated with female body size (means = 9325 ± 279.05, range = 3275–15880; Yu and Sharma, 2012). It is an explosive breeder with typical breeding habitats concentrated along the vegetated edges of large still water bodies and a relatively short breeding season (6-14 days; Wells, 2007; Yu and Sharma, 2012). The tadpoles hatch after two weeks in the breeding ponds. The timing of larval development in natural ponds is about 50-55 days, when water temperature varies from 6 (at night of early–March) to 29 °C (at noon of mid–April, mean temperature less than 25 °C, Yu, personal observations). Rich *Spirogyra* and pondweed grow in pond, and provide food for *B. g. gargarizans* tadpoles in the larval period (Wei et al., 2011).

During the peak period of breeding activity, we collected a total of 25 amplectant pairs in one population in Shihe County (32°08'N, 114°01'E; datum = WGS84), Henan, the central plains of China, in mid–February 2012. Those animals were transported to laboratories close to spawning sites. We kept pairs separately in plastic cask (20 L) filled with approximately 12-15 cm of pond water until the eggs were deposited. Once oviposition was completed, we collected 40 fresh eggs from each of the egg masses from 20 *B. g. gargarizans* females because five female toads did not lay eggs. On the same day, we put all fresh eggs into two 80 L plastic containers with an automatic aerator, and we left them there until hatching. A total of 360 tadpoles (Gosner stage 25; Gosner, 1960) were randomly allocated to six experimental treatments (n = 60). Larvae were fed with commercial fish food (Bieyanghong, Biological Co. Ltd., Hangzhou, China, protein content, PC; protein >45%, lipids >12%, algae >12%, fiber >4%, ash <10%). Tadpoles were exposed to a 13L:11D photoperiod throughout the study period and the water in the containers was changed weekly.

We used a 2×3 factorial design to examine the effects of food level and rearing temperature on larval growth rates and post-metamorphic performance. To evaluate the effects of food level, half of the tadpoles in each temperature treatment were placed at low mass-specific food level (25 mg food/g tadpole per day) and a half on a high food regimen (50 mg food/g tadpole per day) throughout the experiment. These food levels were chosen to be consistent with the previous study on this species by Zhang et al. (2007). For each food regimen, three different temperature levels were kept across the rearing period: low (room) temperature (17.3 ± 1.33 °C; mean ± SD); middle temperature (27.3 ± 1.42 °C); high temperature (31.3 ± 0.56 °C). The "low" and "middle" temperature were chosen because they fall within the range this species experiences in the field, while "high" temperature approached the avoidance temperature (Ma and Long, 2005). Aquarium heaters (Minjiang, BaolaiHD–200, Guangzhou, China) were used to raise the water temperature in the "middle" and "high" treatments. For each temperature treatment, 120 individual vessels with foam board (60 for each diet treatment), each of which is 300 ml, were randomly placed in two rectangular tanks (110 × 90 × 60 cm; L × W × H). To further minimize the possible effects of such heterogeneity, the positions of the 120 tadpole containers within a given temperature were reassigned at random every three days.

After the first metamorph (defined as the emergence of at least one forelimb, Gosner stage 42) was discovered, the six large tanks were checked daily and all metamorphs found were collected and kept individually in plastic vials (8 cm diameter) with sand and 1 mm of water until tail re-sorption was completed (Gosner stage 46). Four variables were measured: (1) age at complete metamorphosis (number of days from the beginning of the experiment until complete metamorphosis, Gosner stage 46); (2) SVL (snout-to-vent length) at complete metamorphosis (SVL was measured with digital calipers to the nearest 0.01 mm; (3) growth rate was measured as the SVL at complete metamorphosis divided by the age; (4) the percentage of surviving tadpoles that metamorphose.

We analysed the length of larval period, SVL at metamorphosis, and growth rate by using a generalized linear model (GLM) with type III mean squares and temperature, food level, and their interaction as fixed factors. We used log-linear model to test survival. If the overall GLM or log-linear model results were significant, the data were analysed with ANOVAs by using post-hoc multiple comparisons (Fisher's LSD) or a Chi-square test to evaluate differences between food levels or between temperatures (SPSS 13.0, SPSS Inc., 2004, Chicago, IL, USA). All given P-values are two-tailed, with values presented as means ± standard error.

The effects of rearing temperature or food level on the length of larval period were significant (temperature, $F_{2, 183} = 266.50$, $P < 0.001$; food level, $F_{1, 183} = 38.61$, $P < 0.001$), as well as their interaction ($F_{2, 183} = 10.08$, P

< 0.001). Tadpoles raised at 17.3 °C took longer to metamorphose than those raised at 27.3 and 31.3 °C (all P < 0.05, Fig 1), while no difference was found between the latter temperature treatments. Tadpoles feeding on high food level reached metamorphosis earlier than those raised at low food level (P < 0.001). A significant interaction revealed that tadpoles raised at 17.3 °C, high food level reached metamorphosis earlier than those raised at low food level (post-hoc tests: P < 0.001), but there were not different at 27.3 or 31.3 °C (both P > 0.05; Fig. 1).

The rearing temperature significantly affected SVL at metamorphosis ($F_{2, 183}$ = 3.68, P = 0.027, Fig. 1), but food level, as well as temperature × food level interaction did not (food level: $F_{1, 183}$ = 2.06, P = 0.153; interaction: $F_{2, 183}$ = 1.37, P = 0.257). Tadpoles reared at 17.3 °C had significantly larger SVL at metamorphosis than those at 31.3 °C (P = 0.016), or marginal significantly than those at 27.3 °C (P = 0.051).

The rearing temperature or food level positively influenced growth rate (temperature, $F_{2, 183}$ = 113.89, P < 0.001; food level, $F_{1, 183}$ = 25.72, P < 0.001, Fig. 1), but the interaction between rearing temperature and food level was not significant ($F_{2,183}$ = 0.37, P = 0.694). Tadpoles feeding on a high food level had a greater growth rate than those raised at low food level (P < 0.05). Tadpoles reared at 17.3 °C had a lower growth rate than those raised at 27.3 and 31.3 °C (both P < 0.001), but there was no difference between the latter two temperature treatments (P = 0.350).

Survival at metamorphosis was affected by food level (Z = -2.65, P = 0.008): tadpoles feeding on a high food level had a higher survival rate than those raised at low food level. In contrast, nor the rearing temperature, nor the interaction between temperature and food level were significant (temperature: Z = -0.81, P = 0.416; interaction: Z =1.52, P = 0.129; Fig. 1).

In anuran amphibians, age and size at metamorphosis may be reduced with increasing temperature (Harkey and Semlitsch, 1988; Newman, 1998; Beck and Congdon, 2000; Merila et al., 2000; Laugen et al., 2003; Palo et al., 2003; Liess et al., 2013; Courtney Jones et al., 2015; Yu et al., 2015). Our results confirmed that *B. g. gargarizans* metamorphosed at a younger age when reared at higher temperature. However, tadpoles reared at low temperature had larger SVL at metamorphosis than those reared at other temperatures, which are consistent with previous studies (Atkinson, 1994, 1996; Angilletta and Dunham, 2003; Arendt, 2011; Yu et al., 2016).

Temperature can affect metamorphic size in two ways. First, temperature is a major proximal factor determining growth and development, which cause large differences in size at metamorphosis (Smith–Gill and Berven, 1979). This is the expected outcome if growth rates are more responsive to temperature than differentiation rates. Second, temperature may influence the extent to which food level can affect growth and development, which in turn influence size at metamorphosis. In our study, food level had no effect on SVL independently from the temperature treatment. However, cold-reared individuals had slow growth rates compared to tadpoles in warmer temperatures. This was also reflected in the age at metamorphosis, which was significantly older at low temperatures compared to higher temperatures. Therefore, we suggested that *B. g. gargarizans* larvae reared at cold temperatures have a longer developmental period but they may also become larger as metamorphs than conspecifics reared at warmer temperatures, independently from the available food level.

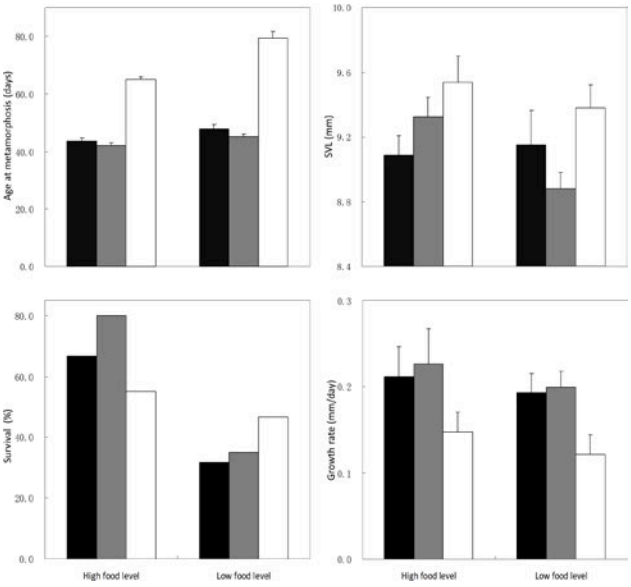

Fig. 1. Influences of temperature and food level on age, SVL, growth rate and survival at complete metamorphosis of *B. g. gargarizans* (Gosner stage 46; black columns = high temperature; grey columns = middle temperature; open columns = low temperature).

The food availability during the larval stage has important effects on timing of metamorphosis (Leips and Travis, 1994). Several experimental studies have demonstrated that high food availability with a large proportion of protein can produce a two-fold effect, accelerating both growth and developmental rates (Nathan and James, 1972; Steinwascher and Travis, 1983; Pandian and Marian, 1985). Laugen et al. (2003) suggested the effect of high food availability for maximum growth and development rates were larger in the warm temperature treatments. In this study, at low temperature, tadpoles raised with high food level reached metamorphosis earlier than

those raised at low food level, but they were not different at middle and high temperature. Thus, the effects of food availability on larval growth were partially dependent on developmental temperature.

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