Evol Ecol (2017) 31:925–936
DOI 10.1007/s10682-017-9920-5

ORIGINAL PAPER

Skin toxin production of toads changes during early ontogeny but is not adjusted to the microbiota of the aquatic environment

János Ujszegi[1] · Ágnes M. Móricz[2] · Dániel Krüzselyi[2] · Attila Hettyey[1]

Received: 3 July 2017 / Accepted: 18 September 2017 / Published online: 30 September 2017

Abstract Many animals display spectacular adjustments of their phenotype to changing environmental conditions in terms of altered behaviour, physiology and morphology, but whether similar plasticity in chemical defences is taxonomically widespread as well has remained unknown. Bufadienolides, toxins produced by some anuran amphibians, are known to be effective against many predators, but they can also contribute to defences against microorganisms. We experimentally investigated if qualitative and/or quantitative changes in the chemical defences of *Bufo bufo* tadpoles are induced by differences in the microbiota of their environment. We reared animals in purified tap water, or in pond water containing lowered or natural quantities of the natural microbiota, and measured the number of compounds and total quantity of bufadienolides at three ontogenetic stages during larval development, as well as 10 days after metamorphosis. We observed a significant decrease in the number of compounds, and a parallel increase in the total quantity of bufadienolides after metamorphosis, independent from treatments. The quantity of individual bufadienolide compounds also changed during ontogeny independently from treatments. Our results, thus, suggest that although bufadienolide production changes during early ontogeny, it is not adjusted to the microbiota present in the environment.

Keywords Bufadienolide · Inducible defence · Natural microbiota · Opportunistic pathogen · Phenotypic plasticity

Electronic supplementary material The online version of this article (doi:10.1007/s10682-017-9920-5) contains supplementary material, which is available to authorized users.

✉ János Ujszegi
ujszegi.janos@agrar.mta.hu

[1] Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó út 15, Budapest 1022, Hungary

[2] Department of Pathophysiology, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó út 15, Budapest 1022, Hungary

Introduction

Phenotypic plasticity is the ability of individual organisms to adjust their phenotype to changing environmental conditions. It evolves if environmental changes are not predictable, and fitness benefits of the response exceed costs in the inducing environment, but not in others (Via and Lande 1985; West-Eberhard 1989; McCall and Fordyce 2010). In animals, phenotypic plasticity has frequently been reported to manifest in behavioural, physiological or morphological changes (Harvell 1990; Van Buskirk 2002; Relyea 2004). Although chemical defences are widespread across several animal taxa, and can act as repellent agents against predators and pathogens (Toledo and Jared 1995; Mebs 2001; Rollins-Smith et al. 2005; Brodie 2009), very little is known about plasticity in their production in response to changing environmental conditions (Hettyey et al. 2014).

Amphibians have a well-developed adaptive immune system, which responds in a plastic and inducible manner, and specifically recognizes and neutralizes pathogens and parasites (Carey et al. 1999). In addition, amphibians synthesize and excrete onto their skin numerous chemical compounds, including antimicrobial peptides (AMP), steroids, alkaloids and biogenic amines, which provide them with an effective first line of defence against many pathogens and parasites (Cunha Filho et al. 2005; Macfoy et al. 2005; Rollins-Smith et al. 2005; Gomes et al. 2007). Because the synthesis of defensive chemical compounds is considered to be metabolically costly (Longson and Joss 2006; Morgenstern and King 2013), and the composition of the microbiota, which includes opportunistic and obligate pathogens, can vary widely and unpredictably in each habitat (e.g., due to different water characteristics, stochastic changes of weather conditions or variation in the density and composition of amphibian communities; Torsvik et al. 2002; Lozupone and Knight 2007; McKenzie et al. 2012; Kueneman et al. 2014), plasticity in the production of these compounds may be expected to evolve. However, we know of only three studies on the effects of changing environmental microbiota on chemical defences of amphibians or animals in general (Hettyey et al. 2014). Miele et al. (1998) reported that experimental exposure to *Aeromonas hydrophila*, an opportunistic amphibian pathogen, induced changes in the AMP production of *Bombina orientalis*. In another study, Simmaco et al. (1998) observed a two- to fourfold increase in AMP secretion in *Pelophylax esculentus* (formerly *Rana esculenta*) experimentally infected with *A. hydrophila*. Finally, AMP secretion in *P. esculentus* decreased in the absence of natural microbiota (Mangoni et al. 2001). We know of no further evidence for direct induction of chemical defences by microbial agents, so that the presence of such phenotypic responses in chemical defences other than AMPs (e.g., bufadienolides) has remained unknown. How generally plastic responses may occur across species has remained obscure as well. Finally, because all previous studies used mature individuals, it remains to be determined if phenotypic plasticity in chemical defences may be expressed already during larval development, which is to be expected, because individuals are exposed to high abundance and diversity of microbial pathogens in the aquatic environment (Pessier 2002; Densmore and Green 2007).

Several species of bufonid toads are known to produce defensive chemicals (biogenic amines, alkaloids and steroid compounds; Daly 1995; Hayes et al. 2009; Macfoy et al. 2005). Bufadienolides are steroid compounds synthesized from early larval development onwards (Hayes et al. 2009; Üveges et al. 2017) with potent antipredator activity due to their antagonistic effect on membrane Na+/K+-ATPase (Daly 1995). In addition, bufadienolides may also contribute to defence against pathogens, as they can have antimicrobial, antiprotozoal and antifungal activity (from plant and animal sources; Taniguchi and Kubo 1993; Cunha Filho et al. 2005; Tempone et al. 2008; Puglisi et al. 2009; Barnhart

et al. 2017; Online Resource 1). In a previous study we found among-population variability in bufadienolide profiles of *Bufo bufo* tadpoles in natural populations, which was not linked to the presence of predators (Bókony et al. 2016). The observed variation in chemical defences may instead be attributed to divergent pathogen communities in the sampled water bodies or to increased risk of disease transmission at locations with high amphibian density (Briggs et al. 2010; Bókony et al. 2016). As bufonid toads lack skin-secreted AMP-s (Conlon 2011), bufadienolides may also function as protective antimicrobial chemicals in these species. Inducibility of bufadienolide production by microbes has, however, never been examined.

We investigated in a controlled laboratory experiment whether differences in the microbiota of the larval environment resulted in quantitative or qualitative changes in the chemical defences produced by *Bufo bufo* (Linnaeus 1758; Anura, Amphibia) tadpoles. We created different microbiotic conditions in the aquatic environment by using filtered tap water, and pond water with original and lowered microbial density. The main defensive chemicals in *B. bufo* are bufadienolides (Mebs et al. 2007), which are already produced during larval development (Üveges et al. 2017). Because toxin composition and quantity varies with age (Üveges et al. 2017), we sampled larvae at three developmental stages. To be able to detect potential effects of the larval environment on the production of defensive chemicals reaching beyond the larval life-stage, we performed an additional sampling 10 days after completion of metamorphosis.

Materials and methods

Experimental procedures

On April 2015 we collected 20 eggs from each of 10 freshly laid egg clutches of the common toad (*Bufo bufo*) from a pond next to Nagykovácsi, Hungary (47.57626 N, 18.86865 E). Eggs were transported to the Experimental Station Júliannamajor of the Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences. We rinsed eggs with reconstituted soft water (RSW; APHA 1985) and then placed them into plastic boxes (24 × 16 × 13 cm) holding 1 L RSW at a constant 19 °C for 5 days and a 12:12 h light: dark cycle. Eggs were separated by families. Because of logistical reasons, we raised the temperature on the 6th day to 25 °C to accelerate the development of hatchlings.

We started the experiment 5 days after hatching, when tadpoles reached the free-swimming state (developmental stage 25; Gosner 1960), with 12 healthy-looking tadpoles from each family. We reared tadpoles individually in opaque plastic boxes (15.5 × 11.5 × 12 cm) and applied three treatments by filling rearing boxes with (1) RSW treated with a germicide light ('RSW' treatment), (2) pond water treated with a germicide light and diluted with RSW 1 to 1 ('Lowered microbial density' treatment), or (3) pond water diluted with RSW 1 to 1 ('Natural microbial density' treatment; for details, see below). Tadpoles were randomly assigned to the three treatments with four replicates per family in each treatment, which resulted in a total of 120 experimental units. We fed tadpoles with slightly boiled spinach ad libitum and changed water twice a week using different nets for each treatment to avoid contamination. Temperature was 19.7 ± 0.85 °C (mean ± SD) during the experiment with a 12:12 h light: dark cycle. We arranged rearing boxes into randomized spatial blocks, each containing 1 replicate from each treatment.

Before each water change we treated the freshly prepared RSW for 24 h by aerating and stirring it with aquarium air pumps, covering the tub with a lid, and irradiating the RSW with a germicide UV lamp (Sylvana G15W/T8, UV-C, 15 W) to keep the RSW close to sterile until water change. At the same time, we transported 40 L of pond water from the tadpoles' site of origin to the laboratory and divided it into two and filtered it by pouring through a plankton net. Pond water was stirred and aerated with aquarium air pumps and both tubs were covered with a lid. In one of the tubs we irradiated the pond water using a germicide UV lamp (for specifications see above) to reduce the number of live microbes. Immediately before water changes, we diluted both types of pond water with UV-treated RSW 1 to 1 to avoid microbial bloom in the tadpoles' rearing boxes.

We monitored survival and development daily. As soon as a tadpole reached developmental stage 42 (forelimb emergence; Gosner 1960), we discarded the water, placed back the metamorphosing individual into the same box, added 100 mL RSW, tilted the box by approximately 10 degrees to provide areas both with and without water, and covered the box with a translucent and punched lid. Once an individual reached developmental stage 46 (complete tail resorption; Gosner 1960), we replaced the water with wet paper towels, and fed the toadlets with small crickets (instar 1–2) ad libitum for 10 days. Date of metamorphosis and tail resorption were registered in each case.

Skin toxin sampling and bufadienolide analysis

We took bufadienolide samples at 4 ontogenetic stages from experimental animals: (1) 7 days after start of the experiment (developmental stage 27.7 ± 0.8, mean $\pm$ SD; henceforth GS 28), (2) 27 days after start of the experiment (developmental stage: 37.9 ± 0.8, mean $\pm$ SD; henceforth GS 38), (3) at the start of metamorphosis (developmental stage 42) and (4) 10 days after completion of metamorphosis. On each occasion, we sampled one randomly selected individual from each family $\times$ treatment $\times$ age combination. We blotted tadpoles dry, measured their body mass to the nearest 0.1 mg using an analytical balance (Ohaus Pioneer PA-114), fixed tadpoles in 1 mL absolute HPLC-grade methanol and stored samples at -20 °C until further processing.

We homogenized samples with a homogenizer (VWR VDI 12) equipped with a dispersing tool (IKA S12N-7S), dried samples under vacuum at 45 °C using a rotary evaporator (Büchi Rotavapor R-134, Flawil, Switzerland), and re-dissolved samples in 1 mL absolute HPLC-grade methanol, aided by brief exposure to ultrasound in a bath sonicator (Tesla UC005AJ1). As a last step of sample preparation, we filtered samples through FilterBio nylon syringe filters (pore size = 0.22 μm).

We analysed bufadienolide compounds by means of high-performance liquid chromatography coupled with diode-array detector and electrospray ionization mass spectrometry (HPLC–DAD–ESI–MS). We identified the chromatographic peaks as bufadienolides based on the UV spectrum (Hayes et al. 2009) and by comparing their retention time and mass spectrum to those of the following commercially available standards: bufalin, bufotalin, resibufogenin, gamabufotalin, areno- and telocinobufagin (Biopurify Phytochemicals, Chengdu, China), cinobufagin (Chembest, Shanghai, China), cinobufotalin (Quality Phytochemicals, New Jersey, USA) and digitoxigenin (Santa Cruz Biotechnology, Dallas, TX, USA) or to compounds established by analysing a sample obtained from an adult male common toad by gently massaging the parotoid glands.

We performed HPLC–MS measurements on a Shimadzu LC–MS 2020 instrument (Shimadzu, Kyoto, Japan) that consists of a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a diode array detector and a single-

quadrupole mass analyser with electrospray ionization (ESI–MS). Chromatographic separations were carried out at 35 °C on a Kinetex C18 2.6 μm column (100 mm × 3 mm i.d.) in series with a C18 guard column (4 mm × 3 mm i.d.) using 10 μL injections. Eluent A was 5% aqueous acetonitrile with 0.05% formic acid and eluent B was acetonitrile with 0.05% formic acid. The flow rate was 0.8 mL/min and the gradient was as follows: 0–2 min, 10.5–21.1% B; 2–15 min, 21.1–26.3% B; 15–24 min, 26.3–47.4% B; 24–25 min, 47.4–100% B; 25–30 min 100% B; 30–31 min 100–10.5% B; 31–35 min 10.5% B. ESI conditions were as follows: desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; drying N_2 gas flow, 15 L/min; nebulizer N_2 gas flow, 1.5 L/min; positive ionization mode. Data was acquired and processed using the programme LabSolutions 5.42v (Shimadzu, Kyoto, Japan).

Statistical analyses

Four tadpoles died during the experiment (two in the RSW treatment, and one in both the Lowered and the Natural microbial density treatments) and we accidentally lost five toxin samples during sample preparation (all in the Natural microbial density treatment). We excluded two further animals from analyses because of slow development (their developmental stage at the relevant sampling occasion was lower than the mean by more than 3 SD one in the RSW treatment, one in the Natural microbial density treatment), and another one because of extremely low body mass (lighter than the mean by more than 3 SD; RSW treatment). This resulted in a total of 108 units in the statistical analyses.

To calculate the number of bufadienolide compounds (NBC) present in each animal, we assumed a compound to be present when its area value was larger than 4000 in the chromatogram, as smaller peaks cannot be identified unambiguously as bufadienolides. We estimated the quantity of each compound from the area values of chromatogram peaks based on the calibration curve of the bufotalin standard, and summed up these values to obtain an estimate of total bufadienolide quantity (TBQ) for each individual. The use of the calibration curve of a specific bufadienolide standard to obtain approximate estimates of bufadienolide quantities has been successfully used before in similar studies (Hagman et al. 2009; Bókony et al. 2016; Üveges et al. 2017). We analysed effects of treatments and sampling time (developmental stage) on toxin content using linear mixed modelling procedures (LMM). To enhance normality of model residuals and homogeneity of variances, we entered square-root transformed values of both NBC or TBQ as the dependent variable. Initial models included treatment and sampling occasion as fixed factors, body mass as a covariate, all possible two-way interactions and the three-way interaction. We entered block and family as random factors. We verified homogeneity of variances using Levene-tests, and normal distribution of model residuals using Shapiro–Wilk tests and by inspecting diagnostic plots. We applied backward stepwise removal (Grafen and Hails 2002) to avoid potential problems due to the inclusion of non-significant terms (Engqvist 2005). We obtained statistics for removed variables by re-entering them one by one to the final model. All tests were two-tailed. Statistics were calculated using SPSS Statistics 17.0 for Windows.

Results

We detected 20 bufadienolide compounds identified as such based on their UV spectra, but none of the compounds used as standards were found to be present (Table 1). The quantity of individual compounds showed varied age-dependent patterns (Fig. 1; Table 1): some bufadienolides were present at all sampling occasions in similar quantities (e.g., compound 9, see Fig. 1i), while others were present in detectable amounts only in tadpoles or only in toadlets (e.g., compound 6 or 12, respectively, see Fig. 1f, l).

The number of bufadienolide compounds (NBC) differed among sampling occasions, but not among treatments (LMM; developmental stage: $F_{3,\ 93} = 9.5$, $P < 0.001$; treatment: $F_{2,\ 63} = 0.47$, $P = 0.63$; Fig. 2).The main effect of body mass, as well as the interactions remained non-significant (body mass: $F_{1,\ 100} = 0.2$, $P = 0.66$; interactions: all $P > 0.09$). Tukey HSD post hoc tests revealed that NBC was lower in toadlets sampled 10 days after metamorphosis than in samples collected in any of the larval stages (all pairwise $P < 0.018$; toadlets: median: 11, range: 9–14, versus larvae at developmental stage 28: median: 13, range: 10–15; stage 38: median: 14, range: 10–16; stage 42: median: 13, range: 9–17.

Table 1 Percentages of studied individuals in which the given bufadienolide compound occurred in various developmental stages, and the analytical properties of detected compounds

Bufadienolide compound	Percent occurrence of bufadienolide compounds				Analytical properties	
	Stage 28	Stage 38	Stage 42	Toadlet	Retention time [min]	m/z $[M + H^+]$
Compound 1	0	0	100	100	3.1	419
Compound 2	11.5	17.2	100	33.3	4.4	403
Compound 3	100	6.9	3.4	62.5	6.7	417
Compound 4	100	100	100	58.3	7.1	401
Compound 5	96.2	100	89.7	0	7.4	701
Compound 6	100	93.1	86.2	0	9.1	731.2
Compound 7	100	100	100	100	10.2	729
Compound 8	65.4	58.6	17.2	0	10	715
Compound 9	96.2	100	100	100	11.6	727
Compound 10	3.8	48.3	10.3	100	13.2	715
Compound 11	100	89.7	48.3	62.5	13.8	729
Compound 12	0	0	0	95.8	14.5	713
Compound 13	100	100	100	100	16.1	715
Compound 14	80.8	96.6	100	100	17.7	713
Compound 15	53.8	93.1	55.2	8.3	18	743
Compound 16	0	3.4	86.2	100	18.2	701
Compound 17	92.3	79.3	75.9	0	18.5	715
Compound 18	100	100	100	100	19.7	757
Compound 19	23.1	55.2	55.2	0	20.4	573
Compound 20	38.5	89.7	3.4	4.2	21.5	571
N	26	29	29	24		

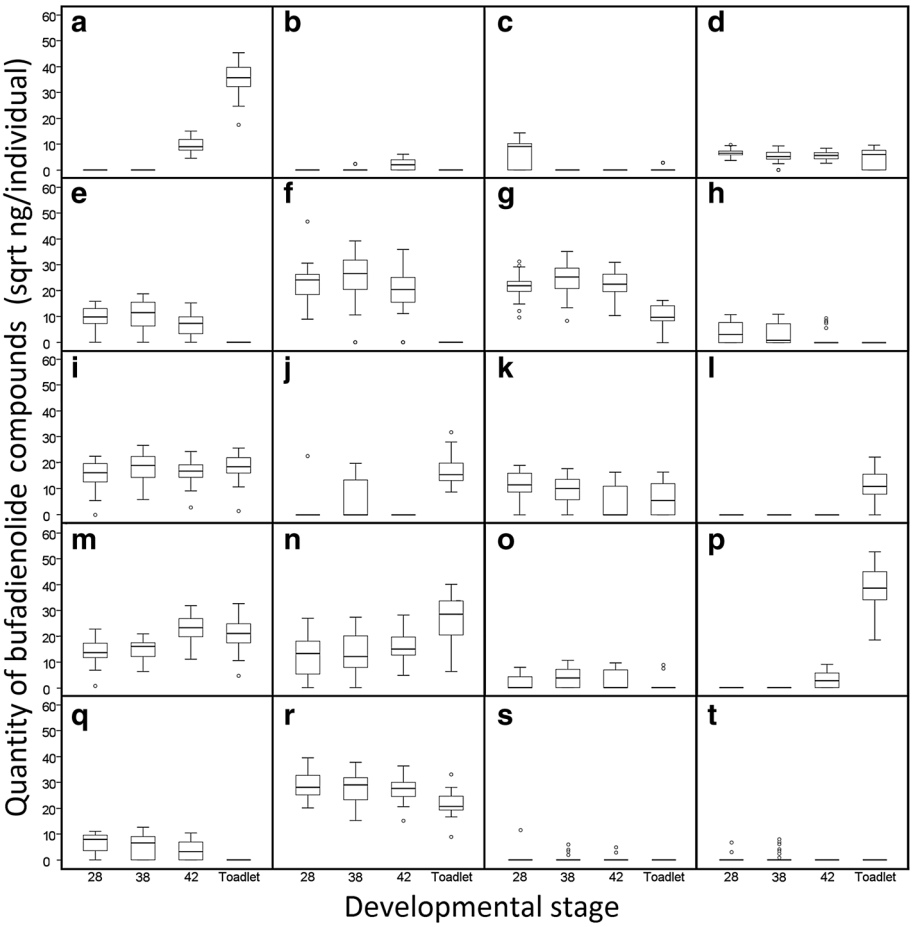

Fig. 1 Ontogenetic changes in the quantity of bufadienolide compounds in *Bufo bufo* (compound 1 is depicted in panel a, compound 2 in panel b, and so forth). Because the effect of treatment was non-significant, treatments are merged within developmental stages. Note that Table 1 shows data on the incidence of certain compounds, whereas these graphs depict toxin quantities calculated as Bufotalin-equivalent. Data are square-root transformed to enhance comparability, vertical lines depict medians, boxes represent interquartiles, bars represent ranges, circles indicate outliers (deviating from the boundary of the interquartile range (IQR) by a factor greater than 1.5)

Total bufadienolide quantity (TBQ) also significantly differed among developmental stages (LMM; $F_{3,\ 92} = 9.26$, $P < 0.001$), but not among treatments ($F_{2,\ 58} = 0.30$, $P = 0.74$; Fig. 3). TBQ was not related to the body mass of individuals ($F_{1,\ 100} = 0.57$, $P = 0.45$), and all interactions were non-significant (all $P > 0.13$). According to Tukey HSD post hoc tests, TBQ was significantly higher in 10 days old toadlets than in larvae of any developmental stage (all pairwise $P < 0.001$; toadlets: 5675 ± 408 ng/individual (mean ± SE), versus larvae at developmental stage 28: 3127 ± 271 ng/individual; stage 38: 3537 ± 320 ng/individual; stage 42: 3176 ± 228 ng/individual.

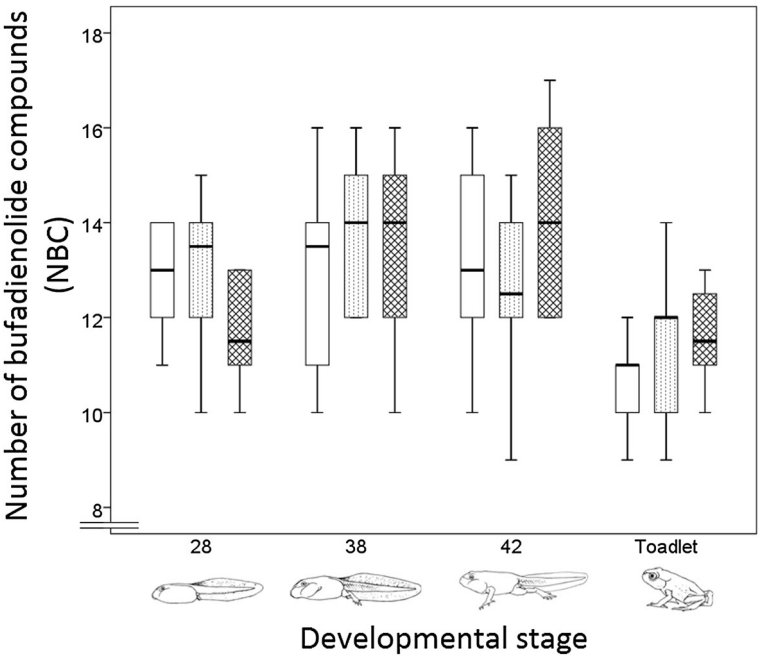

Fig. 2 The number of bufadienolide compounds in the three treatments and across the sampled developmental stages in *Bufo bufo*. Shading represents the three treatments: white: RSW, dotted: lowered microbial density, reticulated: natural microbial density. Vertical lines depict medians, boxes represent interquartiles, bars represent ranges

Discussion

Bufo species do not produce dermal AMP-s (Conlon 2011), so that they have to rely on other agents as a first line of defence against pathogens. Bufadienolides, which are produced in the skin of bufonids already during larval stages (Üveges et al. 2017), have been shown to exhibit potent activity against pathogens (Cunha Filho et al. 2005; Tempone et al. 2008). Here we present the first study testing for inducibility of bufadienolide production by the microbiota in the aquatic environment. We show that although the number of bufadienolide compounds and their total quantity changed during early ontogeny, the surrounding microbiota had no significant effect on these changes in either characteristic of chemical defence (Figs. 2, 3, Online Resource 2).

In a similar experiment, Mangoni et al. (2001) reported a sharp reduction of AMP synthesis in adult frogs (*Pelophylax esculentus*) kept in sterile water compared to conspecifics bathed in pond water containing natural microbiota. This demonstrates that the production of chemical defences secreted in skin glands of amphibians can undergo plastic changes depending on the pathogens present in the environment (also see Miele et al. 1998; Simmaco et al. 1998). Nevertheless, we did not detect similar induced changes in the toxin production of experimental tadpoles. One reason for this result could be the lack of large enough differences between the microbial communities of treatment water types. Even though we did not perform detailed and systematic microbial analyses, this explanation seems unlikely because of the fundamentally different origin of water used in rearing (laboratory-made vs. natural pond). Also, both time until metamorphosis and body mass at

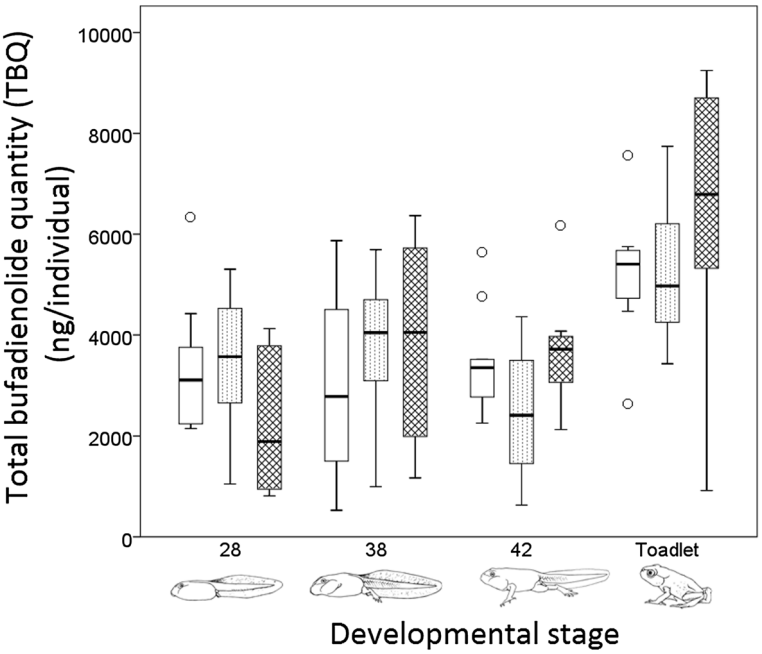

Fig. 3 Total bufadienolide quantity in the three treatments and across the sampled developmental stages. Shading represents the three treatments: white: RSW, dotted: lowered microbial density, reticulated: natural microbial density. Vertical lines depict medians, boxes represent interquartiles, bars represent ranges, circles indicate outliers (deviating from the boundary of the interquartile range (IQR) by a factor greater than 1.5)

metamorphosis were influenced by the treatments (see Online Resource 3). Finally, streaking and microbial culturing of water samples originating from the three treatment types indicated large initial qualitative and quantitative differences (see Online Resource 4). It is, thus, possible that tadpoles indeed do not modulate their bufadienolide production in response to changes in microbial communities.

The question arises why plasticity in chemical defences of the common toad has not evolved in response to the microbiota present in the environment. It is possible that, despite their antimicrobial properties (Cunha Filho et al. 2005; Tempone et al. 2008; Barnhart et al. 2017; Online Resource 1), bufadienolides do not play a major role in defence against infections, but other elements of chemical defences (e.g. alkaloids, biogenic amines) evolved to fulfil this duty in toads (Preusser et al. 1975; Roseghini et al. 1989; Macfoy et al. 2005). Furthermore, the skin microbiome can also contribute to defences against pathogens (Kueneman et al. 2014; Becker et al. 2015). Another possible explanation relies on the observation that natural water bodies and the skin of amphibians are inhabited by several widespread microbial taxa which are opportunistic pathogens of amphibians with low host specificity and become virulent only if their hosts' immune system becomes suppressed (Carey et al. 1999; Taylor et al. 1999; Mauel et al. 2002; Densmore and Green 2007). The omnipresence of opportunistic pathogens in the natural microbiota and the resulting continuous need for effective antimicrobial defence may have led to the fixation of a baseline level of bufadienolide synthesis.

Ten days after metamorphosis, both NBC and TBQ differed significantly from what we observed in larval stages: NBC decreased by ca. 13%, whereas TBQ increased by ca. 36%

(Figs. 2, 3). At the same time, some compounds produced during the aquatic life-stage disappeared and were not present in toadlets, while other compounds only appeared after metamorphosis. These results are in line with the hypothesis that toxin production is adjusted to the switch from the aquatic to the terrestrial environment, which exposes individuals to drastically differing assemblages of predators and pathogens (Alford 1999). Specifically, the decrease in NBC after metamorphosis may be explained by lowered infection probabilities due to lowered conspecific density in terrestrial habitats, compared to aquatic environments (Briggs et al. 2010). A parallel increase in TBQ may have evolved due to larger quantities of toxins necessary to repel terrestrial predators, which are often larger in body size (mainly vertebrates) than their aquatic counterparts (mainly invertebrates) and are encountered singly (lack of dilution effect). The increase in TBQ after metamorphosis is aided by the appearance of differentiated, complex granular glands in metamorphs as opposed to single-celled glands in tadpoles (Chammas et al. 2015) and has been reported for other bufonids as well (Benard and Fordyce 2003; Hayes et al. 2009).

In conclusion, this study documents age-dependent changes in both quality and quantity of skin-associated chemical defences during early life stages of *Bufo bufo*. At the same time, qualitative and quantitative differences in the microbiota present in the developmental environment had no observable effect on bufadienolide production. Therefore, the detected patterns under our experimental conditions are result of an intriguing mixture of constitutive age-dependent changes, mirroring the life-history shift of metamorphosis, and the lack of microbiota-induced plastic responses in chemical defences. Nonetheless, whether the presence of highly infective and virulent, obligate pathogens or developing morbidity also do not induce plastic responses in bufadienolide toxin production remains to be explored.

Acknowledgements We thank Z Pillió and M Szederkényi for assistance during the experiment, V Bókony and B Üveges for help in the statistical analyses, the Pilisi Parkerdő Zrt. for allowing us to use their roads, and V Verebélyi for the graphics depicting tadpole development. The Közép-Duna-Völgyi KTVF issued the permission to conduct the study (KTF:2771-3/2015 and KTF:2771-4/2015) and the Ethical Commission of the MTA ATK NÖVI approved the investigation in accordance with Good Scientific Practice guidelines and national legislation. Research was supported by the Lendület Programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012) and an FP7 Marie Curie Career Integration Grant (PCIG13-GA-2013-631722).

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

References

Alford RA (1999) Ecology: resource use, competition and predation. In: McDiarmid RW, Altig R (eds) Tadpoles: the biology of anuran larvae. University of Chicago Press, Chicago, pp 240–278

APHA (1985) Standard methods for the examination of water and wastewater, vol 16. American Public Health Association, Washington

Barnhart K, Forman ME, Umile TP et al (2017) Identification of bufadienolides from the boreal toad, *Anaxyrus boreas*, active against a fungal pathogen. Microb Ecol. doi:10.1007/s00248-017-0997-8

Becker MH, Walke JB, Cikanek S et al (2015) Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. Proc R Soc B 282:20142881. doi:10.1098/rspb.2014.2881

Benard MF, Fordyce JA (2003) Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. Ecology 84:68–78

Bókony V, Móricz ÁM, Tóth Z et al (2016) Variation in chemical defense among natural populations of common toad, *Bufo bufo*, tadpoles: the role of environmental factors. J Chem Ecol 42:329–338. doi:10.1007/s10886-016-0690-2

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proc Natl Acad Sci USA 107:9695–9700. doi:10.1073/pnas.0912886107

Brodie DEI (2009) Toxins and venoms. Curr Biol 19:931–935. doi:10.1016/B978-0-12-415813-9.00014-3

Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: an immunological perspective. Dev Comp Immunol 23:459–472. doi:10.1016/S0145-305X(99)00028-2

Chammas SM, Carneiro SM, Ferro RS et al (2015) Development of integument and cutaneous glands in larval, juvenile and adult toads (*Rhinella granulosa*): a morphological and morphometric study. Acta Zool 96:460–477. doi:10.1111/azo.12091

Conlon JM (2011) The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tissue Res 343:201–212. doi:10.1007/s00441-010-1014-4

Cunha Filho GA, Schwartz CA, Resck IS et al (2005) Antimicrobial activity of the bufadienolides marinobufagin and telocinobufagin isolated as major components from skin secretion of the toad *Bufo rubescens*. Toxicon 45:777–782. doi:10.1016/j.toxicon.2005.01.017

Daly JW (1995) The chemistry of poisons in amphibian skin. Proc Natl Acad Sci USA 92:9–13. doi:10.1073/pnas.92.1.9

Densmore CL, Green DE (2007) Diseases of amphibians. ILAR J 48:235–254. doi:10.1093/ilar.48.3.235

Engqvist L (2005) The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. Anim Behav 70:967–971. doi:10.1016/j.anbehav.2005.01.016

Gomes A, Giri B, Saha A et al (2007) Bioactive molecules from amphibian skin: their biological activities with reference to therapeutic potentials for possible drug development. Indian J Exp Biol 45:579–593

Gosner KL (1960) A simplified table for staging anuran embryos larvae with notes on identification. Herpetologica 16:183–190. doi:10.2307/3890061

Grafen A, Hails R (2002) Modern statistics for the life sciences. Oxford University Press, Oxford

Hagman M, Hayes RA, Capon RJ, Shine R (2009) Alarm cues experienced by cane toad tadpoles affect post-metamorphic morphology and chemical defences. Funct Ecol 23:126–132. doi:10.1111/j.1365-2435.2008.01470.x

Harvell CD (1990) The ecology and evolution of inducible defenses. Q Rev Biol 65:323–340. doi:10.1086/416841

Hayes RA, Crossland MR, Hagman M et al (2009) Ontogenetic variation in the chemical defenses of cane toads (*Bufo marinus*): toxin profiles and effects on predators. J Chem Ecol 35:391–399. doi:10.1007/s10886-009-9608-6

Hettyey A, Tóth Z, Van Buskirk J (2014) Inducible chemical defences in animals. Oikos 23:1025–1028. doi:10.1111/OIK.01338

Kueneman JG, Parfrey LW, Woodhams DC et al (2014) The amphibian skin-associated microbiome across species, space and life history stages. Mol Ecol 23:1238–1250. doi:10.1111/mec.12510

Longson CG, Joss JMP (2006) Optimal toxicity in animals: predicting the optimal level of chemical defences. Funct Ecol 20:731–735. doi:10.1111/j.1365-2435.2006.01148.x

Lozupone CA, Knight R (2007) Global patterns in bacterial diversity. Proc Natl Acad Sci USA 104:11436–11440. doi:10.1073/pnas.0611525104

Macfoy C, Danosus D, Sandit R et al (2005) Alkaloids of anuran skin: antimicrobial function? Z Naturforsch 60:932–937

Mangoni ML, Miele R, Renda TG et al (2001) The synthesis of antimicrobial peptides in the skin of *Rana esculenta* is stimulated by microorganisms. Faseb J 15:1431–1432

Mauel MJ, Miller DL, Frazier KS, Hines ME (2002) Bacterial pathogens isolated from cultured bullfrogs (*Rana castesbeiana*). J Vet Diagn Investig 14:431–433. doi:10.1177/104063870201400515

McCall AC, Fordyce JA (2010) Can optimal defence theory be used to predict the distribution of plant chemical defences? J Ecol 98:985–992. doi:10.1111/j.1365-2745.2010.01693.x

McKenzie VJ, Bowers RM, Fierer N et al (2012) Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME J 6:588–596. doi:10.1038/ismej.2011.129

Mebs D (2001) Toxicity in animals. Trends in evolution? Toxicon 39:87–96. doi:10.1016/S0041-0101(00)00155-0

Mebs D, Wagner MG, Pogoda W et al (2007) Lack of bufadienolides in the skin secretion of red bellied toads, *Melanophryniscus* spp. (Anura, Bufonidae), from Uruguay. Comp Biochem Physiol C Toxicol Pharmacol 144:398–402. doi:10.1016/j.cbpc.2006.11.009

Miele R, Ponti D, Boman HG et al (1998) Molecular cloning of a bombinin gene from *Bombina orientalis*: detection of NF-κB and NF-IL6 binding sites in its promoter. FEBS Lett 431:23–28. doi:10.1016/S0014-5793(98)00718-2

Morgenstern D, King GF (2013) The venom optimization hypothesis revisited. Toxicon 63:120–128. doi:10.1016/j.toxicon.2012.11.022

Pessier AP (2002) An overview of amphibian skin disease. Semin Avian Exot Pet Med 11:162–174. doi:10.1053/saep.2002.123980

Preusser HJ, Habermehl G, Sablofski M, Schmall-Haury D (1975) Antimicrobial activity of alkaloids from amphibian venoms and effects on the ultrastructure of yeast cells. Toxicon 13:285–289. doi:10.1016/0041-0101(75)90135-X

Puglisi S, Speciale A, Acquaviva R et al (2009) Antibacterial activity of *Helleborus bocconei* Ten. subsp. *siculus* root extracts. J Ethnopharmacol 125:175–177. doi:10.1016/j.jep.2009.06.011

Relyea RA (2004) Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. Ecology 85:172–179

Rollins-Smith LA, Reinert LK, O'Leary CJ et al (2005) Antimicrobial peptide defenses in amphibian skin. Integr Comp Biol 45:137–142. doi:10.1093/icb/45.1.137

Roseghini M, Erspamer Falconieri G, Severini C, Simmaco M (1989) Biogenic amines and active peptides in extracts of the skin of thirty-two European amphibian species. Comp Biochem Physiol Part C Comp Pharmacol 94:455–460

Simmaco M, Mangoni ML, Boman A et al (1998) Experimental infections of *Rana esculenta* with *Aeromonas hydrophila*: a molecular mechanism for the control of the normal flora. Scand J Immunol 48:357–363. doi:10.1046/j.1365-3083.1998.00407.x

Taniguchi M, Kubo I (1993) Ethnobotanical drug discovery based on medicine men's trials in the African savanna: screening of East African plants for antimicrobial activity II. J Nat Prod 56:1539–1546

Taylor SK, Williams ES, Pier AC et al (1999) Mucormycotic dermatitis in captive adult wyoming toads. J Wildl Dis 35:70–74

Tempone AG, Pimenta DC, Lebrun I et al (2008) Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad *Rhinella jimi* parotoid macrogland secretion. Toxicon 52:13–21. doi:10.1016/j.toxicon.2008.05.008

Toledo RC, Jared C (1995) Cutaneous granular glands and amphibian venoms. Comp Biochem Physiol Part A Physiol 111:1–29. doi:10.1016/0300-9629(95)98515-I

Torsvik V, Øvreås L, Thingstad TF (2002) Prokaryotic diversity—magnitude, dynamics, and controlling factors. Science 296:1064–1066. doi:10.1126/science.1071698

Üveges B, Fera G, Móricz ÁM et al (2017) Age- and environment-dependent changes in chemical defences of larval and post-metamorphic toads. BMC Evol Biol 17:137. doi:10.1186/s12862-017-0956-5

Van Buskirk J (2002) Phenotypic lability and the evolution of predator-induced plasticity in tadpoles. Evolution 56:361–370. doi:10.1111/j.0014-3820.2002.tb01346.x

Via S, Lande R (1985) Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution 39:505–522

West-Eberhard MJ (1989) Phenotypic plasticity and the origins of diversity. Annu Rev Ecol Syst 20:249–278