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# Tadpole morphology features of different *Pelophylax esculentus* complex forms

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Complicated relationships between different forms of *Pelophylax esculentus* complex are strongly connected to their reproduction modes. Stability of the hemiclonal population systems including variety of hybrids is provided by balance between gamete production patterns and selective death of offspring portion. A direct way to study such mechanisms is to investigate the ontogeny of different forms – that means studying their tadpoles. However, there are still no suitable methods to morphologically distinguish the known diversity of hybrid forms (2n and 3n of different genomic composition) from the naturally diverse parental species at the tadpole stage. The present work is aimed to investigate external quantitative (measurements-based) and coloration features for tadpoles of parental species (*P. lessonae*, *P. ridibundus* "pure" and triploid-born) and of two *P. esculentus* forms (progeny of unusual LLR-females and diploid hybrids). For this purpose, the set of experimental artificial crossings were established and larvae were reared under similar conditions (equal water volume, light, temperature and feeding regimes). Species and ploidy of experimental frogs were identified using external morphology features, microscopic cytometry of blood cells, karyology of intestine mitotic cells and microsatellite analysis. Coloration of different body parts were scored visually using microscope; measuring was performed by microscopic photographing with scale and further measuring using AxioVision soft. Measurements were analyzed via multidimensional analyses (PCA, discriminant, canonical), and appeared weakly applicable taken both together and separately. It allowed us only to partly separate progeny of two parental species from each other and from progeny of unusual triploid hybrids. States combinations of coloration features appeared to be specific for each form taken into analysis, but only at the particular age range. Specificities of triploid and different *P. ridibundus* groups can be explained by natural variability as well as by peculiar processes in hybridogenetic systems.

**Key words:** *water frogs, larvae, development, progeny, hybrid, triploid.*

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### Introduction

Some *Pelophylax* species possess a rare reproduction mode: their interspecies hybrids transmit the parental genomes clonally, eliminating other genomes in the germ cells, – the "hemiclonal reproduction". It's practiced by edible frog (*Pelophylax esculentus* L., 1758), Italian edible frog (*Pelophylax hispanicus* Bonaparte, 1839) and Graf's hybrid frog (*Pelophylax grafi* Crochet, Dubois, Ohler, Tunner, 1995). Hemiclonal hybrids typically coexist with one or both parental species as they need genomes, deleted in their own germ cells to reproduce (Berger, 2008), these mixed populations are called hemiclonal/hybridogenetic systems. *Pelophylax esculentus* complex is the most studied and complicated among such: it includes pool frog (*Pelophylax lessonae* Camerano, 1882), marsh frog (*Pelophylax ridibundus* Pallas, 1771) and hemiclonal hybrid edible frog (*Pelophylax esculentus* Linnaeus, 1758). This hybrid exists as di- and triploid forms of both sexes; their genome compositions are denoted with L (*lessonae*) and R (*ridibundus*): LL and RR genotypes in parental species, LR and LLR/LRR in hybrids. The significant role in hemiclonality studies belongs to Siverskyi Donets river basin (Eastern Ukraine), named for the huge variety of population systems "The Siverskyi Donets center of diversity of water frogs" (Borkin et al., 2004; Shabanov et al., 2009). The peculiarities here include: absence of one parental species (*P. lessonae*), mass polyploids occurrence (with rare tetraploids; Shabanov et al., 2006), production of two gamete types with different genomes by hybrids ("amphispermy" for males), and regular production of 2n-gametes by 2n-individuals (Biriuk et al., 2016; Borkin et al., 2004; Morozov-Leonov et al., 2009).

The hemiclonal systems have intricate mechanisms of sustainable existence. Parental species and hybrids have different reproductive contribution in terms of transmitted genome. And predominance of form producing particular gametes, would lead the system to collapse without some internal balance mechanisms (e.g. different gamete production patterns, survival, maturation, fertility, and ontogenetic strategies in different forms) (Shabanov et al., 2020; Shabanov et al., 2015; Usova et al., 2015). The shifted survival rate of particular forms is observed among tadpoles (Pruvost, 2013), that explains the absence of adult forms which should appear from known produced gametes (Christiansen et al., 2010; Reyer et al., 2015), like the absence of *P. lessonae* in Siverskyi Donets center where many hybrids produce L-gametes (Biriuk et al., 2016). Gamete production is linked to the elimination of genomes in the hybrid germ cell line, which occurs exactly during tadpole development (Dedukh et al., 2017; Dedukh et al., 2020; Haczkiewicz et al., 2017). Thus, the crucial questions on the hybridogenetic complexes are connected with the frogs' ontogeny study. Nothing makes the ontogeny study easier for species complex case, than the known distinctive characteristics of their tadpoles. By now, only adult *Pelophylax* frogs can be distinguished based on morphological features, and even this approach is still imperfect and applicable for diploids mostly (Plotner, 2005), demanding cytogenetics and molecular methods for confirmation (Dedukh, Krasikova, 2017). Morphological differences of *Pelophylax* tadpoles remain a big question, though their morphology was studied for a long time.

The studying of tadpole ontogeny started mostly as the part of developmental biology (early XX century). Many Anuran development tables were dedicated to former *Rana* members (Pollister, Moore, 1937; Shumway, 1940; Taylor, Kollros, 1946, Dettlaff, Vassetzky, 1991), though the question of any hybridogenesis was not raised then. A famous "simplified table for staging Anuran embryos" by Gosner (1960) is still popular, but mainly for the description of development. As the *Pelophylax* is the common object in water quality studying, the toxical investigations are also the wide source of developmental and morphology data on tadpoles (e.g. Johari et al. 2015), but usually don't concern the hybridogenesis, and so provide little for our main topic. The huge contribution to tadpole research was the comprehensive book "Tadpoles" (McDiarmid, Altig, 1999), summarized known morphology and physiology principles for Anuran larvae; though, it provided characteristics at the minimal level of family, which is insufficient for the interspecies studies. The same authors contributed abundantly to the tadpoles structure investigation (Altig, 1970; Altig, McDiarmid, 2015). Among the earliest notes on East-European frogs' tadpoles identification was a key by Terentyev (1950): with *Rana*, but without hybrids. The larvae morphometry of European frogs (*R. temporaria* and *R. dalmatina*) was studied for possible distinguishing between these species (Ilić et al., 2016; Ilić et al., 2019). Grosjean (2005) drawn attention to the variation of some morphological traits, recommending the usage of larvae at particular stages for taxonomical descriptions.

The most relevant are works dedicated to *Pelophylax esculentus* complex tadpoles: unfortunately, they often concern only single species (Arifulova, Chirikova, 2018; Amanat Behbahani et al., 2014). There are two works of the most interest. A comprehensive study of Ukrainian Anurans by Tkachenko (2019) included detailed descriptions on *Pelophylax* larvae, but only on parental species without hybrids. However, it contained only comparative (and relative) morphometry of body parts along with duration of features presence. Also, the way of species identification was not clearly described in this work. Considering the hybrid phenotypic diversity, it may be hard to surely identify water frogs by only morphology without molecular methods, even for such a qualified researcher. The study by Günther (1978) was similar and the first with morphometry of both species' and hybrids' larvae. According to it, the only suitable markers to distinguish the species are the set of ratios between only a few external measurements. Identification doubts are fair in this case too, considering the wider variety of hybrids known by now. So, such narrow criteria can barely help to distinguish all the forms.

By the nowadays, the problem of distinguishing of *Pelophylax esculentus* complex tadpoles remains complicated. Most of the modern data on their tadpoles are restricted to usage of the tadpoles as model objects. Their morphological diversity is still not sufficiently described in connection with diversity of hybridogenetic systems. Main questions about *Pelophylax esculentus* complex and evolution of their hemiclonal systems, that are up to investigate yet, strongly connected to its peculiar reproduction mode. Studying of reproduction is clearly incomplete without direct ontogeny investigation. Such an investigation demands suitable and sure tadpole identification.

The present work is aimed to find the morphological traits, suitable enough to distinguish the tadpoles of some forms of *Pelophylax esculentus* complex.

**Materials and methods**

The crossing method was chosen to obtain a set of progenies of particular genotypes. We artificially crossed the adult frogs of known form, and then morphologically analyzed offspring. All adult frogs were originated from the Siverskyi Donets center of water frog diversity.

*Identification methods*

- Species and sex of adult frogs were primarily identified by song criteria and morphological features (Shabanov, 2015)
- Rough ploidy identification of adult frogs was performed by measuring the average size of erythrocytes on dry blood samples (Bondareva et al., 2012). Blood was taken from the fingers.
- Karyological analysis for exact ploidy identification was performed for adult frogs. It included dropping and staining (with Ag or Giemsa stain) of hypotonized intestine epithelium according to (Birstein, 1984 with modifications; Bondareva et al., 2013).
- Species and form of studied individuals were also confirmed by microsatellite analysis of tissue samples of parents (fingers) and tadpoles (tail tips or the whole specimens). Analysis was performed in collaboration with Glib Mazepa (University of Lausanne, Lausanne, Switzerland). For primers and procedure details see Leuenberger et al. (2014).

*Origin of parental frogs*

All parental frogs were caught by hand during the night using a flashlight. Frogs were kept in plastic tanks with air holes, water boxes and foam rubber mats, and were fed by cockroaches poured with calcium carbonate. Data on the parents' origin, codes, genotypes and progeny groups are presented in the Table 1. Brief description:

- *P. lessonae* female and male originated from Krasnokutsk vicinity (Kharkiv region, Ukraine; 50°4'25.7844"N, 35°11'40.2036"E). This is an unique locality for Kharkiv region because of both our *Pelophylax* species and hybrids presence (Shabanov et al., 2017). Work code for their progeny was **L** ("pool frog" progeny).
- *P. ridibundus* female and male originated from R-E system in Kharkiv River (Kharkiv, Ukraine; 50°1'17.886"N, 36°18'47.2176"E). Work code for their progeny was **RR** ("pure marsh frog").
- A female of next pair originated from Dobrytskyi pond (49°33'23.2914"N, 36°18'34.1748"E), whose complex hemiclonal system contains various hybrid forms (Meleshko et al., 2014). It was identified as LRR-hybrid by the results of erythrocyte cytometry and microsatellite analysis. Triploids from Siverskyi Donets are known to typically produce haploid gametes with that genome, which they have in two copies – gametes with R-genome in this case (Biriuk et al., 2016). Male was undoubtedly identified as *P. ridibundus* because of its host Brusivka system (Donetsk region, Ukraine; 48.900284, 37.784786) is known to consist only of both sexes of *P. ridibundus* and quite distinctive LLR-females (Drohvalenko et al., 2017). As both female and male produced R-genome, progeny was considered to be *P. ridibundus* and got code **R** ("triploid-born marsh frog").
- We also analyzed two progenies from *P. esculentus* LLR-females and *P. ridibundus* males originated from the aforementioned Brusivka system. Triple ploidy of these females was confirmed karyologically. As Brusivka triploid hybrids live without any other hybrid form, they should have a peculiar reproduction mode giving all-triploid progeny – or peculiar ontogeny with high selective death rate. According to the first assumption, whole progeny of both crossings was considered as triploids, got the code **T1** and **T2** and was analyzed as two homogenous samples ("triploid hybrids").
- A few larvae from crossings between *P. esculentus* LRR-female and *P. esculentus* LR-male from Dobrytskyi pond were used as reference for interspecies morphological comparisons. Genotypes of parents and diploid (LR) genotype for the entire progeny were identified by microsatellite analysis. They got work code **LR** ("diploid hybrids").

*Artificial crossing method*

Crossing method was aimed at parents' staying alive. For assurance of gametes maturing, animals were caught at spawning season. Each frog was stimulated by 2.5 ml of "Surfagon" (gonadotropic hormone synthetic analogue) injecting into a subcutaneous lymphatic sack (abdomen side). Approximately 2 hours are enough for males and about a day is necessary for females to get stimulated. Mature eggs presence is easy to check by slightly squeezing and palpating the frog's abdomen. Sperm was obtained using water rinsing of male cloaca; eggs were obtained by gently squeezing of female's abdomen. Drop of acquired sperm suspension was examined for the presence and amount of active (motile) spermatozoids using simple light microscopy. Sperm suspensions were then mixed with eggs and water and left in Petri dish for

fertilization for about a day. Progenies from Brusivka LLR-females were obtained by natural crossings through amplexus, though occurring in semi-natural conditions: water-filled boxes under the outdoor conditions. After spawning, frogs were removed from the boxes to let the eggs develop undisturbed.

**Table 1. Parents and progeny in research**

| Parents | | | Progeny | | |
|---|---|---|---|---|---|
| Sex | Species | Genotype | Genotype | Species | Code |
| F<br>M | *P. lessonae*<br>*P. lessonae* | LL<br>LL | LL | *P. lessonae* | **L** |
| F<br>M | *P. ridibundus*<br>*P. ridibundus* | RR<br>RR | RR | *P. ridibundus* | **RR** |
| F<br>M | *P. esculentus*<br>*P. ridibundus* | LRR<br>RR | RR | *P. ridibundus* | **R** |
| F<br>M | *P. esculentus*<br>*P. ridibundus* | LLR<br>RR | LLR | *P. esculentus* likely 3n | **T1, T2** |
| F<br>M | *P. esculentus*<br>*P. esculentus* | LRR<br>LR | LR | *P. esculentus*<br>2n | **LR** |

*Notes: Letters 'L' and 'R' stand for species genomes, their combinations denotes corresponding ploidy (see Introduction section); letters 'F' and 'M' denote females and males respectively.*

*Larvae rearing*

Clutches were moved into 4-litres boxes with aged tap water in a day after crossing. A lower temperature limit of 22-23 $^{o}$C was controlled by thermocontroller "Tetra HT200", installed underwater externally of rearing boxes. "Atman AT-7500" air-pump provided equal aeration to each box. Nature sunlight was equally available for the all clutches. After a couple of days some undeveloped eggs were removed to prevent poisoning the live embryos. Density of larvae in all boxes was adjusted to approximately equal. Right after the most larvae hatched, the food was started to add daily (in excessive amounts). We chose "Tetramin" for bottom-feeder fish, because tadpoles have feeding mode very similar with such fish and due to its suitability (confirmed earlier by us; also used in Grosjean, 2005). Boxes cleaning and water changing were performed once a day during tadpoles' early developmental stages (quick growth) and once in two days during later stages.

*Fixation scheme*

Fixation started at the second day of development, when it became possible to identify the living embryos. We used plastic Pasteur pipettes to sample the individuals; only alive larvae were randomly caught and fixed. Larvae were fixed in 96%-ethanol (which does not affect the coloration) once a day till 12-day age (2-12 days age samples were thus obtained). The next four fixations were done every three days. We had only 1 fixation of "triploid hybrids" progeny (T1, T2) at the age of 10 days, and "diploid hybrids" progeny (LR) at the age of 8 days. Total numbers of specimens collected: 362 with majority of L, R and RR group (130, 100 and 100 respectively), equal size of T1 and T2 group (15 each) and only 2 LR representatives.

*Tadpole studying*

Data collection included the analysis of two classes of features: qualitative features (external body coloration) and quantitative features (size of external body parts). The microscope МБС-9 with camera ToupCam AMA075 and ToupView soft were used for visual assessment and photographing. Each specimen was placed on a millimeter paper and shot from below, top and side. Metal pins and Petri dish with foam plastic bottom bed were used to ease manipulation with underwater immersed larvae. Morphometry was performed using AxioVision (Carl Zeiss Vision) soft package. Each image was scaled according to individual millimeter paper marks (to avoid deviation that would occur with unified scale).

We chose as many direct measurements as it could be possible to measure confidently on each image; for explanation of abbreviations see the note for Figure 1. Proportions of these parameters were also taken into analysis, in order to analyze to shape of larvae bodies without the actual size variation. Names and abbreviations of the features in present paper are derived from those in different works on tadpole morphology (e.g. Altig, 2007; Haas, Das, 2011; McDiarmid, Altig, 1999) (Fig. 1).

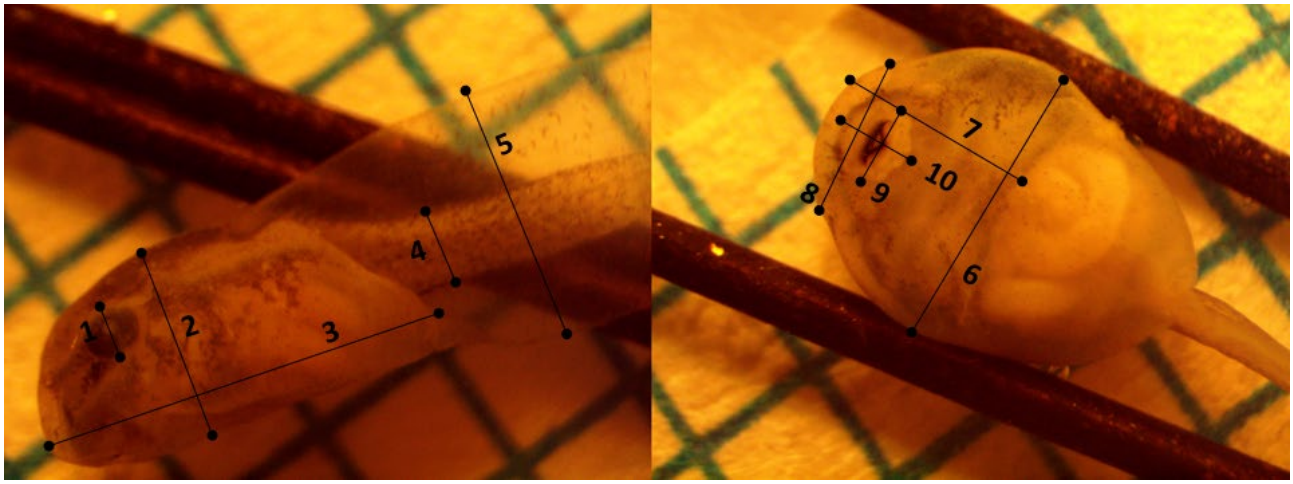


**Figure 1. Measurements used in research: 1 - orbital height (OH), 2 - head height (HH), 3 - body length (BL), 4 - tail muscle (TMH), 5 - height of tail (TH), 6 - head width (HW), 7 - head length (HL), 8 - interorbital width (IOW), 9 - mouth width (MW), 10 - mouth length (ML).** Head margin were considered at the margin of buccal cavity, visible if tadpole is placed on its back

*Statistics*

The Kruskal-Wallis test and multidimensional tests (PCA, discriminant with canonical analyses) were used for assess the role of different features. Kruskal-Wallis test was chosen, as the normality examining (Shapiro-Wilk test) demonstrated no normal distribution of data samples, so ANOVA should not be applied.

*Disclaimer*

*Pelophylax* species used are listed by the IUCN Red list as “Least concern”, and are not listed by CITES. Techniques used in the capture, breeding, tissue sampling and euthanasia sought to minimize animal suffering according with recommendations of the Directive 2010/63/EU of the European parliament and of the council on the protection of animals used for scientific purposes (2010).

## Results

*Qualitative features*

For all analyzed tadpoles we described the set of coloration features, each with two distinctive states (Fig. 2):

- Coloration of adhesive glands, which had remained during development till stage 25, after which started to disappear: light (A) or dark (B).
- Body background: light (A) or dark (B).
- Back pattern: clear pigmented segments (A) or blurred even coloration (B).
- Abdomen pattern: “arc-like” image (visible with enough light; A) or plain even background (B).
- Iris coloration: pale (A) or bright (B).

We found that each analyzed group had the unique combination of these features’ states. Those combinations also allowed us to ensemble the groups according to the similarity with some parental form (Table 2). For example, *P. lessonae* and triploid tadpoles (supposed to be LLR-hybrids) had a ‘*lessonae*’-like feature combinations, and tadpoles of both *P. ridibundus* forms had ‘*ridibundus*’-like combinations. Diploid hybrids demonstrated the intermediate combination between these groups.

Unfortunately, those combinations were usable only between the ages of 6 and 10 days: for elder larvae features became too blurred for sure diagnosis, for younger larvae not all of them were developed enough. For instance, tadpoles started to totally lose their adhesive glands while reaching the 10 days age. Worth noting: tadpoles, which did and did not lose their glands, could remain in the same 25-th developmental stage.


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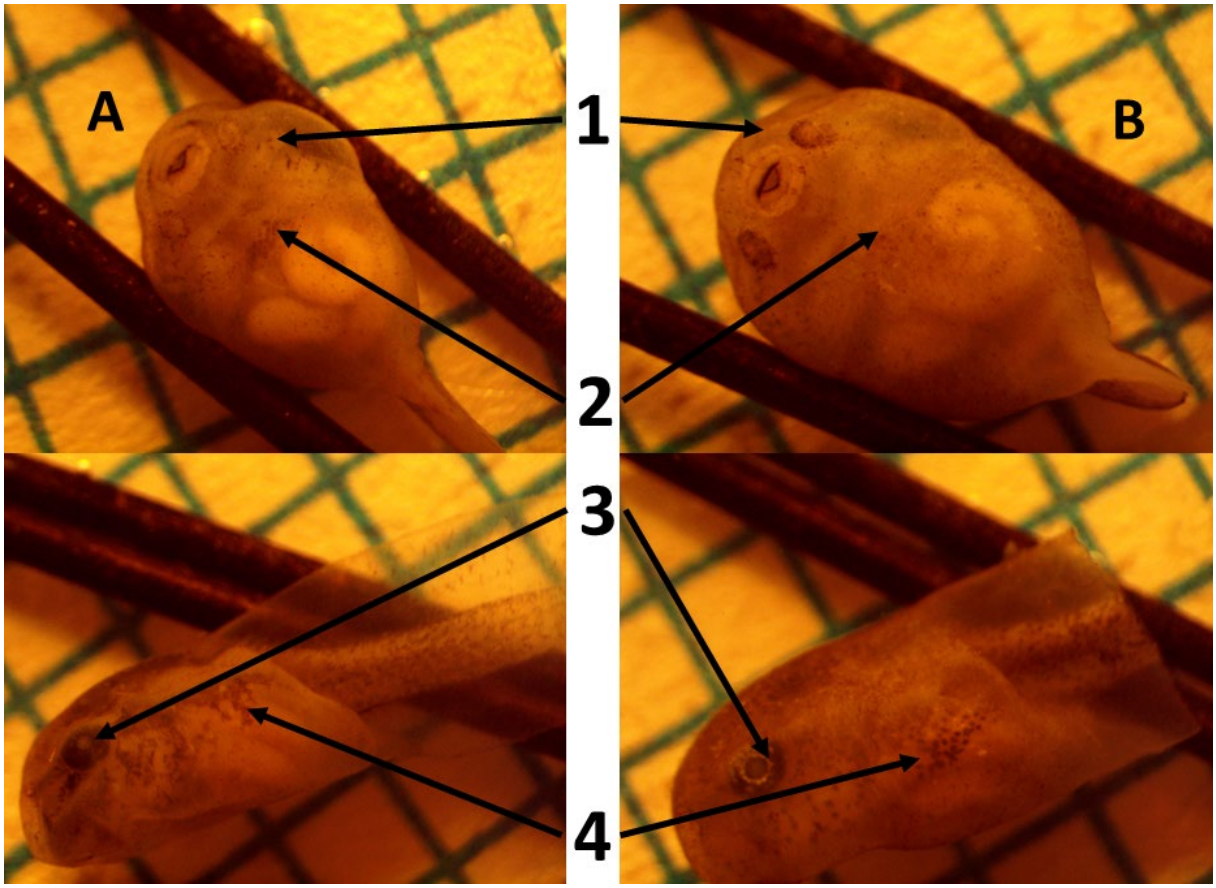


**Figure 2. Features of external coloration: 1 – adhesive glands, 2 – abdomen coloration, 3 – iris brightness, 4 – back pattern and overall background**

**Table 2. Morphology features of 6-10 days age tadpoles**

| Feature | Group | | | | |
|---|---|---|---|---|---|
| | L | T1+T2 | LR | RR | R |
| Adhesive glands | Light | Light | Dark | Dark | Dark |
| Body background | Light | Light | Dark | Dark | Light |
| Back pattern | Clear | Clear | Blurred | Blurred | Blurred |
| Abdomen pattern | Clear arc | Plain | Clear arc | Plain | Plain |
| Iris brightness | Pale | Pale | Pale | Bright | Bright |
| Feature combination | '*lessonae*'-like | | 'intermediate' | '*ridibundus*'-like | |

*Notes: Group codes, feature names and feature states are explained in the text.*

*Quantitative features analysis*

Table 3 presents the range of each feature measured in the same ages, for which coloration criteria were applicable (6-10 days).

To test the suitability of each direct measurement and some proportions for tadpole groups distinguishing we applied the multivariate non-parametric rank analysis (Kruskal-Wallis test). We tested

only L, RR and R groups (as the most numerous) at the same age range, for which qualitative criteria were applicable (6-10 days). The results are presented in the Table 4. After proper adjustment (Bonferroni-Holm), it appeared that after the age of 7 days no suitable measurement or proportion left.

**Table 3. Ranges of values for studied features**

| Group, age | | Stage | HL | HH | OH | BL | TMH | TH | HW | IOW | ML | MW |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L | 6 | 21-21 | 0.96-1.21 | 0.93-1.12 | 0.26-0.36 | 2.21-3.11 | 0.42-0.53 | 0.98-1.48 | | | | |
| | 7 | 22-24 | 1.11-1.47 | 1.03-1.24 | 0.24-0.39 | 2.47-3.25 | 0.48-0.54 | 1.54-1.82 | | | | |
| | 8 | 24-25 | 1.20-1.83 | 1.16-1.56 | 0.34-0.50 | 2.64-3.41 | 0.48-0.60 | 1.55-2.09 | 1.42-2.01 | 0.63-0.95 | 0.39-0.56 | 0.42-0.56 |
| | 9 | 25 | 1.44-1.76 | 0.95-1.38 | 0.28-0.43 | 2.50-3.09 | 0.48-0.59 | 1.45-1.94 | 1.81-2.14 | 0.67-1.00 | 0.42-0.60 | 0.52-0.61 |
| | 10 | 25 | 1.42-1.86 | 1.21-1.54 | 0.32-0.46 | 2.81-3.57 | 0.55-0.65 | 1.76-2.10 | 2.03-2.65 | 0.84-1.16 | 0.47-0.59 | 0.58-0.83 |
| RR | 6 | 20-22 | 0,89-1,25 | 1.02-1.22 | 0.26-0.43 | 2.87-3.40 | 0.38-0.50 | 0.94-1.49 | | | | |
| | 7 | 21-23 | 1.23-1.46 | 1.14-1.31 | 0.3-0.41 | 3.00-3.50 | 0.34-0.63 | 1.31-1.75 | | | | |
| | 8 | 23-24 | 1.34-1.68 | 1.14-1.51 | 0.34-0.46 | 2.73-3.00 | 0.52-0.66 | 1.55-1.97 | 1.48-1.94 | 0.74-0.96 | 0.32-0.48 | 0.35-0.52 |
| | 9 | 24-25 | 1.50-1.83 | 1.25-1.46 | 0.38-0.45 | 2.82-3.59 | 0.52-0.63 | 1.60-1.88 | 1.88-2.27 | 0.72-0.99 | 0.42-0.52 | 0.41-0.59 |
| | 10 | 25 | 1.68-1.88 | 1.38-1.52 | 0.40-0.49 | 3.31-3.75 | 0.55-0.72 | 1.60-2.07 | 2.10-2.56 | 1.06-1.16 | 0.40-0.57 | 0.57-0.71 |
| R | 6 | 20-22 | 0.97-1.21 | 1.05-1.36 | 0.29-0.39 | 2.90-3.47 | 0.37-0.58 | 1.07-1.80 | | | | |
| | 7 | 20-24 | 1.03-1.43 | 1.22-1.36 | 0.30-0.43 | 2.67-3.25 | 0.39-0.63 | 1.21-1.99 | 1.64-1.73 | 0.83-0.87 | 0.35-0.45 | 0.37-0.48 |
| | 8 | 22-24 | 1.16-1.85 | 1.08-1.42 | 0.28-0.44 | 2.67-3.435 | 0.43-0.63 | 1.42-2.01 | 1.64-2.10 | 0.66-0.94 | 0.36-0.50 | 0.31-0.50 |
| | 9 | 24-25 | 1.29-1.90 | 1.23-1.44 | 0.37-0.43 | 2.70-3.24 | 0.52-0.64 | 1.46-1.95 | 1.61-2.31 | 0.81-1.10 | 0.40-0.54 | 0.43-0.60 |
| | 10 | 25 | 1.73-2.10 | 1.51-1.74 | 0.44-0.51 | 3.09-3.72 | 0.60-0.65 | 1.82-1.92 | 2.02-2.53 | 0.97-1.23 | 0.40-0.53 | 0.51-0.67 |
| T1 | 10 | 24-25 | 1.25-1.55 | 1.20-1.40 | 0.33-0.43 | 2.54-2.86 | 0.51-0.61 | 1.50-1.96 | 1.41-1.87 | 0.55-0.83 | 0.36-0.48 | 0.40-0.50 |
| T2 | 10 | 24 | 1.22-1.59 | 1.18-1.38 | 0.33-0.43 | 2.41-2.76 | 0.50-0.61 | 1.69-1.85 | 1.43-1.66 | 0.74-1.02 | 0.35-0.49 | 0.41-0.51 |
| LR | 8 | 23 | 1.30 | 1.38 | 0.41 | 2.92 | 0.59 | 1.64 | 1.76 | 0.93 | 0.44 | 0.51 |
| | 10 | 25 | 1.82 | 1.60 | 0.47 | 3.65 | 0.74 | 2.04 | 2.43 | 1.20 | 0.49 | 0.71 |

*Notes: Ranges presented as min-max. The single value means the uniformity of this feature for entire sample or presence of the only one specimen measured; empty cells mean the absence of such a feature for these samples. For groups and features abbreviations see Materials and Methods section.*

Discriminant analysis was also used to test, which the measurements are able to divide existing groups. First, we applied it to the same three groups (L, RR and R) and all measurements. Variables appeared to be the significant for the group dividing, were the next: TMH, TH and IOW. So, no matches with Kruskal-Wallis test results analysis were found. Specimens having been plotted by the first two discriminant functions (canonical roots) are shown on the Figure 3 (left). First root allowed us almost surely distinguish L and RR+R groups, while RR and R overlapped by both axes.

Then these groups were analyzed along with triploid progenies (T1, T2), but only in correspondent age (10 days). In this case discriminant analysis showed the significance of HH, BL, IOW and MW. Here the partial match with Kruskal-Wallis test results was found as well as with previous discriminant analysis step. First canonical root separated diploids from triploids, and the second root divided two species.

Also, the PCA was used with the same age, progenies and measurements set. However, principal components method, having analyzed the total distribution of specimens without groups, just allowed us divide triploids from diploids without their internal dividing (Fig. 4).


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**Table 4. Significance of differences by each parameter between tadpoles groups at different age**

| Age, days | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|
| HL | 0.457 | 0.077 | 0.498 | 0.275 | 0.224 |
| HH | **0.0003** | **0.0004** | 0.261 | 0.012 | 0.063 |
| OH | 0.087 | 0.913 | 0.626 | 0.008 | 0.231 |
| BL | **0.003** | 0.017 | 0.309 | 0.041 | 0.230 |
| TMH | 0.260 | 0.457 | 0.230 | 0.188 | 0.779 |
| TH | 0.089 | 0.428 | 0.703 | 0.996 | 0.467 |
| HW | | | 0.866 | 0.332 | 0.683 |
| IOW | | | 0.318 | 0.543 | 0.147 |
| ML | | | 0.012 | 0.597 | 0.068 |
| MW | | | 0.013 | 0.072 | 0.669 |
| HL/HH | 0.008 | **0.002** | 0.150 | 0.069 | 0.293 |
| HL/HW | | | 0.392 | 0.765 | 0.570 |
| HH/HW | | | 0.527 | 0.049 | 0.064 |
| BL/HL | 0.018 | 0.587 | 0.238 | 0.081 | 0.768 |
| BL/HW | | | 0.987 | 0.106 | 0.061 |
| TMH/TH | 0.459 | 0.336 | 0.005 | 0.132 | 0.087 |
| ML/MW | | | 0.030 | 0.759 | 0.505 |

*Notes: Names of measurements are explained in the text (see Materials and Methods). Bold underlined font highlights p-values for Kruskal-Wallis analysis results, considering significant after Bonferroni-Holm adjustment. Empty cells indicate measurements not used for particular age (were not defined surely for small larvae).*

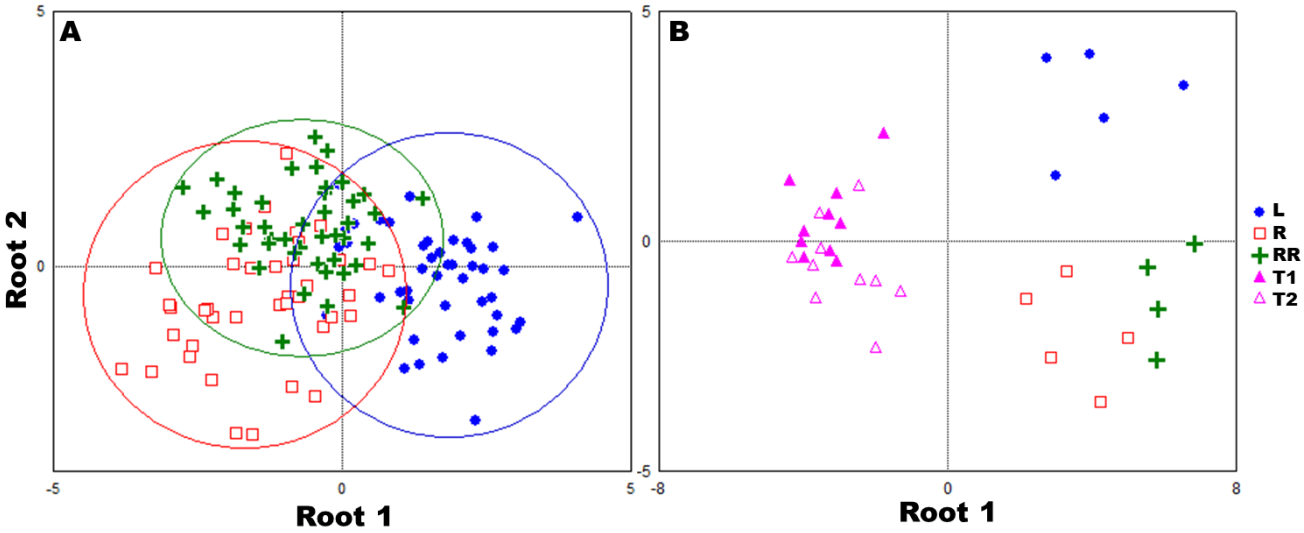


**Figure 3. Samples plotted by two first canonical roots as the results of discriminant analysis of L, RR and R groups (A) and all groups (B) by all measurements.** Circles mark areas of corresponding points. Explanations are in the text

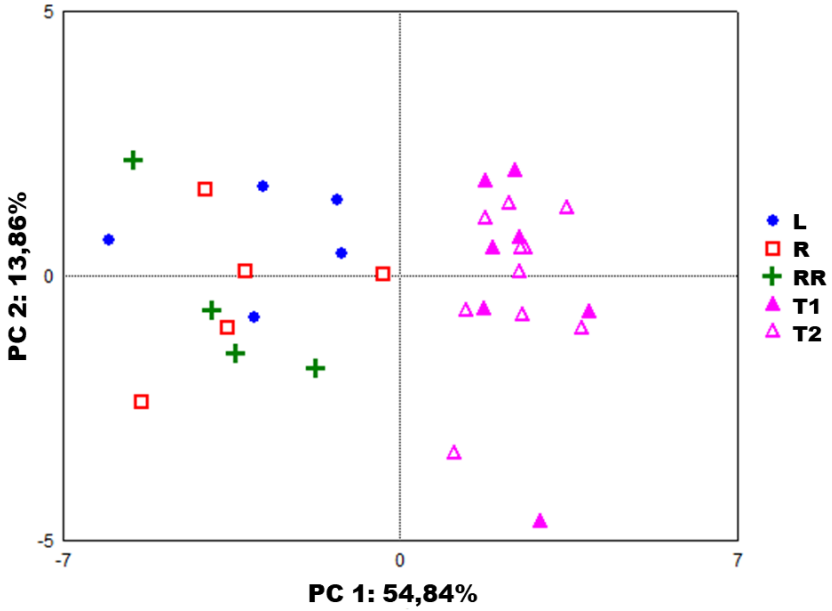


**Figure 4. Result of principal components analysis of parameters for all larvae forms (two first components)**

**Discussion**

We found, that the qualitative features of experimental tadpoles distributed respectively to their genetic groups. Morphometric features, although, did not demonstrate enough power to distinguish the same groups. Some indirect results were obtained via discriminant analysis and PCA. But, PCA divided larvae only by the ploidy, while discriminant analysis works with pre-assigned groups, and did not indicate the set of significant parameters in this case.

The origin of morphological differences between *Pelophylax esculentus* complex larvae remains hypothetical. We consider some hypothetic explanation for observed differences.

One of the most logical ways to explain them is to suspect the different ecological niches for different genetic forms. Coloration could reflect some metabolic differences, found for tadpoles from this hybridogenetic complex (Plenet et al., 2000), either directly via pigmentation synthesis, or indirectly, via light and heat perception. Some evidence of thermoregulation role of body color was presented by the Rodríguez-Rodríguez et al. (2020) along with capability of tadpoles to change body color depending on background color. Additionally, natural interactions with predators are also reported to be the driver for morphology divergence of tadpoles (Johnson et al., 2015; McCollum, Leimberger, 1997). All these hypotheses are weakly applied for our case, since all the tadpoles were reared under the similar temperature conditions, light regime and background color, and surely with no predators. Some works suggest more complex factors affecting tadpole coloration, like present or future adaptive fitness and behavioral properties (see Thibaudeau, Altig, 2012). Unfortunately, genus *Pelophylax* has so ecologically close larvae, that by now we don't have enough data to assume connections between their ecological and morphological variety.

We also suppose, that origin of observed differences may relate to ontogenetic development of adult features. Adult frogs have well-distinctive morphology, which they derive during metamorphosis of tadpoles. Ontogeny processes leading to adult features formation hypothetically begin already in larva. Therefore, observed differences between tadpoles of different groups can supposedly present the ways of adult morphology developing, and don't relate to tadpoles' lifestyle directly. It is known, that adult hybrids morphology is connected with parental genomes dosage (Kierzkowski et al., 2011). Thus, "*lessonae*"-like and "*ridibundus*"-like combinations of qualitative features along with close position of R and RR samples on graphs could be the confirmation of such hypothesis.

The ways, how *P. esculentus* individuals derive their phenotype, are intricate. It could be expected that hybrids inherit their features from parental species – just because they have common genomes and


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share habitats with them. From this point of view, the morphology of diploid hybrids (LR) is the easiest to explain: having one genome from each parental species, they just have intermediate combination of parental features.

Two groups of *P. ridibundus* of different origin (RR and R) were almost identical by coloring and placed close to each other in multidimensional analyses. Similarity is obviously provided by the same genotype RR. That single coloration difference (lighter body background) can be the result of just natural variability – this explanation requires more experiments to be tested (with more individuals of various origins). But assumingly it can also reflect changes in the *P. ridibundus* genomes, connected to evolution of their clonal transmitting. Among such changes introgressions between L and R genomes, provided by rare recombination events, are known (e.g. Mazepa et al., 2018). Mother of the R-group was LRR-triploid and transmitted haploid gametes with sexually recombined R genomes. Triploids per se usually emerge in the offspring of diploid hybrids, occasionally producing diploid LR-gametes (Biriuk et al., 2016). Thus, R genome from LRR-female could have recombined with L-genome in previous generations of diploid hybrids, where it came from. And noted difference between *P. ridibundus* tadpoles – feature state of another species, – might be a trace of such recombination event in the past.

"Triploid hybrids" individuals (T1, T2) could share the similarity simply due to same genotype, containing two L-genomes and making them look "lessonae"-like. However, other researches revealed the possible presence of diploids (of both sexes and undefined genotype) among the progeny of Brusivka triploids (Fedorova, Pustovalova, 2019). If our T1 and T2 groups actually contained only triploids, it would mean that T1/T2 feature combination reflects LLR triploids feature combination. Their morphology differences can be partially explained by different cell size (Hermaniuk et al., 2016), which can affect both body shape and metabolic processes. If the studied groups contained diploids (with undefined ratio), it would mean the peculiarity of exactly Brusivka HPS. It has a strict selection of unknown mechanism among the offspring of LLR-females, eliminating all forms but LLR-females among adults. By some way, morphology of larvae would be a side effect of so complex ontogeny, not connecting with ploidy directly. Data of these progeny might be extrapolated to other triploid tadpoles with great care only. Worth saying, both T1 and T2 were placed very close on both PCA and discriminant functions graphs by morphometry – this can be counted as indirect evidence of the last hypothesis.

The notability of differences only via multidimensional analyses is not surprising (similar in Ilić et al., 2016; Ilić et al., 2019). This hints at inapplicability of the separate linear measurements only. So, perhaps, the future approach should include more powerful methods like geometric morphometry (described in the last paper) to extract more size-independent data.

The role of natural variability should not be excluded from the future experiments. Zhao et al. (2017) showed that tadpoles even from one species (*Scutiger boulengeri* in their work) can vary between geographically distant populations. The same work connects the separate morphology trait with the particular ecological adaptation, which this trait favors for. Such approaches can be considered for *Pelophylax* tadpoles also in order to estimate their inter- and intraspecific variability.

**Conclusion**

Overall study showed only partial effectiveness of external morphology for *Pelophylax esculentus* complex species distinguishing at tadpole developmental stage. Combinations of coloration features were the only key to unbiasedly separate all the studied groups, though remaining effective in particular age range only. We consider this as perspective direction for future investigations of tadpole morphology within this and other species complexes. Body measuring appeared not to be reliable method for this purpose. Significance of measurements for group dividing varied by applied test (Kruskal-Wallis, discriminant analysis). Being analyzed without pre-assigned groups, they did not divide these groups (PCA).

The questions on nature of particular group differences (e.g. offspring of triploids) apparently demand additional surveys with wider samples. Taking into account the complex relationships between species and hybrids at the many levels (genome, organism, population) and their genetic diversity, such sampling should cover both different HPS and variability of each form.

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## Морфологічні ознаки пуголовків різних форм *Pelophylax esculentus* complex

**М.О. Дрогваленко**

Складна взаємодія між різними формами *Pelophylax esculentus* complex тісно пов'язані зі способами їхнього розмноження. Стабільність геміклональних популяційних систем, що включають в себе різноманіття гібридів, забезпечується балансом між характером продукування гамет та вибірковою смертністю частини потомства. Прямий шлях до вивчення таких механізмів – це вивчення онтогенезу різних форм – що означає вивчення їхніх

пуголовків. Втім, досі не існує жодного надійного способу морфологічно відрізнити відоме різноманіття гібридних форм (2n та 3n різного геномного складу) від природно різноманітних батьківських видів на стадії пуголовка. Ця робота націлена на дослідження зовнішніх кількісних ознак (основаних на промірах) та ознак забарвлення у пуголовків батьківських видів (*P. lessonae*, *P. ridibundus* «чисті» та «з потомства триплоїда») та двох форм *P. esculentus* (потомство незвичайних LLR-самиць та диплоїдних гібридів). Задля цього було проведено ряд експериментальних штучних схрещувань, а личинки були вирощені за однакових умов (однаковий об'єм, світловий, температурний режими та годування). Вид та плоїдність експериментальних жаб були визначені за допомогою ознак зовнішньої морфології, мікроскопічної цитометрії клітин крові, каріології мітотичних клітин кишечнику і мікросателітного аналізу. Ознаки забарвлення різних частин тіла фіксувалися візуально під мікроскопом; проміри здійснювалися завдяки фотографуванню під мікроскопом разом з масштабом та подальшим вимірюванням за допомогою програми AxioVision. Проміри було проаналізовано за допомогою багатовимірних аналізів (PCA, дискримінантний та канонічний), але вони виявилися слабко застосовуваними як поодинці, так і узяті разом. Вони дозволили нам лише частково розділити потомство двох батьківських видів одне від одного та від потомства незвичайного триплоїдного гібрида. Комбінації станів ознак забарвлення виявилися специфічними для кожної проаналізованої форми, але лише на певному діапазоні віку. Особливості триплоїдних груп та різних груп озерних жаб можуть бути пояснені як природною мінливістю, так і специфічними процесами у гібридогенетичних системах.

**Ключові слова:** *зелені жаби, личинки, розвиток, потомство, гібрид, триплоїд.*

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