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First report of overwintering tadpoles in the endemic Italian agile frog *Rana latastei*

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Abstract. Climate change is affecting organism seasonality patterns, and this can drive phenological shifts in key traits, including breeding activity. Here, we report the first record of overwintering tadpoles in the Italian agile frog (*Rana latastei*), a threatened endemic species of Northern Italy. This species usually breeds between late January and April, with tadpoles reaching metamorphosis by early summer. In February 2025, alongside the first freshly-laid egg-clutches of the usual breeding season, we observed several large-sized tadpoles at a reproductive site of *R. latastei* within Como province. A few days after hatching, six newly hatched tadpoles and six large-sized individuals at a later developmental stage were captured, photographed, and measured. Concurrently, a recently metamorphosed froglet was found at the same site. Morphological analyses revealed that individuals showed typical traits of *R. latastei*. Moreover, while newly hatched tadpoles were at Gosner's development stages 25-26, large-sized individuals were visibly bigger and at a late developmental stage, which was incompatible with the classical late-winter breeding timing of this species. Our study provides the first evidence of overwintering tadpoles in *R. latastei*, suggesting that potential shifts in development timing (prolonged larval time) or altered breeding habits (autumnal reproduction) may be occurring. Although the mechanisms behind this unusual observation remain unclear, such a phenological shift may have been favoured by variation in climatic regime.

Keywords. Amphibians, anurans, breeding, climate change, global warming, froglet, phenology, reproduction.

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Global warming is causing unprecedented quick phenological shifts in animal populations (Horton et al., 2020). These shifts can involve breeding activities, including both advances (Parmesan, 2007; Ficetola and Maiorano, 2016) and delays (Dalpasso et al., 2023) in the onset of breeding season after the winter. Additionally, due to the general increase in winter temperatures, the end of the activity season in autumn is expected to be postponed (Lang et al., 2025). This may influence voltinism, potentially favouring an increase in reproductive events within a year in some populations. However, this may also produce survival risks for early stages, when suitable conditions for development are not matched (Benard, 2015; Bison et al., 2021). For instance, in temperate regions several amphibian species have been shown to extend or shift their breeding season by days or weeks in the last decades (Todd et al., 2011), with some cases of amphibian and reptile species reproducing outside their typical breeding season reported (Graña and Martínez-Freiría, 2020; Rodriguez-Muñoz et al., 2020).

In Europe, most anurans breed in late winter-spring, with juveniles metamorphosing in late spring-summer. This timing allows them taking advantage of spring rain that fills temporary wetlands and exploiting the warm season for larval growth and development. However, breeding patterns can be different in some species in Southern Europe. For instance, in Sicily, the green toad (*Bufo boulengeri* Laurenti, 1768) can also breed in autumn and winter, with tadpoles from autumnal mating overwintering and metamorphosing in early spring (Sicilia et al., 2006). In this case, autumnal breeding has been interpreted as an adaptation to arid environments, where waterbodies filled by autumn rain are more likely to retain water long enough to enable toadlets attaining metamorphosis (Sicilia et al., 2006). Less evidence for autumnal breeding and tadpole overwintering is available for amphibians living in Northern Italy. Among them, the Italian agile frog, *Rana latastei* Boulenger, 1879, is endemic to the lowlands of Northern Italy and adjacent areas and breeds from late January to mid-April (Ambrogio and Mezzadri, 2018; Ficetola et al., in press). In principle, changes in temperature

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and/or precipitation patterns related to climate change might affect breeding phenology of this species, but information on autumn breeding and/or overwintering tadpoles is so far lacking. Here, we report the first observation of overwintering tadpoles in the Italian agile frog. The reproductive site is a small artificial permanent pond (roughly 2 × 1 m; 0.3 m depth) located in a small wooded area in the foothills of the Como district (Inverigo, Lombardy, Italy). The site is located approximately 350 m a.s.l. and hosts numerous water bodies supporting a diverse amphibian community, including three urodele and six anuran species. This area is part of a long-term amphibian monitoring program covering the past two decades (Ficetola et al., 2009; Falaschi et al., 2021), revealing the presence of a stable meta-population for *R. latastei* across interconnected breeding sites (Manenti et al., 2020). The permanent pond where overwintering tadpoles were found has been consistently surveyed multiple times during spring since 2010 and is a stable breeding site of *R. latastei*, where no other amphibian, except the fire salamander (*Salamandra salamandra* Linnaeus, 1758), has been reported reproducing. On 27 January 2025, ~5 days after the first mid-winter rainfalls that usually trigger amphibian breeding in the area, the site was monitored along with other ones in the surroundings. This survey revealed the presence of a single freshly laid egg-clutch of *R. latastei* (the first clutch of the breeding season found in the area). The site was then monitored on January 30 (one new clutch), February 6 (no new clutches), February 11 (one new clutch), and on February 26 (four new clutches). The first egg hatch was observed on February 11. On February 6, the presence of three large-size tadpoles showing *R. latastei* traits was observed. On February 26, we collected six large-sized tadpoles and six small-sized tadpoles (Gosner's stage >25) for measuring (Fig. 1). Concurrently, a newly metamorphosed froglet was found (Fig. 2). Individuals were captured by gently netting the pool, then briefly kept in a small plastic tank, and photographed on graph paper using a 100 mm macro lens to minimize distortion. Pictures were taken by a single operator using a standardized photographic setting. After the photoshoot, the individuals were immediately released (permits listed in acknowledgements). Froglet and tadpoles were measured from scaled

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pictures using ImageJ software (Schneider et al., 2012) following standard procedures by a single operator (Relyea, 2001; Melotto et al., 2020, 2021). Tadpole developmental stages were determined based on Gosner's tables (Gosner, 1960). Although never observed breeding in the study site, another *Rana* Linnaeus, 1758 species with similar ecology, the agile frog (*Rana dalmatina* Fitzinger in Bonaparte, 1838), is present in the nearby woods, and tadpoles of this frog sometimes show phenotypes difficult to tease apart from *R. latastei* (Barbieri et al., 2000). Hereafter, along with morphometric and stage comparison between tadpole cohorts, key phenotypic traits differing between the two frog species are highlighted (Lanza et al., 2009; Ambrogio and Mezzadri, 2014).

We obtained tadpole's measurements from both dorsal and side pictures (Table 1A, Fig. 1A-D) and included total length (Totl), body length (BL), body height (BH), body width (BW), tail length (TL), tail height (TH), tail muscle height (TMH), and tail muscle width (TMW). Moreover, we used dorsal pictures to calculate minimum eye distance (ED) and minimum nostril distance (ND). ND was available for large-sized tadpoles only, as nostril position was not clearly identifiable in newly hatched tadpoles. All tadpole measurements, except for ND, were included in a principal component analysis to ascertain the existence of distinct size classes among tadpoles from different cohorts and if they matched the eye-based and Gosner's stage identification. Moreover, we calculated eye-distance nostril-distance ratio (ENDr) from ED and ND measurements for the perspective overwintering tadpoles (large-sized individuals). This ratio is one of the key traits that differ between tadpoles of *R. dalmatina* and *R. latastei*, being around 2 for the first one and rarely >1.5 for *R. latastei* (Lanza et al., 2009). Finally, we used ventral pictures of the froglet to take some morphological measurements (Table 1B): total length (TL), body width (BW), jaw width (JW), and few left hindlimb lengths, including proximal hindlimb (LPHL), distal hindlimb (LDHL), tarsus (LTL), and foot (LFL). Additionally, froglet ED and ND were obtained from dorsal picture (Table 1B).

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Overall, all tadpoles showed characters matching the typical features of *R. latastei* [absent, poor, or incomplete ventral colouration and visible guts (Fig. 1E); EDNr of large-sized tadpoles ranged from 1.15 to 1.43 (average: 1.29) (Lanza et al., 2009; Ambrogio and Mezzadri, 2014)]. All the small-sized individuals were between Gosner's stage 25 and 26, showing functional oral canal but no trace of hindlimb formation. Instead, large-sized tadpoles were at the Gosner's stage 40 (four individuals; hindlimb with clearly differentiated toes and tubercles, cloacal tail piece visible), or between 37 and 39 (evident toes with no tubercles). The principal component analysis revealed that the first axis explained 99% of variation, identifying three distinct size classes corresponding to the three Gosner's stages identified, clearly distinguishing as different classes large-sized individuals and newly hatched ones (Fig. 3), also evident from picture comparison (Fig. 1E).

The newly metamorphosed individual measured 1.87 cm (Table 1B) and presented the typical traits of *R. latastei* (Lanza et al., 2009): the light stripe running above upper lip suddenly stops under the eye (while it is generally prolonged in *R. dalmatina*), and nostril distance was shorter than eye distance (Fig. 2A-B, Table 1B); moreover, chest and throat looked diffusely spotted and a "T" shaped stripe is noticeable between the throat and the forelimb junction (Fig. 2C), while ventral colouration is generally homogeneously pale and unspotted in *R. dalmatina*.

Taken together, our observations confirm that all individuals belong to *R. latastei.* At the same time, the size and late stage of the large tadpoles along with the presence of a froglet and the comparison with newly hatched tadpoles, suggest that large-sized individuals originated from reproductive events occurred in the previous year and underwent overwintering at larval stage.

In absence of a direct observation of autumnal reproductive events, only speculations can be made concerning the breeding period. Overwintering tadpoles are not rare in anurans (McDiarmid and Altig, 1999) and can be occasionally observed in other Northern-Italian species [e.g., green frogs, *Pelophylax* synkl. *esculentus* (Linnaeus, 1758)] and in some *Rana* species in other areas (Walsh et al., 2008; Lanza et al., 2009). However, this is generally associated with

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anurans facing shorter growing seasons, such as late-breeding species, or species and populations from high latitude or elevation (McDiarmid and Altig, 1999), as overwintering can extend the growth period and allow individuals attaining larger size at metamorphosis (Walsh et al., 2008; Iwai, 2024). This is not the case for *R. latastei*, which is an early breeder, whose tadpoles typically reach metamorphosis in June-July (Lanza et al., 2009).

We suggest these tadpoles originated from one or more breeding events that occurred in late summer or autumn 2024. Tadpoles exposed to low temperatures typically incur in metabolic depression, which slows down growth and development (McDiarmid and Altig, 1999; Enriquez-Urzelai et al., 2022). However, autumn and winter temperatures of 2024–2025 have been among the mildest in recent decades and 2024 has been the warmest year since consistent temperature monitoring began (https://climate.copernicus.eu/). A similar increase in temperature was recorded at the study site, where precipitation showed large variation with peaks of autumn rainfall in the last two years (see Supplementary material: Table S1; Fig. S1). This could have allowed larval development during the winter months, similarly to what happens in anurans breeding in warmer regions (Sicilia et al., 2006). Additionally, previous records of *R. latastei* males calling in autumn have been reported for nearby areas (Grossenbacher et al., 2000). While the presence of calling males does not guarantee that breeding activities are occurring, milder autumn and winter temperatures may favour egg deposition and allow tadpole development during this time.

An alternative hypothesis might be that large-sized tadpoles originated from a typical late-winter deposition event in spring 2024. The monitored breeding site is partially shaded by canopy cover, and 20 egg-clutches were laid by February 2024 (the last observed deposition). We cannot exclude that tadpole density and cold water temperatures might have induced delayed development in some individuals at the breeding site. However, tadpole density was comparable to other nearby sites monitored where *R. latastei* metamorphosed as usual in early summer. Additionally, June–

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September temperatures of 2024 (mean air temperature ± SD: 29.7 ± 5.1 °C) were markedly higher than those experienced over the typical growing season (March–June temperatures: 21.4 ± 6.1), making it extremely unlikely that overwintering individuals originated from a late-winter 2024 deposition that experienced developmental delays due to low temperatures.

Whatever the period of deposition, the present report reveals an unusual case of shift in breeding phenology for *R. latastei*, which calls for further investigations assessing the frequency of these events and unravelling drivers and potential implications of such a shift. Indeed, variation in reproductive timing can result in crucial consequences for individual life-history and survival (Bison et al., 2021; Enriquez-Urzelai et al., 2022). For instance, amphibians developing during winter may face mass mortality risks due to freezing or desynchronization with trophic resources and increase competition (Li et al. 2013); moreover, in explosive breeders, such as wood frogs, these shifts can also trigger cascading effects on amphibian populations and freshwater community dynamics, with complex and rarely predictable outcomes (Todd et al., 2011). This first record of overwintering tadpoles in the Italian agile frog may represent an anomalous or isolated event, but correlation between increasing temperatures, altered precipitation patterns, and shifts in amphibian breeding period have been observed in multiple species as a response to global warming (Todd et al., 2011; Ficetola and Maiorano, 2016). Under the ongoing climate change scenarios, amphibian phenology is expected to be considerably impacted worldwide, and variations in their reproductive activity should deserve particular attention. Further research should aim at unravelling physiological mechanisms regulating shifts in breeding phenology and their implications on amphibian life-history traits and fitness. In light of the present case, autumnal monitoring of the study site and surrounding areas will be planned during the incoming years to ascertain the occurrence of *R. latastei* depositions outside the typical reproductive season. This study case also stresses the importance

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of constant monitoring of amphibian activity and phenology in a climate change scenario, calling for increased surveillance to implement appropriate conservation strategies.

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Table 1. Morphological assessment of *Rana latastei* tadpoles and froglet. A) measures and approximated Gosner's development stage of tadpoles of different cohorts are reported together with individual identification code and size class; B) measures of the newly metamorphosed froglet found. Measurements abbreviations hereafter: body height (BH), tail height (TH), tail muscle height (TMH), body length (BL), tail length (TL), total length (TotL), body width (BW), tail muscle width (TMW), eye distance (ED), nostril distance (ND), jaw width (JW), left distal hindlimb length (LDHL), left proximal hindlimb length (LPHL), left tarsus length (LTL), left foot length (LFL), eye-nostril distance ratio (ENDr).

ID	size class	Gosner	BH	TH	TMH	BL	TL	TotL	BW	TMW	ED
GIR1	large	37 - 39	0.884	1.042	0.510	1.858	3.149	4.525	1.089	0.349	0.34
GIR2	large	40	0.912	0.810	0.405	1.563	2.320	3.489	1.049	0.301	0.26
GIR3	large	40	0.811	0.858	0.403	1.539	2.450	3.626	1.027	0.289	0.26
GIR4	large	40	0.847	0.826	0.375	1.550	2.347	3.467	0.917	0.363	0.27
GIR5	large	40	0.857	0.983	0.423	1.563	2.461	3.698	1.076	0.312	0.32
GIR6	large	37 - 39	0.992	1.069	0.606	1.850	3.483	4.841	1.136	0.379	0.33
GIR7	small	25 - 26	0.374	0.423	0.178	0.633	1.285	1.772	0.395	0.128	0.11
GIR8	small	25 - 26	0.370	0.403	0.172	0.624	1.194	1.685	0.404	0.091	0.13
GIR9	small	25 - 26	0.347	0.387	0.168	0.662	1.206	1.740	0.455	0.095	0.15
GIR10	small	25 - 26	0.362	0.404	0.167	0.683	1.258	1.696	0.431	0.091	0.14
GIR11	small	25 - 26	0.297	0.344	0.166	0.582	1.201	1.585	0.367	0.091	0.12
GIR12	small	25 - 26	0.361	0.404	0.171	0.700	1.299	1.781	0.404	0.082	0.12

D	TL	BW	JW	LDHL	LPHL	LTL	LFL	ED	ND	ENDr
froglet	1.867	0.888	0.819	0.968	0.952	0.480	0.899	0.437	0.195	2.241

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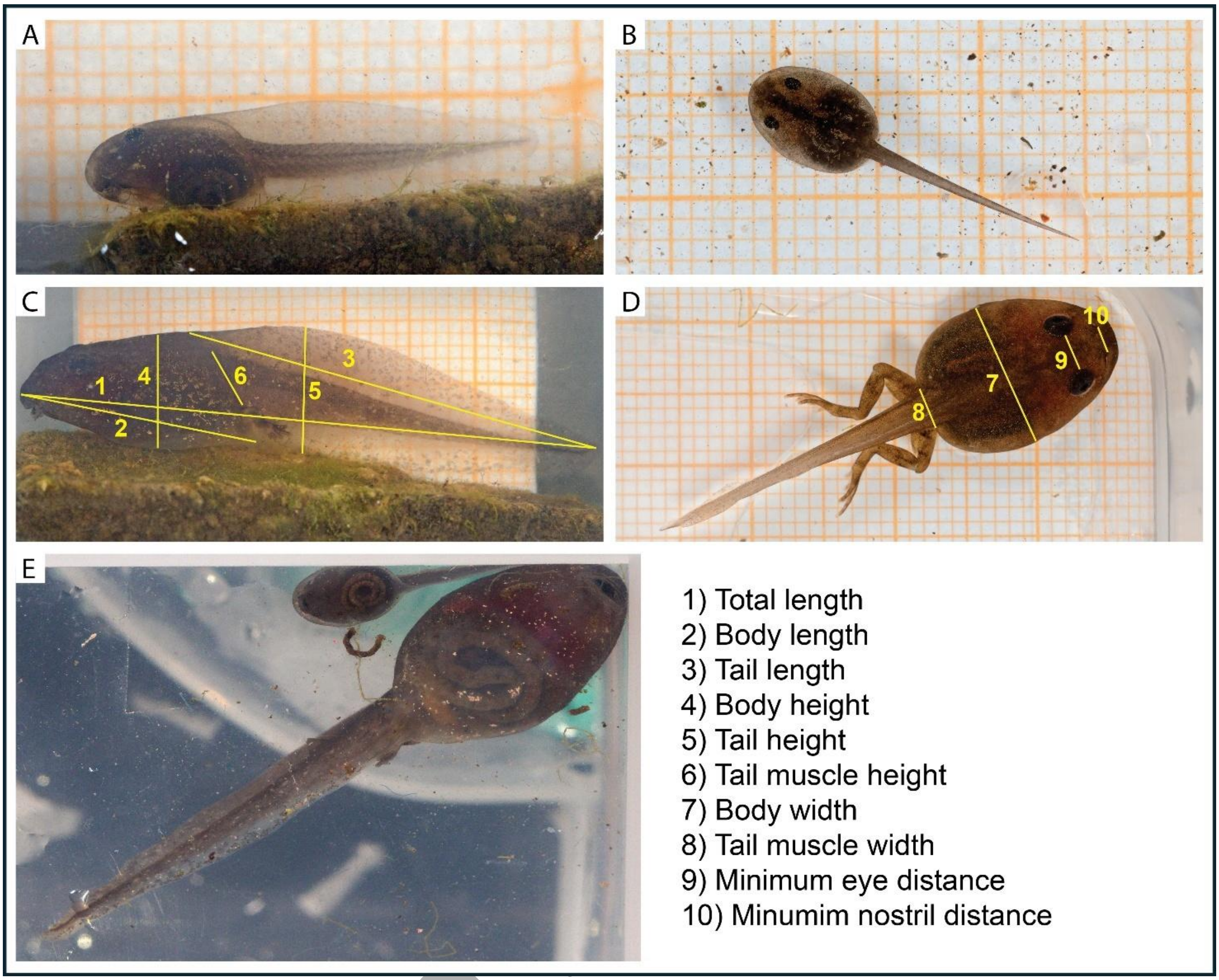

Figure 1. Tadpoles of *Rana latastei*. A-B) Lateral (A) and dorsal (B) view of newly hatched individuals (small-sized); C-D) lateral (C) and dorsal (D) view of overwintering individuals (large-sized); E) ventral view showing size difference between a newly hatched and an overwintering individual. For C e D, examples of measures performed are provided.

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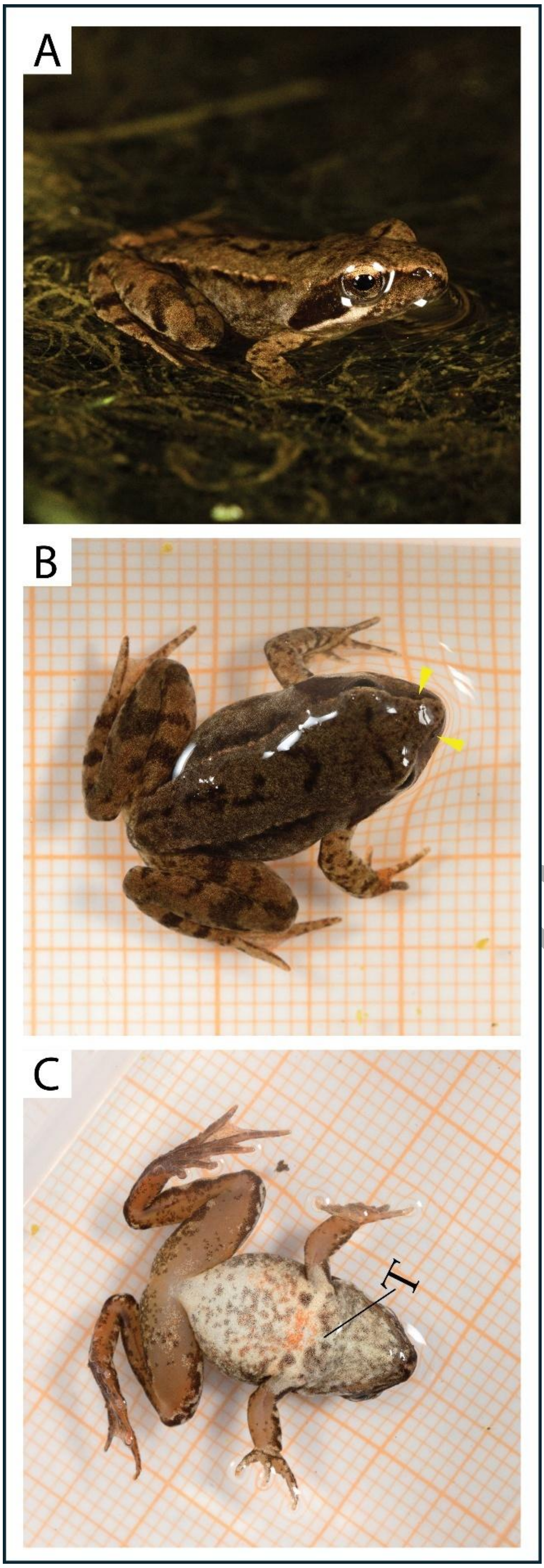

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Figure 2. Newly metamorphosed *Rana latastei* individual. A) lateral view, white stripe stopping under the eye visible; B) dorsal view, nostrils are highlighted by yellow arrow-heads; C) ventral view, T shape between throat and forelimb junction highlighted.

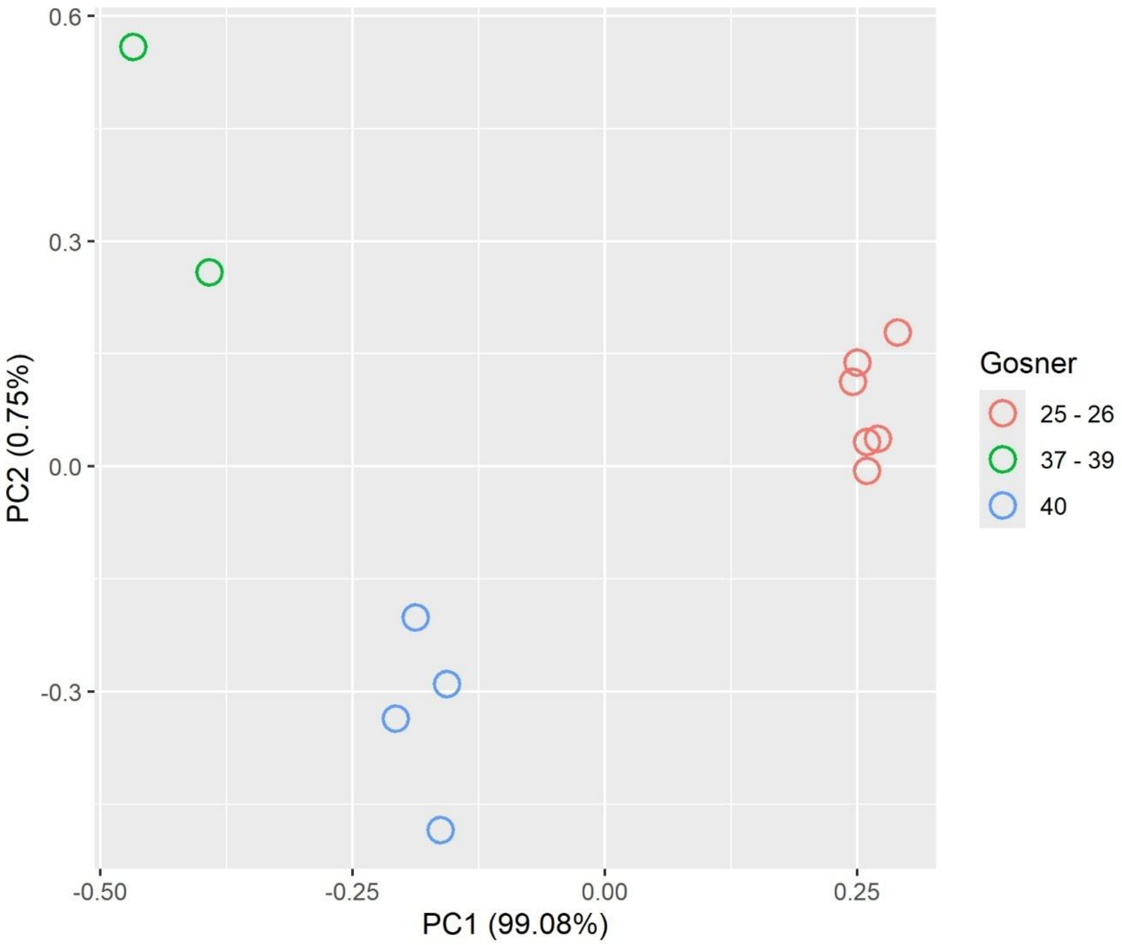

Figure 3. Principal component analysis performed using tadpole measures in Table 1A, excluding nostril distance (ND), which were not measured for Gosner's 25 – 26 tadpoles. Colours represent Gosner's developmental stages, as indicated in Table 1A.

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