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# Larval development and breeding ecology of Ziegler's Crocodile Newt, *Tylototriton ziegleri* Nishikawa, Matsui and Nguyen, 2013 (Caudata: Salamandridae), compared to other *Tylototriton* representatives

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***Abstract.*—We describe for the first time the larval development and stages of the recently described Ziegler's Crocodile Newt (*Tylototriton ziegleri*), an endemic species to northern Vietnam. Diagnostic morphological characters are provided for Grosse (1997, 2013) stages 27–32, 35–36, and 44–45, as well as comparisons with larval stages of other *Tylototriton* representatives. In addition, natural history data and an ecological assessment of the breeding niche are presented for *T. ziegleri* as well as for *T. vietnamensis*, from whom the former species was only recently taxonomically separated. We provide data extending the known breeding season of these two cryptic species in the North of Vietnam, which in fact lasts from April until July. On average, the clutches of *T. ziegleri* consisted of 67 ± 32 eggs, were found on rock and soil substrates with a distance of 50 ± 28 cm from water, whereas the clutches of *T. vietnamensis* were significantly smaller (43 ± 19 eggs), found only on soil and were further distant from water (80 ± 41 cm). The known maximum altitudinal distribution of *T. vietnamensis* is herein increased to 980 m above sea level. Based on the examples of *T. ziegleri* and *T. vietnamensis*, this study highlights how important it is to uncover cryptic species, define their exact distribution range, and investigate potential differences in ecological adaptations in order to assess the conservation status, develop proper conservation planning and provide suitable conditions for potential *ex situ* breeding programs.**

**Keywords.** Vietnam, Crocodile Newts, cryptic species, developmental biology, larval staging, microhabitat characterization, conservation, captive breeding

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## Introduction

The genus *Tylototriton* currently consists of 22 species with a distribution from Nepal, Bhutan, and India eastwards to China and southwards to Indochina (Nishikawa et al. 2013a). Phylogenetic analyses divided the genus into the *T. asperrimus* group (Fei et al. 2005) or the subgenus *Yaotriton* (Dubois and Raffaëlli 2009), which includes: *T. asperrimus, T. broadoridgus, T. dabienicus, T. hainanensis, T. liuyangensis, T. lizhenchangi, T. notialis, T. podichthys, T. panhai, T. vietnamensis, T. wenxianensis, and T. ziegleri*; and the *T. verrucosus* group (Fei et al. 2005) or the subgenus *Tylototriton* (Dubois and Raffaëlli 2009), which includes: *T. anguliceps, T. himalayanus, T. kweichowensis, T. pseudoverrucosus, T. shanjing, T. shanorum, T. taliangensis, T. uyenoi, T. verruco-*

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**Fig. 1. A.** Adult male of *Tylototriton ziegleri*; **B.** Habitat type in Bao Lac district, Cao Bang Province; **C.** Adult male of *Tylototriton vietnamensis*; **D.** Habitat type in Tay Yen Tu Nature Reserve, Bac Giang Province. *Photos M. Bernardes.*

*sus,* and *T. yangi* (Khatiwada et al. 2015; Le et al. 2015; Nishikawa et al. 2014; Phimmachak et al. 2015; Yang et al. 2014).

In Vietnam, this genus is currently represented by *T. asperrimus, T. notialis, T. anguliceps,* as well as by two endemic species, viz. *T. vietnamensis,* and *T. ziegleri*. Specimens of Ziegler's Crocodile Newt were previously referred to *T. asperrimus* (Sparreboom et al. 2011, Yuan et al. 2011) or *T.* cf. *vietnamensis* (Stuart et al. 2010). *Tylototriton ziegleri* was subsequently described as a distinct species by Nishikawa et al. (2013b) based on morphological and molecular differences from *T. vietnamensis*. The latter species has been evaluated as Endangered in the Vietnam Red Data Book (Tran et al. 2007), and in the IUCN Red List (IUCN SSC Amphibian Specialist Group 2016).

*Tylototriton vietnamensis* inhabits secondary evergreen lowland forests on granite parent rock material consisting of hardwood, bamboo and shrubs and is known from lower elevations in Bac Giang, Quang Ninh, Lang Son, and Phu Tho provinces (Bernardes et al. 2017; Nguyen et al. 2009). In contrast, *T. ziegleri* is known from primary forests on limestone parent rock material at higher elevations characterized mainly by bamboo vegetation in Cao Bang and Ha Giang provinces (Nishikawa et al. 2013b) (Fig. 1).

In-depth studies focusing on distinctive features and thus on the taxonomic status of closely related or at least similar, potential taxa which are either threatened and/or have a limited range, as in the case of the species pair *T. vietnamensis* and *T. ziegleri*, are important for proper identification and suitable conservation actions. Since both species are distributed at different elevations and occupy distinct geological areas, we expected to find ecological, morphological, and developmental differences to support their discrimination. Herein, we document for the first time the larval development of *T. ziegleri*, in comparison with information on the development of other *Tylototriton* species. We also provide data on the ecological niche of *T. ziegleri*, in particular microhabitat preferences associated with reproduction, and compare this with our own field data for *T. vietnamensis*.

## Materials and Methods

**Field surveys.** Field surveys were conducted by M. Bernardes, C.T. Pham and H.T. An during the rainy season between 10 April and 11 July 2010, 8 June and 7 August 2012, 13 June and 28 July 2013, and 15 May and 28 June 2014 in northern Vietnam. The surveys were conducted in Son Dong and Luc Nam districts in Bac Giang Province, Hoanh Bo district in Quang Ninh Province, and Mau Son district in Lang Son Province for *T. vietnamensis*; and in Quan Ba and Bac Quang districts in Ha Giang Province, and Bao Lac district in Cao Bang Province for *T. ziegleri*. Daytime visits to breeding sites were conducted for an average time of 20 minutes and varied between two and eight times, sometimes in repeated years. Besides coordinates and elevations recorded with Garmin GPS MAP62, a defined set of several abiotic variables were collected and recorded in order to classify each study site according to their environmental conditions. The physical characteristics of each pond (area and maximum depth) were determined by use of a measuring rope of precisely known size. A pH meter (Hanna HI 98129) calibrated for 25 °C was used to measure the pH and record water temperature. Water chemical parameters were taken for an analysis of pollution (concentration of nitrate [$NO_3$-] and nitrite [$NO_2$-]) and water hardness (carbonate [KH] and total hardness [gH], measurements following the German degree) using drop-by-drop color tests from JBL (Testlab, Germany). Since some pools occurred only temporary the hydroperiod was also taken into account. Weather data consisting of temperature, humidity, and atmospheric pressure were recorded with a weather station (Krestel 3500) at each breeding site. The tree canopy cover above the pond water was assessed visually and classified in five levels ranging from 0–100% cover.

Adult animals were searched on the bottom of the water body using visual survey encounter techniques followed by subsequent capture with a sweep of a 25 cm dip-net (JBL, Germany). The surrounding shore of the pond was searched for the presence of egg clutches, efts, and additional adults until a maximum distance of three meters from the water line. All captured adults were counted, photographed, and subsequently released. The distance of the egg clutch to the water source was measured and the total number of eggs present in the clutches counted with minimum disturbance. The diameter and the weight of seven randomly picked eggs (in the case of *T. ziegleri* belonging only to Bao Lac district, Cao Bang Province) were measured by using a digital calliper to the nearest 0.1 mm and weighted with a digital scale to the nearest 0.01 g.

**Larval staging, morphological description and comparisons with congeners.** One clutch of *T. ziegleri* was collected on the 17th July 2014 in Bao Lac district, Cao Bang Province, Vietnam to observe the larval development and for further morphological comparisons. The clutch was collected together with associated substrate and foliage coverage from the shore of a breeding pond. The clutch was kept inside an open-air plastic box and regularly sprayed with water to keep up the suitable humidity level. Due to the thickness of the gelatinous layer (albumen) it was not possible to observe and document the developing larvae inside; therefore we preserved few eggs for morphological analysis. Every one or two days one egg was randomly selected (in total 23), transferred to a 4% formalin solution for fixation and subsequently preserved in 70% ethanol. This procedure was repeated until hatching of the first larva. The remaining larvae were later transferred to the Me Linh Station for Biodiversity to contribute to a captive breeding program. In addition, seven swimming larvae were collected at the breeding pond for a morphological description of more advanced developing stages. The larvae were anaesthetized with ethyl acetate, fixed in 40% ethanol, and later transferred to 70% ethanol for preservation. Preserved individuals subsequently were deposited in the collections of the Institute of Ecology and Biological Resources (IEBR), Hanoi, Vietnam, with the catalogue numbers: IEBR A.2016.19-A.2016.31 and of the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK), Bonn, Germany, with the catalogue numbers: ZFMK 98792-ZFMK 98796.

Preserved eggs were cut open to examine the developing larvae inside. A detailed description of the development and measurements of the ovum in early stages could not be performed as the jelly layers could not be opened without destroying the fragile content. Staging was performed under a magnifying loupe and by support from a digital microscope (Keyence VHX-500F) when extra magnification, photographs, and morphological measurements were needed. To complement the characterization process (e.g., for characterizing the coloration in life), additional photographs of hatched larvae were taken during field work by M. Bernardes or at the Me Linh Station for Biodiversity by T. Ziegler by placing single larvae into a water filled glass vessel.

Larval stages were identified according to Grosse (1997, 2013) and the morphological terminology followed Nishikawa et al. (2013b). The following measurements were taken: snout-vent length (SVL), from tip of snout to posterior edge of the vent; head length (HL), from posterior edge of right parotid to snout tip; maximum head width (HW); head height (HH), measured above the eyes; snout length (SL), from right nostril to right posterior corner of mouth; interocular distance (IoD), from anterior corner of eyes; internostril distance (InD); eye-nostril distance (EnD), from right nostril to anterior corner of right eye; forelimb length (FlL), from right anterior limb measured from point of body insertion to tip of longest finger; hind-limb length (HlL), right posterior limb measured from point of body insertion to tip of longest toe; axillar distance (AD), from axilla to groin on right side; width of tail base (TW), measured at posterior edge of vent; maximum tail height (TH); tail length (TaL), from posterior edge of vent to tail tip; total length (TL), from tip of snout to tail tip.

For morphological comparisons, data from literature was included for the following species: *T. anguliceps*, *T. asperrimus*, *T. broadoridgus*, *T. hainanensis*, *T. himalayanus*, *T. kweichowensis*, *T. liuyangensis*, *T. podichthys*, *T.* cf. *shanjing*, *T. shanorum*, *T. taliangensis*, *T. uyenoi,* and *T. wenxianensis* (see Bourret 1942; Khatiwada et al. 2015; Kuzmin et al. 1994; Mudrack 2005; Nishikawa et al. 2013a, 2014, 2015; Phimmachak et al. 2015; Shen et al. 2012; Sparreboom 2014; Yang et al. 2014; Zhao 1988; Ziegler et al. 2008). For detailed comparisons with *T. vietnamensis* we included our own field data and pictures

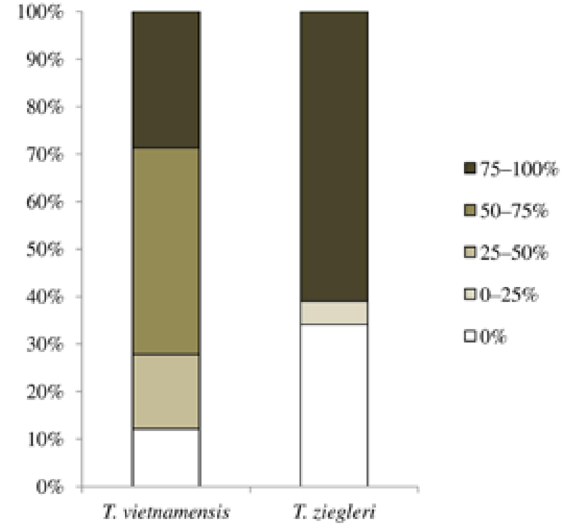

**Fig. 2.** Percentage of the number of adults of *Tylototriton vietnamensis* and *T. ziegleri* found at each interval of percentage of canopy cover measured above the water of the breeding site.

of larvae (in stages 33, 35, 41, and 42) photographed either *in situ* during our field work in Bac Giang Province by M. Bernardes or *ex situ* at the Me Linh Station for Biodiversity by T. Ziegler.

**Statistical analysis.** Comparisons between *T. ziegleri* and *T. vietnamensis* regarding the area and depth of the different ponds, clutch sizes, and distance to water, as well as the regression between the clutch and egg sizes within the genus were examined with Student's *t*-test after confirming a normal distribution of the data. Analyses were performed in R version 3.2.3.

**Molecular analysis.** For species identification, we sequenced a partial mitochondrial gene, the NADH dehydrogenase subunit 2 (ND2), for the egg / larval tissue samples (IEBR A.2016.19–A.2016.21) from the clutch of *T. ziegleri* collected on the 17th July 2014 in Bao Lac district, Cao Bang Province, Vietnam, which was used for larval staging, using the primer pair, Sal_ND2_F1 and Sal_ND2_R2 (Nishikawa et al. 2013b). Tissue samples were extracted using DNeasy blood and tissue kit, Qiagen (California, USA). Extracted DNA from the fresh tissue was amplified by PCR mastermix (Fermentas, Canada). The PCR volume consisted of 21 µl (10 µl of mastermix, five µl of water, two µl of each primer at 10 pmol/µl, and two µl of DNA or higher depending on the quantity of DNA in the final extraction solution). PCR condition was: 95 °C for five minutes to activate the taq; with 40 cycles at 95 °C for 30 s, 50 °C for 45 s, 72 °C for 60 s; and the final extension at 72 °C for six minutes.

PCR products were subjected to electrophoresis through a 1% agarose gel (UltraPure™, Invitrogen). Gels were stained for 10 minutes in 1X TBE buffer at two pg/ml of ethidium-bromide, and visualized under UV light. Successful amplifications were purified to eliminate PCR components using GeneJET™ PCR Purification kit (Fermentas, Canada). Purified PCR products were sent to Macrogen Inc. (Seoul, South Korea) for sequencing.

Sequences generated in this study were aligned with one another using the De Novo Assemble function in the program Geneious v.7.1.8 (Kearse et al. 2012). They were then compared with other sequences using the Basic Local Alignment Search Tool (BLAST) in GenBank.

## Results

**Molecular analysis.** Three sequences of 987 bps were obtained. The sequences were almost identical, except in two positions, and 99% to 100% similar to the sequence with the GenBank's accession number AB769542 of *T. ziegleri* (voucher VNMN 3389). The results confirm the samples collected in Cao Bang Province are conspecific with *T. ziegleri.*

**Distribution, ecological niche and microhabitat use of *T. ziegleri* and *T. vietnamensis*.** *Tylototriton ziegleri* was found in Cao Bang Province at elevations between 1,325 and 1,420 m above sea level, in Ha Giang Province, Bac Quang district between 868 and 932 m above sea level, and in Quan Ba district between 1,080

**Table 1.** Results of water chemical analysis conducted during field work in the habitat of *Tylototriton vietnamensis* and *T. ziegleri* during 2010, 2013, and 2014. Values are presented as min. – max. (mean ± standard deviation).

| Species | Province | district | pH | °KH | °gH | NO$_2^-$(mg l-1) | NO$_3^-$(mg l-1) |
|---|---|---|---|---|---|---|---|
| *T. vietnamensis* | Bac Giang | Son Dong | 4.65–6.43 | 1–5 | 1–5.5 | 0–0.5 | 0–10 |
| | | | (5.48 ± 0.48) | (1.80 ± 1.11) | (2.46 ± 1.63) | (0.04 ± 0.11) | (3.39 ± 2.38) |
| | Quang Ninh | Uong Bi | 7.36–7.51 | 4–8 | 5–6 | 0–0.4 | 5–15 |
| | | | (7.43 ± 0.11) | (6 ± 2.83) | (5.5 ± 0.71) | (0.2 ± 0.28) | (10 ± 7.07) |
| *T. ziegleri* | Cao Bang | Bao Lac | 7.08–7.28 | 6–6 | 7–7 | 0.03–0.03 | 0.03–20 |
| | | | (7.18 ± 0.14) | | | (10.01 ± 14.12) | |
| | Ha Giang | Bac Quang | 6.36–7.05 | 1–1 | 1–2 | 0–0.05 | 1–1 |
| | | | (6.63 ± 0.37) | | (1.33 ± 0.58) | (0.03 ± 0.03) | |
| | Ha Giang | Quan Ba | 6.41–7.94 | 1–8 | 1 –9 | 0.05–0.4 | 0–1 |
| | | | (7.30 ± 0.61) | (5.33 ± 2.88) | (5 ± 3.41) | (0.13 ± 0.14) | (0.79 ± 0.4) |

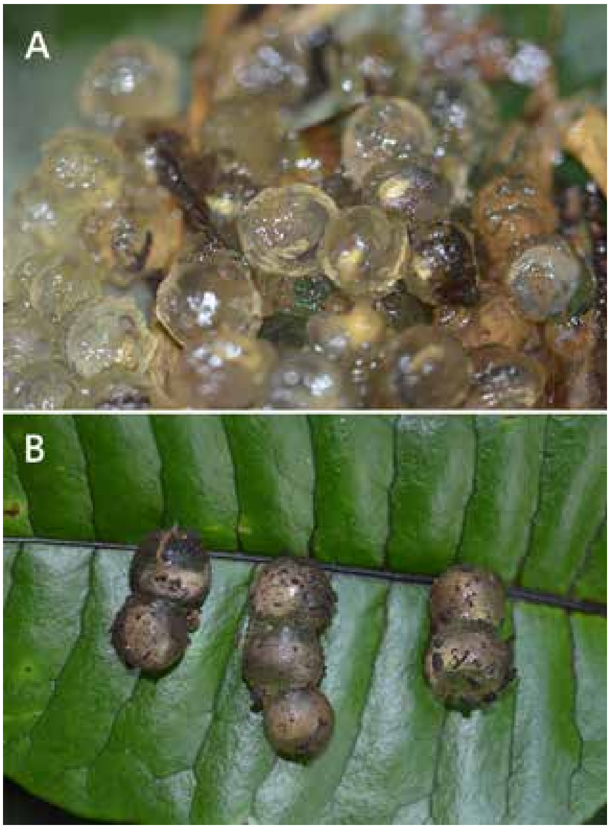

**Fig. 3. A:** Typical clutch of *Tylototriton ziegleri* composed by single eggs; **B:** an exceptional case of "stickiness" where eggs were aggregated in groups of 2–4. *Photos M. Bernardes.*

and 1,369 m above sea level. *Tylototriton vietnamensis* was found between 181 and 512 m above sea level in Bac Giang and Quang Ninh provinces, and between 840 and 980 m above sea level in Lang Son Province. Spawning sites consisted of small ponds for both species, although in the district Quan Ba, Ha Giang Province we also found clutches of *T. ziegleri* in the slopes of a slow flowing forest stream, suggesting that this species can also breed in this type of habitat. A physical evaluation of ponds during our field work showed that the ones inhabited by *T. ziegleri* were significantly deeper ($F_{1,42} = 25.11$, $P < 0.001$; mean 79 ± 58 cm, $n = 19$, range between 10 and 200 cm) than those inhabited by *T. vietnamensis* (mean 25 ± 14 cm, $n = 81$, range between 3 and 60 cm), while the area was roughly the same ($F_{1,44} = 0.004$, $P = 0.95$; *T. ziegleri:* mean 84 ± 165 $m^2$, range between 2.5 and 510 $m^2$; *T. vietnamensis:* mean 82 ± 102 $m^2$, range between one and 460 $m^2$). Most adults (61% of 82 individuals of *T. ziegleri* and 72.2% of 255 individuals of *T. vietnamensis*) were found in breeding sites with 50% or more canopy cover, although still 34.1% of all *T. ziegleri* and 12.2% of all *T. vietnamensis* were found in breeding sites with no canopy cover (Fig. 2).

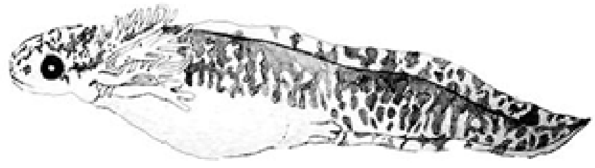

**Fig. 4.** Drawing of a formol-preserved larva of *Tylototriton ziegleri* at stage 35. *Drawing C. Michel.*

A comparison of water quality showed that *T. ziegleri* occurred in ponds with pH values between 6.4 and 8 (mean 7 ± 0.5; throughout Cao Bang and Ha Giang provinces), while *T. vietnamensis* occurred in ponds with pH values ranging from 4.7 to 7.5 (mean 5.6 ± 0.7; throughout Bac Giang and Quang Ninh provinces). Following US Geological Survey standard for water hardness classification both *T. ziegleri* and *T. vietnamensis* varied between soft (0–1 °KH) and hard (8 °KH), with *T. ziegleri* distributed over an average of 4 ± 3 °KH and *T. vietnamensis* over an average of 2 ± 2 °KH. The general hardness was also on average higher for *T. ziegleri* (1–9 °gH; mean 4 ± 3 °gH) compared to *T. vietnamensis* (1–6 °gH; mean 3 ± 2 °gH). Concentration of nitrite ranged from 0–0.4 mg l-1 for *T. ziegleri* and from 0–0.5 mg l-1 for *T. vietnamensis*, while concentrations of nitrate ranged from 0–20 mg l-1 and 0–15 mg l-1, respectively (Table 1). Environmental data revealed higher humidity levels for *T. ziegleri* (mean 100 ± 0%) than those for *T. vietnamensis* (mean 94 ± 9%, range between 68 and 100%), and slightly higher temperature oscillations for *T. vietnamensis* 24.2–34.2 °C (mean 28.6 ± 2.2 °C) than those for *T. ziegleri* 26–34.4 °C (mean 27.4 ± 3.3 °C).

**Mating and egg deposition of *T. ziegleri* and *T. vietnamensis*.** During the breeding season of these two species (April–July) reproductive males that were otherwise terrestrial, moved into the water at the breeding sites and waited for the females. When precipitation was lacking and breeding sites dried out, adults were forced to maintain their terrestrial life mode. However, if climatic conditions were favorable, males preferably were found inside the water. From a total of 547 captured adults of *T. vietnamensis*, and 101 adults of *T. ziegleri*, only five (0.91%) and two (1.98%), respectively, were found on land. Females seem to join the males in the water for a very short period, since only 12 females of *T. vietnamensis* and one female of *T. ziegleri* were captured during field work. After courtship and reproduction, gravid females laid large eggs at the shore of the water body and returned to the forest. Due to their slight stickiness, the eggs aggregated in egg masses (Fig. 3A) which were subsequently covered by the females with leaf litter from the forest ground. The clutch size was significantly different for the two species ($F_{1,71} = 11.29$, $P < 0.001$). For *T. ziegleri*, it ranged between 10 and 109 eggs (mean 67 ± 32 eggs, $n = 10$), with clutches found between rock (83.3%) and soil (16.7%) substrates, while for *T. vietnamensis* ($n = 63$) it ranged between 5 and 85 eggs (mean 43 ± 19 eggs) and clutches were always found on soil substrate. Clutches of *T. ziegleri* and *T. vietnamensis* differed significantly in their distances to the nearest water

**Fig. 5.** Metamorph of *Tylototriton ziegleri* at stage 44, with an additional finger on left hand, collected in Ha Giang Province in 2012 and preserved in ethanol. *Photo M. Bernardes*.

body ($F_{1,50} = 5.32$, $P < 0.01$). Clutches of *T. ziegleri* were between 10 and 100 cm away from water (mean 50 ± 28 cm, $n = 11$), while the ones from *T. vietnamensis* were found at a distance between 17 and 188 cm (mean 80 ± 41 cm, $n = 41$) from the water.

In one exceptional case a clutch of *T. ziegleri* consisted of eggs sticking so strongly together that they were no longer solitary but formed small aggregations of two to four eggs (Fig. 3B).

**Egg description, developmental stages and larval morphology of *T. ziegleri*.** From a total of 80 eggs in the collected clutch of *T. ziegleri*, 34 (42%) did not show normal development. The same count was done in the field with one other clutch consisting of 107 eggs and revealed that 23% of the eggs had ceased to develop.

We estimated that the collected egg clutch was around two days old at the time of collection (16th June 2014). The diameter of the preserved eggs ranged between 8.7 and 11.2 mm (mean 10.1 ± 0.8 mm, $n = 23$), while the diameter of eggs measured randomly in the field (all measurements of the eggs from Bao Lac district, Cao Bang Province) ranged between 7.2 and 8.9 mm (mean 8.1 ± 0.8 mm, $n = 28$). The associated weight for the eggs measured in the field ranged between 0.2 and 0.4 g (mean 0.3 ± 0.1 g, $n = 28$).

In general, the egg shape was round and the surface of the jelly layers uneven. Most eggs cointained a clear gelatinous layer, few were slightly more opaque. The liquid inside was clear. The capsular chamber contained the embryo or in less developed stages the ovum which was either attached or not attached to the inside of the chamber. An outer observation of the ovum in early stages identified a round ovum with a creamy yellowish white color. The size of three measured ovae varied between 3.87 and 4.78 mm. Larvae in an advanced stage of development showed a more slender shape, curved to fit inside the capsular chamber. While some stages were represented by multiple specimens, stages 33, 34, and 37–43 were not found and thus could not be examined. At stage 27 (IEBR.A.2016.22) gill and forelimb buds were discernible. Stage 28 (IEBR.A.2016.23) was characterized by further development of the forelimb buds.

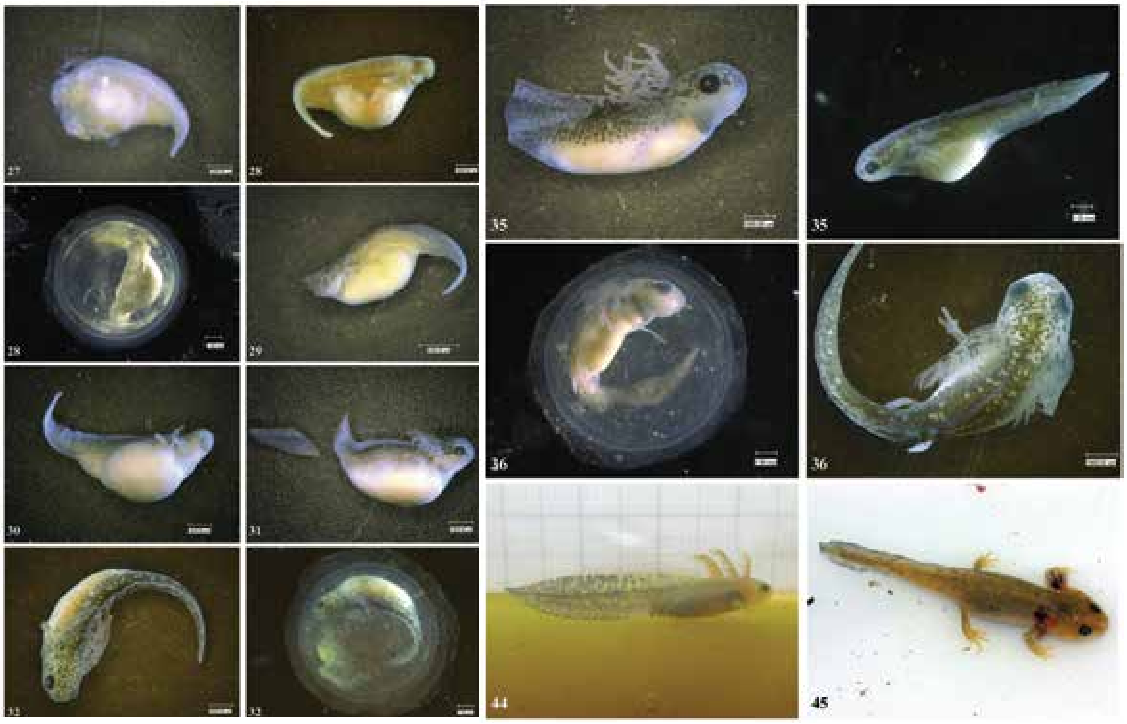

**Fig. 6.** *Tylototriton ziegleri* larvae from Bao Lac district, Cao Bang Province with indication of the corresponding developmental stage and scale. Photographs of stages 27 to 36 are from preserved eggs photographed under a digital microscope (photos *C. Michel*) and photographs of stages 44 and 45 are from individuals in life kept at the Me Linh station (photos *T. Ziegler*).

This was also the case for stage 29 (IEBR.A.2016.24 and ZFMK 98792) along with the growth of fimbriae. Stage 30 (IEBR.A.2016.25) could be determined by the dome shaped distal tip of the forelimb. The forelimb was cone shaped at stage 31 (IEBR.A.2016.26 and ZFMK 98793). Two digits were formed at stage 32 (IEBR.A.2016.27). At stage 35 (IEBR.A.2016.28 and ZFMK 98794) the forelimb had a joint and a hand with three digits. Additionally the hind limbs started to develop and in some cases already showed toe buds (see Fig. 4). At this stage, around 20 days after the assumed egg deposition date (4th July), the first larvae hatched, while some still remained inside the egg. At hatching time larvae had an average total size of 14.65 ± 0.77 mm (size ranged between 13.78 and 15.22 mm, *n* = 3). Stage 36 (IEBR.A.2016.29 and ZFMK 98795) was characterized by a forelimb with four digits and a hind limb with three toes and a knee joint. The yolk sac was prominent in stages 27–30 and was evident until stage 35. At stages 44 (IEBR.A.2016.30) and 45 (IEBR.A.2016.31 and ZFMK 98796), larvae were black and had well developed limbs with four fingers and five toes, and the gills atrophied. In one individual at stage 44 we observed the splitting of one finger in two, resulting in five fingers on the left hand (Fig. 5). No juve-

**Table 2.** Developmental stages, morphological description and coloration of *Tylototriton ziegleri* from stages 27 to 45; stage diagnostic characteristics according to Grosse (1997, 2013) are italicized. Specimens from stages 27–36 originated from the egg clutch while data on stages 44 and 45 were gathered from hatched larvae collected inside the breeding pond.

| Stage | Morphology | Coloration |
|---|---|---|
| **27**<br>(*n* = 1) | Head trapezoidal and sloping in profile, snout short and flat, no labial fold visible, eyes distinguishable but unobtrusive. Gills upright, shorter than head. No balancers. Dorsal and ventral fins about the same height, higher than head; dorsal fin starts at last third of the trunk; tail short; tail-tip round. Forelimb-buds start developing; yolk mass twice as high as body, nearly round. Larva clearly visible through egg jelly and can be moved inside the capsular chamber. | Ground color white-yellowish, with yolk mass more yellow. Dark, irregularly distributed pigmentation on dorsum head and flanks, getting lighter towards the ventrum until total disappearance on ventral side. Eyes white without pigmentation. |
| **28**<br>(*n* = 1) | Head trapezoidal and sloping in profile, snout short and flat, no labial fold visible; gills nearly head high. Dorsal fin higher than head, starting at last third of the trunk; ventral fin shorter than dorsal fin; tail stretching; tail-tip round. Forelimb-buds clearly visible, yolk mass big and round. | Pigmentation similar to stage 27, additionally longitudinal lateral stripe on rib area without pigmentation. Slightly pigmented rim around the eyes forming circle; forelimb-bud base with slight pigmentation on dorsal side. |
| **29**<br>(*n* = 2) | Head more pronounced, labial fold distinct at posterior half of upper jaw; gills developing fimbriae and higher than head; tail getting longer; yolk is less round and oval shaped; forelimb-buds longer with rounded tip; mouth is located on ventral side of head, beneath the snout tip. | Pigmentation getting darker, particularly in the eyes, also slight pigmentation underneath the gular fold. |
| **30**<br>(*n* = 2) | Shape similar to stage 29, forelimb-buds are slightly longer with a dome shaped distal tip; gill rami and fimbriae more developed, dorsal and ventral fin have become larger. | No change in pigmentation. |
| **31**<br>(*n* = 2) | Gills growth; labial folds distinct at posterior half of upper jaw; forelimb cone shaped; tail and fins well developed; dorsal fin starts at middle of the trunk; yolk mass receding. | Pigmentation getting darker, denser pigmentation on dorsum behind head; eyes nearly fully black with white pupil, small, unpigmented stripe from pupil towards ventral side; gill rami slightly pigmented on upper side; fimbriae without pigmentation. |
| **32**<br>(*n* = 1) | Dorsal and ventral fin growing; gular fold clearly visible; mouth orientation is more rostral; two digits developing as small knobs on distal edge of forelimb with a notch forming in the middle. | Pigmentation getting denser on dorsum forming a dark stripe with unpigmented spots; head pigmentation less dense; eyes except for pupil fully pigmented; few dark spots on dorsal and ventral fin and forelimb. |
| **35**<br>(*n* = 11) | Head more depressed, sloping in profile; mouth more pronounced with nares clearly visible; hand with three digits is visible beyond the end of the gills; digits round at the tip; the middle digit the longest; limb with joint, bending at the elbow; yolk mass has almost completely receded; gut getting tubular. Hind limb buds discernible and in some cases elongated, indentation between first two toes in some larvae. | No change in pigmentation. |
| **36**<br>(*n* = 3) | Lateral line organs visible on ventral side of head; mouth open with well-developed teeth; four digits have formed on hand, forelimb turned, palm is facing ventrally. Hind limb with three toes and a knee joint starting to form. | No change in pigmentation. |
| **44**<br>(*n* = 4) | Skin mostly smooth with some warts starting to form; tail long and pointed; limbs well developed with four fingers and five toes; no remains of yolk; head trapezoidal, wide and depressed with a short and flattened snout; dorsal and ventral fin receding; dorsal fin beginning on the first quarter of back and ventral fin beginning above the cloaca; caudal fin higher than head; gills higher than body, with fimbriae still clearly visible. | Pigmentation black and dense over the whole body; lighter on underside of head and ventral side; tip of toes and fingers and labial folds are unpigmented. |
| **45**<br>(*n* = 3) | Skin gets less smooth and more granular and warty; teeth well developed; fins receding; gills atrophy (only stumps left). | Similar to stage 44, but tip of toes and fingers are colored in yellow. |

**Table 3.** Morphological measurements of larvae and respective eggs of *Tylototriton ziegleri* from Ha Giang sorted by stage (mean ± standard deviation, range in parenthesis, in mm). N: Number of individuals, D: diameter. For abbreviations see Material and Methods. Internostril distance, eye-nostril distance, and snout length were not discernible for stages 27–30 and hind limb length could only be measured from stage 25 onwards.

| **Stage:** | **27** | **28** | **29** | **30** | **31** | **32** | **35** | **36** | **44** | **45** |
|---|---|---|---|---|---|---|---|---|---|---|
| (N) | (1) | (1) | (2) | (2) | (2) | (1) | (11) | (3) | (4) | (3) |
| SVL | 7.3 | 5.30 | 5.94 ± 0.21<br>(5.73–6.15) | 5.41 | 5.95 ± 0.14<br>(5.85–6.05) | 7.87 | 7.6 ± 0.56<br>(6.32–8.28) | 6.89 ± 0.18<br>(6.76–7.02) | 21.39 ± 9.37<br>(10.92–31.94) | 34.62 ± 0.25<br>(34.44–34.8) |
| Tal | 2.36 | 1.67 | 3.71 ± 0.19<br>(3.53–3.9) | 3.99 | 3.13 ± 0.73<br>(2.61–3.65) | 5.59 | 6.49 ± 0.85<br>(5.16–8.7) | 5.56 ± 2.54<br>(3.76–7.36) | 14.04 ± 7.04<br>(5.69–23.33) | 24.89 ± 1.37<br>(23.92–25.86) |
| TL | 9.66 | 6.97 | 9.65 ± 0.52<br>(9.02–10.05) | 9.39 | 9.08 ± 0.5<br>(8.67–9.38) | 13.46 | 14.09 ± 1.17<br>(12.17–16.31) | 12.45 ± 3.77<br>(10.53–15.86) | 35.43 ± 16.42<br>(17.25–55.11) | 59.512 ± 3.4<br>(57.82–62.62) |
| HL | 0.73 | 0.92 | 0.97 ± 0.03<br>(0.94–1) | 1.20 | 1.23 ± 0.03<br>(1.21–1.25) | 1.95 | 1.57 ± 0.26<br>(1.23–2.14) | 1.82 ± 0.07<br>(1.77–1.86) | 5.15 ± 1.97<br>(3.07–7.64) | 6.03 ± 1.15<br>(5.22–6.84) |
| HW | 0.89 | 0.95 | 1.01 ± 0.18<br>(0.83–1.19) | 1.05 | 1.36 ± 0.01<br>(1.35–1.37) | 1.68 | 1.99 ± 0.16<br>(1.78–2.24) | 2.22 ± 0.01<br>(2.21–2.22) | 5.54 ± 2.37<br>(2.8–8.36) | 8.84 ± 0.04<br>(8.81–8.87) |
| HH | 0.66 | 0.94 | 0.92 ± 0.06<br>(0.86–0.98) | 1.16 | 1.21 ± 0.01<br>(1.2–1.22) | 1.62 | 1.5 ± 0.22<br>(1.13–1.82) | 1.65 ± 0.1<br>(1.58–1.72) | 2.75 ± 1.06<br>(1.37–4.03) | 4.65 ± 0.41<br>(4.36–4.94) |
| IoD | 0.77 | 0.75 | 0.88 ± 0.12<br>(0.76–0.99) | 1.14 | 1.09 ± 0.03<br>(1.07–1.11) | 1.34 | 1.62 ± 0.09<br>(1.46–1.79) | 1.81 ± 0.07<br>(1.76–1.86) | 3.62 ± 1.62<br>(1.75–5.55) | 5.67 ± 0.51<br>(5.31–6.03) |
| InD | – | – | – | – | 0.43 | 0.57 | 0.78 ± 0.18<br>(0.57–1.28) | 0.76 ± 0.06<br>(0.72–0.8) | 1.69 ± 0.76<br>(0.76–2.61) | 2.25 ± 0.48<br>(1.91–2.59) |
| EnD | – | – | – | – | 0.38 | 0.44 | 0.68 ± 0.08<br>(0.53–0.78) | 0.84 ± 0.01<br>(0.83–0.85) | 1.63 ± 0.68<br>(0.9–2.42) | 2.2 ± 0.56<br>(1.81–2.6) |
| SL | – | – | – | – | 0.32 | 0.47 | 0.78 ± 0.21<br>(0.51–1.4) | 1.03 ± 0.08<br>(0.97–1.09) | 2.18 ± 1.8<br>(0.44–4.14) | 3.21 ± 0.53<br>(2.84–3.59) |
| TH | 0.86 | 1.00 | 1.11 ± 0.03<br>(1.08–1.13) | 1.22 | 1.15 ± 0.08<br>(1.09–1.21) | 1.85 | 1.95 ± 0.32<br>(1.49–2.55) | 2.32 ± 0.23<br>(2.16–2.47) | 3.46 ± 1.75<br>(1.9–5.76) | 5.29 ± 1.32<br>(4.36–6.23) |
| TW | 0.75 | 0.69 | 0.77 ± 0.04<br>(0.73–0.8) | 0.74 | 0.65 ± 0.05<br>(0.61–0.68) | 0.87 | 1.04 ± 0.18<br>(0.81–1.44) | 1.1 ± 0.09<br>(1.04–1.16) | 2.57 ± 1.32<br>(1.05–4.08) | 3.28 ± 1.51<br>(2.21–4.35) |
| FlL | 0.16 | 0.36 | 0.43 ± 0.06<br>(0.37–0.48) | 0.00 | 0.73 ± 0.01<br>(0.73–0.73) | 0.82 | 1.65 ± 0.26<br>(1.2–2.24) | 2.37 ± 0.13<br>(2.28–2.47) | 6.84 ± 2.53<br>(3.98–9.97) | 10.04 ± 0.35<br>(9.79–10.29) |
| HlL | – | – | – | – | – | – | 0.66 ± 0.35<br>(0.24–1.41) | 1.41 ± 0.17<br>(1.29–1.54) | 6.4 ± 2.79<br>(3.69–9.69) | 9.63 ± 0.39<br>(9.35–9.91) |
| AD | 2.44 | 2.94 | 3.22 ± 0.48<br>(2.73–3.7) | 3.48 | 3.24 ± 0.01<br>(3.23–3.25) | 3.91 | 3.75 ± 0.43<br>(3.19–4.75) | 3.26 ± 0.36<br>(3.01–3.52) | 11.74 ± 5.2<br>(6.25–17.53) | 19.49 ± 0.31<br>(19.27–19.71) |
| egg D | 8.8 | 9.55 | 10.63 ± 0.81<br>(10.05–11.2) | 10.57 | 10.86 ± 0.14<br>(10.76–10.96) | 10.72 | 9.78 ± 0.77<br>(8.67–11.18) | 10.31 ± 0.65<br>(9.85–10.77) | –<br>– | –<br>– |

niles of *T. ziegleri* were found in the field, but at stage 45 with total lengths between 57.82–62.62 mm larvae still had not reached metamorphosis.

The detailed descriptions of the available larval stages of *T. ziegleri* are given in Table 2 and respective photographs in Fig. 6. For detailed morphological measurements of the larval stages see Table 3. The overall shape and pigmentation of the larvae remained similar through all stages: head wider than long (with the exception of stages 30 and 32); interocular distance bigger than internostril distance; eye-nostril distance very similar to internostril distance; width of tail base smaller than tail height; tail length smaller than snout-vent length. There was no evidence of balancers throughout the development.

**Coloration in life:** Body with golden yellowish-brown ground color; venter whitish-transparent. Fingers and toes yellow. Golden spots scattered on dorsal head and trunk, lateral body, tail fin and axilla to throat. Gills yellow with an orange-reddish hue at the edges and on the fimbriae. Ground color turned darker with age. Shortly after metamorphosis the skin was totally black except for yellow fingers, toes, and ventral ridge of tail. At this time the skin started to become less smooth and more granular and warty.

## Developmental biology of *T. vietnamensis*

***Eggs*:** The record of one egg directly after deposition had a diameter of 11.97 mm and 0.73 g of weight, while one

**Fig. 7.** Dark and light phenotypic variations of *Tylototriton vietnamensis* found at the type locality. *Photo M. Bernardes.*

egg ready to hatch measured 10.10 mm and weighted 0.56 g. Measurements from random eggs in the field showed an egg diameter ranging between 6.06–13.58 mm (mean 9.73 ± 1.61 mm, $n = 133$) and weight ranging from 0.19–1.15 g (mean 0.48 ± 0.21 g, $n = 133$). Eggs were transparent and clear shortly after egg deposition and later changed to brownish transparent.

***Body shape and size of hatched larvae*:** range of body length at hatching time was 15.59–17.85 mm (mean 17.04 ± 0.85 mm, $n = 5$). Dorsal fin well developed and higher than head, starting at the middle of trunk; ventral fin shorter than dorsal fin; body long and slender; snout short and flat; gills well developed. At stage 33 two fingers were visible in the forelimb and the hind limb bud was already visible. Toes, fingers, and joints were fully developed at stage 41. At stage 44 gills started to atrophy. Efts started to move to land at a size of 44.15 mm with 0.6 g of weight.

***Coloration in life*:** ground color light yellowish ochre; dark pigmentation on dorsal flanks, tail and head; venter slightly transparent to creamy white with no pigmentation; yellow spots scattered on dorsal side of head, body and tail; fingers and toes transparent to yellowish; gills light orange; eyes golden with black pupils. Pigmentation got darker with age turning black shortly before metamorphosis; toes and finger tips remained yellow as well as ventral ridge of tail. However, during field work at the type locality of this species we came across slight phenotypic variations, where larvae were also totally white at older stages (Fig. 7).

***Comparison with T. ziegleri*:** The diameter of the gelatinous layer of the egg was bigger in *T. vietnamensis*, as well as sizes of hatchlings. However, the estimated size at metamorphosis is likely bigger in *T. ziegleri*. The development and body shape of larvae of *T. vietnamensis* were very similar to *T. ziegleri*, with the exception that in *T. vietnamensis* the body is more slender and elongated and the gills more orange than reddish.

**Comparisons between the development of *T. ziegleri* and its congeners.** *Tylototriton ziegleri* showed terrestrial oviposition, while *T. taliangensis* and *T.* cf. *shanjing* showed aquatic oviposition and *T. kweichowensis* and *T. himalayanus* showed both. In *T. podichthys* and *T. panhai* eggs were laid adhered to vegetation, while in *T. ziegleri* eggs were oviposited on the ground. One exceptional clutch of *T. ziegleri* showed eggs in small aggregations, like in *T. podichthys. T. ziegleri* had similar clutch sizes compared with *T. hainanensis*, but they were smaller than clutch sizes of *T. kweichowensis* and *T. taliangensis* and bigger than those of *T. vietnamensis*, *T asperrimus*, *T. wenxianensis,* and *T. himalayanus*. Eggs of *T. ziegleri* were transparent in coloration when young and turned to yellow-brownish when older, like in *T. vietnamensis*, while in *T. himalayanus* eggs were greenish-yellow in color. The comparison between sizes of ovae showed larger diameters for *T. ziegleri* in relation to *T. kweichowensis*, *T. asperrimus,* and *T. podichthys*. In relation to the diameter of the gelatinous layer, *T. ziegleri* had similar diameters to those of *T. liuyangensis* and *T. wenxianensis*, which were bigger than those of *T. taliangensis, T. kweichowensis,* and *T. podichthys,* and smaller than those of *T. asperrimus*. *T. vietnamensis, T.* cf. *shanjing,* and *T. himalayanus* showed a wider range of egg diameter, both bigger and smaller than those of *T. ziegleri*. Furthermore egg size was related to clutch size, as species with smaller eggs had bigger clutches and vice-versa ($y = -29.68x + 313.64$; $F_{1,5} = 66.85$, $P < 0.001$; $r^2 = 91.7\%$). At hatching time *T. vietnamensis* had the largest larvae, followed in size by larvae of *T. ziegleri*, *T. kweichowensis,* and lastly by *T. himalayanus*. Size at metamorphosis seemed the smallest for *T. shanorum* and *T. vietnamensis*, followed by *T.* cf. *shanjing*, *T. kweichowensis*, *T. broadoridgus*, and *T. himalayanus*, while in comparison *T. uyenoi* and *T. taliangensis* had the largest sizes at metamorphosis (Table 4).

Generally, the larvae of *T. ziegleri* can be distinguished from the described larvae of the genus *Tylototriton* by having: 1) a broad head (longer in *T.* cf. *shanjing*); 2) the interorbital distance wider than internostril distance (similar distances in *T.* cf. *shanjing*); 3) a pointed tail tip (round in *T. uyenoi*, *T. taliangensis*, *T.* cf. *shanjing,* and *T. liuyangensis*); 4) the absence of balancers (versus present in *T. uyenoi* and *T.* cf. *shanjing*); 5) dorsal fin higher than ventral fin (almost identical height in *T. liuyangensis*); 6) tail shorter than SVL (tail longer than SVL in *T. himalayanus*); 7) reddish gills (versus orange in *T. vietnamensis*); 8) advanced larval stages with dark ground color with the exception of yellow digits and ventral fin (versus orange digits and fin in *T. broadoridgus*; yellow at head,

**Table 4.** Reproduction data of *Tylototriton* representatives (after Bourret 1942; Khatiwada et al. 2015; Kuzmin et al. 1994; Mudrack 2005; Nishikawa et al. 2013a, 2014, 2015; Phimmachak et al. 2015; Shen et al. 2012; Sparreboom 2014; Yang et al. 2014; Zhao 1988; Ziegler et al. 2008; and after own data for *T. vietnamensis* in comparison with *T. ziegleri*). * – based on the description of a single larva; ** – based on the description of two juveniles. All measurements in mm. For abbreviations see Material and Methods.

| | Clutch size (Egg nrs.) | Oviposition site | Jelly layer diameter | Ovum diameter | TL hatchling | Body shape and size of larvae | Larvae coloration | Differences to *T. ziegleri* |
|---|---|---|---|---|---|---|---|---|
| **Subgenus *Yaotriton*:** | | | | | | | | |
| *T. asperrimus* | 30–52 | – | 10 | 3.0–3.4 | – | – | – | bigger diameter of gelatinous layer; ovae diameter and clutch size smaller |
| *T. broadoridgus* | – | – | – | – | – | gills start to atrophy at 62 mm TL | orange coloration on digits and ventral fin | yellow coloration on digits and ventral fin in *T. ziegleri* |
| *T. hainanensis* | 58–90 | – | – | – | – | – | – | similar clutch sizes |
| *T. kweichowensis* | 121–141 | in water, on moist soil or under large stones nearby water | 6.2–6.5 | 2.3–3.4 | 12 | ~ 62 mm TL at metamorphosis | – | bigger clutch size; diameter of gelatinous layer, ovae and hatchling size smaller, in *T. ziegleri* only terrestrial oviposition |
| *T. liuyangensis* | – | on land | 7.8–8.1 | – | – | – | dorsal and ventral fins almost identical in height; tail tip rounded | dorsal fin higher and tail tip pointed in *T. ziegleri* |
| *T. panhai* | – | adhered to sticks slightly above the water surface | – | – | – | – | – | egg deposition terrestrial, mostly on rock substrate in *T. ziegleri* |
| *T. podichthys* | – | adhered to vegetation (individually or in groups of up to three) | 5.0 ± 0.3 | 2.9 ± 0.2 | – | – | – | eggs not adhered to vegetation and egg aggregations only in one exceptional case in *T. ziegleri*; gelatinous layer and ovum diameter larger |
| *T. vietnamensis* | 5–85 | on land | 6.06–13.58 | – | 15.59–17.85 | 44.15 mm at metamorphosis; slender and elongated body | orange colored gills | smaller clutch sizes; diameter of gelatinous layer and hatchling sizes bigger; body more slender and elongated; estimated TL at metamorphosis for *T. ziegleri* bigger; gills more reddish than orange in *T. ziegleri*. |
| *T. wenxianensis* | 56–81 | on land or in transition to water | 7–8 | 3 | – | – | – | clutch size slightly smaller |

parotids, vertebral ridge, rib nodules, limbs and tail in *T. uyenoi* and *T. shanorum*; brighter coloration laterally in the rib area in *T.* cf. *shanjing*); and 9) being less slender than larvae of *T. vietnamensis.*

## Discussion

*Tylototriton ziegleri* occurred at elevations congruent with the data provided by Nishikawa et al. (2013b). Occurrences at higher elevations were also found in Cao Bang Province, but not as high as the 1,600 m above sea level reported by Sparreboom (2011) at Mt. Pia Oac in Nguyen Binh district, Cao Bang Province. *Tylototriton vietnamensis* was always found at lower elevations, however, the population from Lang Son Province at 980 m above sea level was found higher than previous records for this species, setting a new elevational record.

The breeding season of *T. ziegleri* in northern Vietnam was previously thought to last from April to May (Nishikawa et al. 2013b); based on our new findings this period lasts longer, from April to July. Likewise, the breeding season of *T. vietnamensis* was recorded to last from June to July (Böhme et al. 2010), but our records show that it starts already in April.

We present for *T. ziegleri* a broader range for the distance of clutches to water with 10–100 cm instead of the 50–60 cm reported by Nishikawa et al. (2013b). The average amount of eggs in a clutch unable to produce viable offspring is still unknown, but might strongly increase by mycosis infection, as observed in one clutch

**Table 4 (continued).** Reproduction data of *Tylototriton* representatives (after Bourret 1942; Khatiwada et al. 2015; Kuzmin et al. 1994; Mudrack 2005; Nishikawa et al. 2013a, 2014, 2015; Phimmachak et al. 2015; Shen et al. 2012; Sparreboom 2014; Yang et al. 2014; Zhao 1988; Ziegler et al. 2008; and after own data for *T. vietnamensis* in comparison with *T. ziegleri*). * – based on the description of a single larva; ** – based on the description of two juveniles. All measurements in mm. For abbreviations see Material and Methods.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Subgenus *Tylototriton:*** | | | | | | | | |
| *T. anguliceps* * | – | – | – | – | – | dorsal fin higher than ventral fin; tail tip pointed | fingers and toes yellow | very similar body shape and coloration of larvae |
| *T. himalayanus* | 26–60 | in water or on land | 6–10 (greenish-yellow in color) | – | 10.9 ± 0.62 | TaL ≤ SVL; 62.5 ± 2.67 mm at metamorphosis | – | clutch size smaller; wider range of egg diameter; smaller hatchlings; in *T. ziegleri* only terrestrial oviposition, eggs transparent and later brownish, and SVL > TaL |
| *T.* cf. *shanjing* | – | in water (e.g., on submerged vegetation) | 6–10 | – | – | HL > HW; IoD > EnD; InD ~ IoD; TaL ≤ SVL; rounded tail tip; presence of balancers; ~ 52 mm at metamorphosis | lack of dark pigmentation in the rib area, which in older larvae might generate the colorful flank tubercles | wider range of egg diameter; larvae with presence of balancers and lighter ground color; in *T. ziegleri* terrestrial oviposition, HW > HL and tail tip pointed |
| *T. shanorum* ** | – | – | – | – | – | 34 and 43 mm (juveniles) | bright yellow coloration on dorsal head, lips, parotids, vertebral ridges, rib nodules, limbs, vent region and whole tail | estimated TL at metamorphosis bigger for *T. ziegleri*; juveniles of *T. ziegleri* completely black except for yellow coloration on fingers and tail fin |
| *T. taliangensis* | 250–280 | in water (individually on water plants) | 2–2.2 | – | – | dorsal and ventral fins almost in parallel; tail tip rounded; larvae overwinter and metamorphose the following year with 59–72 mm sizes | – | oviposition terrestrial in *T. ziegleri*; egg diameter smaller; clutch size bigger; tail tip pointed and dorsal and ventral fins not parallel in *T. ziegleri* |
| *T. uyenoi* * | – | – | – | – | – | presence of balancers in early stages; tail tip round | anterior head, parotids, vertebral ridge, rib nodules, limbs and tail yellow in advanced larval stages | absence of balancers, tail tip pointed and yellow coloration only on fingers and tail fin in *T. ziegleri* |

in Bao Lac district.

Aquatic breeding sites with alkaline pH values and higher carbonate hardness (Quang Ninh, Cao Bang and Ha Giang provinces) were associated with the geological substrate of the areas, mainly limestone rock (Sterling et al. 2006). These karst limestone areas are characterized by thin layers of surface soils, periods of severe soil dryness due to quick drainage of water and erosion of subsurface rock material, resulting in extensive cave systems with underground streams. Firstly, this explains the significantly deeper ponds found in habitats of *T. ziegleri* and secondly the deficiency of soil, leaving the parent rock exposed and explaining the choice of substrate for oviposition. *Tylototriton vietnamensis* on the other hand occurs on soils with granite parent rock material (Bernardes et al. 2017) which are more acidic and have low cation exchange capacity (Ulrich 1991). Ponds inhabited by *T. ziegleri* had higher nitrite and nitrate concentrations than ponds inhabited by *T. vietnamensis*. High levels of these nutrients have usually an anthropogenic origin, like leaching of nitrogen from manure and mineral fertilizers from upstream villages or agricultural fields. High concentrations of nitrate and nitrite can have negative effects on aquatic amphibian larvae, although there are interspecific differences in species’ sensitivity (Marco et al. 1999), which at this point do not seem to negatively affect the investigated species.

The additional finger found in one individual at stage 44 can have numerous causes, as malformations in amphibians have not yet been fully investigated (Blaustein and Johnson 2003). Polydactyly was, for example, associated with ultraviolet radiation (Ankley et al. 2000, 2002), chemical contamination (Burkhart et al. 1998; Gardiner and Hoppe 1999), and parasitic infection (Johnson et al. 1999; Sessions and Ruth 1990). However, developmental abnormalities found in amphibian populations have typical frequencies of 0–3% and are only considered abnormally high when exceeding 5% (Piha et al. 2006). Although field observations revealed that this type of malformation is quite common among adults of *Tylototriton* in Vietnam (M. Bernardes, pers. obs.), more studies have to be conducted to conclude whether these abnormalities are above natural levels.

The diagnostic characteristics of the different developmental stages in *T. ziegleri* corresponded well to the staging system adopted by Grosse (1997, 2013). Noticeable differences concern the earlier development of hind limbs (at stage 36 hind limbs had already developed three toes, while according to Grosse the hand development is finished before hind limb buds occur) and the absence of balancers. Balancers are usually present in salamander larvae that develop in lentic habitats sustaining the hypotheses that these structures are adaptive to still waters and non-functional in flowing waters (Crawford and Wake 1998). This theory does not seem to be verified in *T. vietnamensis*, a species that breeds in lentic habitats, but could explain the absence of balancers in *T. ziegleri,* a species able to reproduce both in lentic as in lotic habitats.

The body length of hatchlings of *T. ziegleri* was on average 30% smaller than the 21–22 mm reported by Sparreboom et al. (2011). According to these authors the larvae completed their metamorphosis at sizes of 43–62 mm. In our collection the biggest larva had not completed metamorphosis at stage 45 with 60 mm total length. However, length at metamorphosis seems to be a variable feature in *Tylototriton* representatives and apparently also depends on parameters such as feeding regime, temperature, etc. Total lengths in eight metamorphlings of *T. vietnamensis* reared in captivity by F. Pasmans varied between 49.9 and 65.8 mm. Further, observations in captive reared *T. wenxianensis* larvae revealed large variation in the stages at which the larvae hatched and consequently also in the total length at hatching (F. Pasmans, pers. obs.).

The morphological similarity within the *T. asperrimus* group in terms of body shape and coloration of adults makes it especially difficult to tell species apart (Stuart et al. 2010). Morphological similarity is even higher in larvae as this study shows. The larval development of *T. ziegleri* is still not completely recorded, as several stages still are unknown. Nevertheless our data allow clear morphological comparisons of developmental stages within this genus. Egg capsule diameter seems to be larger in species with terrestrial oviposition compared to species with aquatic oviposition. We therefore hypothesize that eggs of species with terrestrial oviposition are characterized by an extra thick gelatinous layer to prevent exsiccation of the developing larva, and therefore this trait might be a good indicator for the species' behavior. *Tylototriton ziegleri* had the biggest clutches of all land-laying species and amongst the sub-genus *Yaotriton*, while clutches of *T. vietnamensis* are among the smallest. The wide ranges in clutch sizes seen in *T. vietnamensis* and *T.* cf. *shanjing* might be related to the big sample size in the first case and associated with hidden cryptic diversity in the second. These comparisons must be regarded with caution, since accumulating evidence suggests that the description of the larval development of *T.* cf. *shanjing* by Ziegler et al. (2008) was based on a species complex (e.g., Nishikawa et al. 2013a). More information on the larval development in the genus *Tylototriton* is certainly needed.

## Conclusion

*Tylototriton ziegleri* is a small-ranged species known only from four localities in the North of Vietnam, none of them within protected areas. The species is not yet listed in the IUCN Red List, although it is likely under threat of extinction. Like *T. vietnamensis* it is in demand for the international pet trade as well as the Chinese market (Rowley et al. 2016). Despite morphological conservatism in particular within larval stages, our data clearly confirm contrasting habitat requirements between these cryptic species, both in adults and larval stages. Therefore, our results provide useful guidance to establish proper captive conditions for these two species with strongly deviating breeding requirements. This is in particularly important as *Tylototriton* is known for its cryptic diversity, as it can be seen for example with what was in the past thought to be the single species *T. shanjing*. From this morphological cryptic group several species have been described, like *T. panhai* and *T. uyenoi* (Nishikawa, Khonsue, Pomchote, and Matsui, 2013), *T. anguliceps* (Le, Nguyen, Nishikawa, Nguyen, Pham, Matsui, Bernardes, and Nguyen, 2015), and *T. podichthys* (Phimmachak, Aowphol, and Stuart, 2015), while *T. v. pulcherrima* was considered to be conspecific (Nishikawa et al. 2013a). Meanwhile the *T. shanjing* complex is widely distributed in zoological gardens, but origin and specific identification is in most cases uncertain, as well as information about potential captive hybridization. It is virtually impossible to identify representatives of the *T. shanjing* group without a comprehensive genetic screening. This negative impact on proper conservation breeding measures is yet aggravated by the lack of information regarding origin, natural history data, and data on different habitat adaptations in the field.

Our study describes the different ecological adaptations to strongly contrasting environmental conditions of two morphologically similar species. We highlight the necessity to improve the knowledge on the natural history of the *Tylototriton* species, not only for enhanced *ex situ* measures (viz. husbandry and conservation breeding, see Ziegler et al. 2016), but also for *in situ* approaches, such as supporting the establishment of new reserves, or extending the area of existing ones, as the populations of *T. ziegleri* from Quan Ba and Bac Quang districts occur in the vicinity of protected areas (Bat Dai Son Nature Reserve in Ha Giang Province and Cham Chu Nature Reserve in Tuyen Quang Province, respectively) but are not included in one.

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