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Reproductive biology of *Tylototriton yangi* (Urodela: Salamandridae), with suggestions on its conservation

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***Abstract.*—Despite the long-term establishment and the species richness of the knobby newt genus *Tylototriton*, taxonomy of its members remained controversial, and little is known about the reproductive biology of its members, especially about their courtship behavior. Here we provide information on the reproductive biology of the Tiannan Knobby Newt, *T. yangi*, including the pre-spermatophore-deposition courtship behavior both in the field and in captivity, morphology of its eggs and larvae, and breeding habitat at the type locality. We compare different aspects of the reproductive biology interspecifically within the *T. verrucosus* group, and provide suggestions for future behavioral studies. In addition, with information about the reproductive biology of the species, we offer recommendations for its conservation accordingly.**

Keywords. Comparative ethology, courtship behavior, development, habitat, larvae morphology, sexual isolation

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Introduction

Although most biologists embrace the evolutionary species concept, wherein a species is defined as an independent evolutionary lineage, species delimitation can be difficult in practice using standard morphological and molecular approaches, especially for organisms with conservative morphologies and complex evolutionary histories (Sites and Marshall 2004; Marshall et al. 2006; Barley et al. 2013). The knobby newts of the genus *Tylototriton* Anderson, 1871 represent a classic example of such a challenging species-complex. Despite the establishment of the genus *Tylototriton* for more than a century, the species boundary of its type species, *Tylototriton verrucosus* Anderson, 1871, remains controversial to date, mostly due to the unsettled issue regarding its type specimens (Nussbaum et al. 1995; Chanda et al. 2000; Nishikawa et al. 2013, 2014; Phimmachak et al. 2015). As a consequence, species boundaries and taxonomic validity of remaining members of the *T. verrucosus* group remain unclear (Nishikawa et al. 2013, 2014; Phimmachak et al. 2015).

In contrast to the traditional morphological approach, ecological and ethological approaches, which examine reproductive ecology and courtship behavior, may provide additional evidence to delimit species boundaries and reveal insights into the evolutionary histories of organisms (Töpfer-Hofmann et al. 2000; Rundle and Nosil 2005; Marshall et al. 2006). In salamanders, courtship behavior patterns and pheromones used during courtship are known to be species-specific, and differences in courtship behavior and courtship chemicals can lead to sexual isolation among sympatric species, as well as among conspecific but allopatric populations (Verrell and Mabry 2003; Rissler and Apodaca 2007). Therefore, assessing behavioral differences during courtship among congeners of the genus *Tylototriton* may provide critical insights on its complex systematics and taxonomy.

However, much information on the reproductive biology, including courtship behavior, is lacking for many members of the genus *Tylototriton*, particularly species that were recently described (Nishikawa et al. 2015; Hernandez 2016). One such example is the Tiannan Knobby Newt, *Tylototriton yangi* (Hou, Li, Lv, 2012). First described by Hou et al. (2012) from the *T. verrucosus* group, limited detailed information was known regarding its typical habitat and reproductive ecology since its original description (Fei et al. 2012; Hernandez 2016).

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Fig. 1. Location of the study site (the type locality of *Tylototriton yangi*) at Gejiu, Honghe Prefecture, Yunnan Province, PR China. Numbered locations of potential breeding pools (abbreviated as PBP) are shown in yellow.

Understanding the reproductive biology of *T. yangi* in a comparative framework will facilitate future studies to investigate the evolution of reproductive biology of the genus. Furthermore, since the known distribution range of *T. yangi* overlaps greatly with that of major tin-mining sites in China, it is imperative that we understand its habitat requirements and reproductive biology so that effective conservation efforts can be developed and applied.

Here we provide detailed descriptions of the breeding habitats, pre-spermatophore courtship behavior both in the field and captivity, and morphology of eggs and larvae of the Tiannan Knobby Newt, *T. yangi*. In addition, we compare our descriptions to those available for other species in the *T. verrucosus* group, provide directions for future behavioral and ecological studies of the species group, and suggest conservation strategies.

Materials and Methods

Field observations

Field observations were conducted at the type locality of *T. yangi* in mixed plantations near Gejiu, Honghe Prefecture, southern Yunnan Province, from May 16th to May 18th, and from May 27th to May 28th 2014 (Fig. 1). Detailed locality information is not provided here to prevent potential poaching. Potential breeding pools (PBP) were located and surveyed twice during each day (first during the day, second from dusk until midnight). Plants and other animals around and within the PBPs were collected and photographed. These samples were later identified to species after fieldwork. Behavioral observations and recordings were made at night when the newts were active. Behavior patterns were recorded using a Nikon D7000 digital camera.

Observations in captivity

Five males and five females of *T. yangi* were collected from areas around Gejiu and Mengzi of Honghe Prefecture, Yunnan, China on May 28th. Collecting permits were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences, and animal care followed the Animal Welfare Protocol of Kunming Institute of Zoology, Chinese Academy of Sciences. Sexes were separated and housed in same-sex groups in four 60 × 30 × 40 cm plastic containers with five cm of water and live aquatic plants. Newts were fed live bloodworms and were allowed to acclimate to the captive environment for four days prior to the staging of heterosexual encounters. For the heterosexual encounters, two trials, with two replications each, were conducted at different water depth to determine whether water depth influences courtship behavior. For the first trial, two active males and one of the largest females were placed in a circular plastic container (diameter one m) filled with 15 cm of water and observed at 1 a.m. on June 5th and again on June 6th. All interactions among individuals were observed for 60 minutes, and courtship behavior patterns were recorded using a Nikon D7000 digital camera. For the second trial, the same animals were placed into the same plastic containers with only five cm of water observated at 1 a.m.

Fig. 2. Habitat in which *Tylototriton yangi* was found at the type locality of Gejiu. Examples of typical breeding pools are shown at the right corner (from left to right, PBP#17 and PBP#12), and positions of other pools are indicated by white arrows. *Photographs by Kai WANG.*

on both June 5th and June 6th. Pre-spermatophore deposition courtship behavior patterns were recorded using the same equipment as in the first trial. After the observation sessions, all adults were released back to the wild.

Eggs and larval morphology

Embryos produced by females in captivity were maintained until hatching. Larvae were fed with live bloodworms and housed in five plastic containers. Photographs were taken at different developmental stages until larvae completed metamorphosis. Juveniles were kept for one week after metamorphosis and then released into the wild at the type locality.

Results

Breeding habitat

The dominant habitat type was secondary mixed forest with scattered water sources. Seventeen potential breeding pools were located around a reservoir, including one natural pool along a stream (potential breeding pool number 5, abbreviated as PBP#5) and sixteen artificial irrigation pools for agriculture (PBP#1–4, PBP#6–17) (Fig. 1). The irrigation pools were scattered along the forest edge in mix-crop plantations, and most pools were shallow (water depth from 5–30 cm, the deepest one, PBP#14, 90 cm) with aquatic vegetation. Shores of the pools consisted of either rocky walls with crevices or dense terrestrial vegetation (Fig. 2). No newts were found in the reservoir, moving streams, or pools that were connected to streams (PBP#5). In addition, no newts were found in the mining sediment pools or pools close to the tin mining site (PBP#2). These same habitats were occupied by other amphibian species, including *Aquixalus* sp., *Dianrana pleuraden*, *Duttaphrynus melanostictus*, and *Kaloula verrucosa*. In addition, loaches (*Misgurnus anguillicaudatus*) were found in some pools (PBP#14, 16, and 17).

Field behavioral observations

Six males and one female of *T. yangi* were observed after dusk from 20.00h May 17th to 01.00h the next day, in which all males were found at the bottom of irrigation pools of plantations (one in PBP#11, one in PBP#12, and four in PBP#13), while a female was found crossing the newly plowed plantation not far from pool #13. No behavior patterns that might be interpreted as territorial or aggressive (such as biting or chasing) were observed among males in pool #13; and interactions were limited to nudging (and perhaps sniffing) one another's snouts and bodies. After placing the female into pool #13, the closest male soon approached her and made several brief contacts with his snout to her head. He then moved forward to a position in front of the female, coiling his body into a "C"-shape and holding it next to his body. The female showed no interest and moved away (Fig. 3).

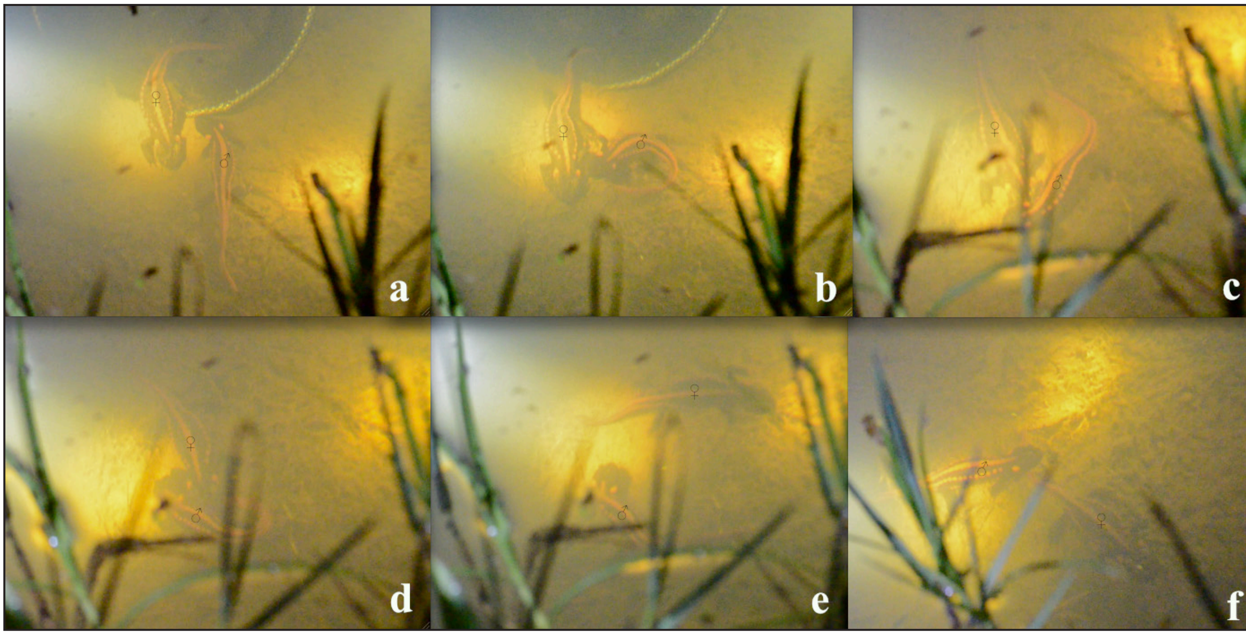

Fig. 3. Heterosexual encounters of *Tylototriton yangi* in the breeding pools near Yangjiatian Reservior, Gejiu, Yunnan Province, China. Clockwise from top left: **a)** male approaching a much larger female; **b)** male following the female; **c)** male coiling up and blocking female's path; **d)** male folding its tail toward the female; **e)** female swimming away; **f)** male following. *Photographs by Kai WANG.*

Another seven males were observed at night from May 27th to May 28th (two in pool#11, one in #13, and four in #14), all of which were on the substrate in water and not on land, and five larvae were found in pool #17.

Captive behavioral observations

As with all newts, sperm transfer in *Tylototriton* is accomplished by means of a spermatophore, placed on the substrate by the male and then is taken up into the cloaca of the female (Houck and Arnold 2003). Pre-spermatophore deposition courtship behavior patterns were identical to those observed in the field, and were the same for the two captive trials despite differences in water depth. Males were not observed to clasp females in amplexus. Here we provide an ethogram of the behavior patterns observed before spermatophore deposition in our two-males/one female trios (actual deposition was not observed) (Fig. 4).

(1) Swim away: the female turns or moves away from an approaching male.
(2) Nudging among males: males get distracted by other males' movements and nudge (sniff?) the head and lateral body of other males; but they quickly lose interest and move away from each other.
(3) Follow: the male rapidly moves after the female as she moves away from him.
(4) Approach: the males move toward the female when she is stationary.
(5) Male touch: the male makes repeated contacts with his head to the female's head, lateral body, especially her orange warts, and the lateral aspect of the proximal portion of her tail.
(6) Female nudge: with the pair in close proximity, the female turns her head toward the male and nudges (sniffs?) him with her snout.
(7) Male rub: the male repeatedly rubs his snout and cheek horizontally and laterally on the head and lateral aspect of the female's body, especially her orange warts.
(8) Tail tremble: the female trembles her tail when the male rubs her body with his cheek.
(9) Tail fan: the male moves forward and turns to place his body in front of the female. The male then curls the posterior part of his body and folds his tail inward in a "S"-shaped posture, with the tip of his tail is close to its base. He then rapidly undulates or fans the distal portion of his tail laterally in a fluid movement toward the female for 3–4s.

Eggs and larval morphology

Eggs were laid individually, not adhered to plants, on the floor of the container, or to one another, even though alternative oviposition materials were available in the containers. The animal pole was dark and the vegetal pole was white (Fig. 5a), and cleavage was observed in most embryos about 24 hours after their initial discovery. Since different sexes were kept separately except during the heterosexual encounter trials, and no actual mating occurred during the heterosexual encounters, females must have mated and so acquired sperm in the field prior to capture. At room temperature (20–25 °C), the hatchling period was 15 days.

Newly hatched larvae were between 10–12 mm in total length with large eyes; one pair of balancers was present on the lower aspect of the sides of the head; small

Fig. 4. Pre-spermatophore courtship behavior pattern of *Tylototriton yangi* in captivity. Clockwise from top-left: **a)** male nudging the side of the female's head with his snout; **b)** male nudging the side of the female's body; **c)** male blocking female's path and beginning to fold his tail; and **d)** male fanning the tip of his tail toward the female's head. *Photographs by Kai WANG.*

forelimb buds were present with very indistinctive toes; individuals had large abdominal yolk sacs; three pairs of gills were present, all of which were well-developed and were the same length as the head; tail fins were relatively deep (dorsal fin began from anterior part of the body, which runs for about three-fourths of the total length; ventral fin began from the posterior edge of the yolk sac, which runs about one-third of the total length). The dorsal surface of the body was yellowish brown and speckled with small dark dots, which formed two lateral bands running along the dorsal midline as well as the mid-lateral line. Speckled patterns also occurred on the tail fins. The gills were light pink and somewhat translucent, and the yolk sac was bright yellow with very few speckled patterns on the upper edges (Fig. 5b).

About five days after hatching, three toes showed on the distal end of the forelimbs and the tail fins were more developed (Fig. 5c). Through the development, the coloration of the larvae got darker, and the gills and the tail fin continued to grow. Later-stage larvae were brownish yellow with dark speckled patterns, possessed high tail fins and long gills, which were also speckled (Fig. 5d). Older pre-metamorphic larvae began to show some adult morphology, in which the head was less pointed, dorsal coloration became dark brown with developing light-colored patches along dorsolateral line, and the tail fins and gills were less translucent (Fig. 5e). Right before metamorphosis, larvae resembled adults in morphology: coloration became black, the head broadened and showed some trace of ridges, mid-dorsal orange ridge started to show, and a series of small orange warts became distinct dorsolaterally (Fig. 5f). Gills eventually disappeared, and the metamorphosis was completed in approximately 115 days (Fig. 5g).

Discussion

Review of courtship behaviors of Tylototriton verrucosus group

Significant differences in pre-spermatophore-deposition courtship behavior have been reported among different populations of *Tylototriton verrucosus sensu lato* from India (Roy and Mushahidunnabi 2001; Deuti and Hedge 2007), upper Myanmar (Boulenger 1920), southwest China (unpubl. data), and from the pet-trade with unknown locality (Sparreboom 2014). For the Indian populations, Roy and Mushahidunnabi (2001) reported that individual newts display extensive nose rubbing, tail fanning, and ventral amplexus (the male clasps the female's forelimbs with his forelimbs, with his dorsal side facing her ventral side). Similar amplexus behavior was also observed for the upper Myanmar population (Boulenger 1920). However, Sparreboom (1999, 2014) reported only tail fanning behavior in *T.* cf. *verrucosus* for pet-trade individuals from an unknown locality, and he did not observe extensive nose rubbing or ventral amplexus. For the topotypic individuals of *T. verru-*

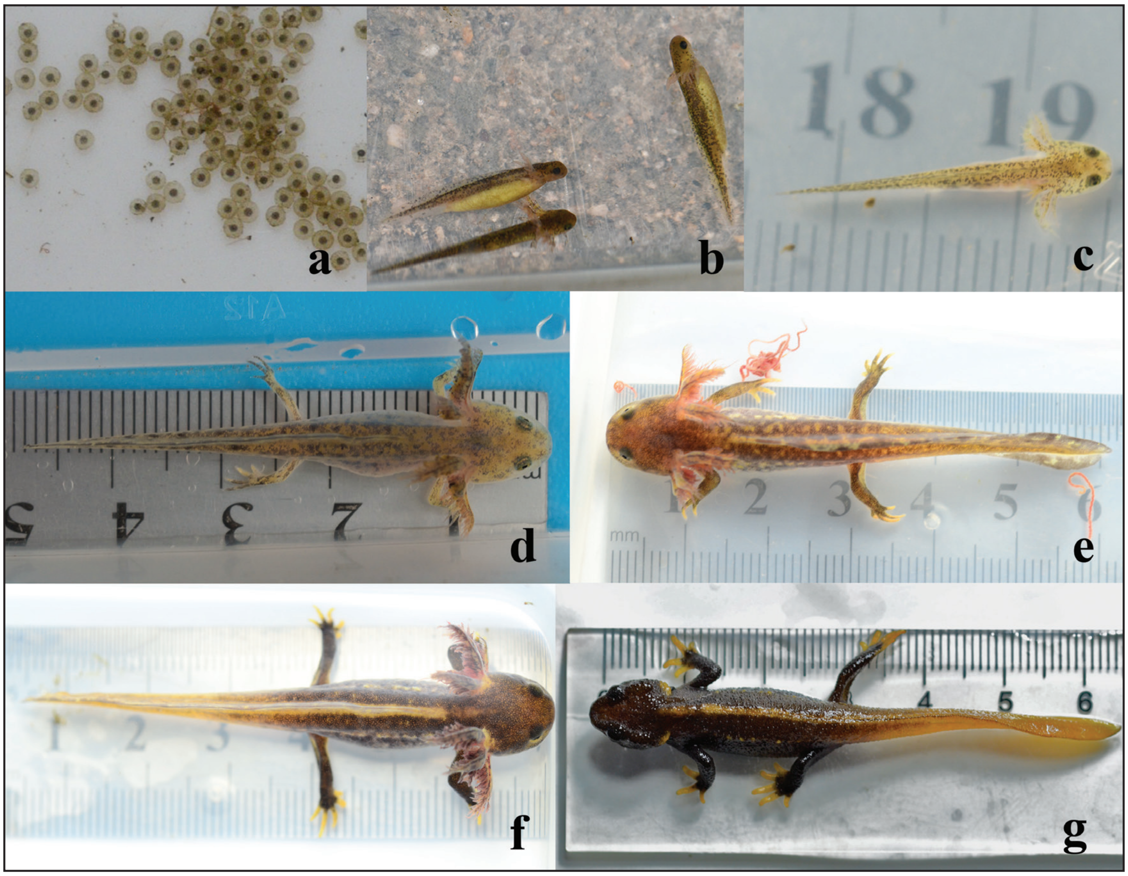

Fig. 5. Developmental series from fertilized embryos to newly metamorphosed juvenile of *Tylototriton yangi*. Clockwise from the upper left: **a)** fertilized embryos of *T. yangi*; embryos sank to the bottom of water, and were not adhesive to plants, the bottom of the container, or to one another; **b)** newly hatched larvae with one pair of balancers 6-day post-hatch; **c)** larva 17-days post-hatch, in which the forelimbs became visible; **d)** larva 50-days post-hatch; **e)** larva 75-days post-hatch; **f)** pre-metamorphic larva 95-days post hatch; **g)** newly metamorphosed individual 115-day post hatch. *Photographs by Kai WANG and Guangyu LI.*

cosus from southwestern Yunnan Province, China, Yuan observed nose-rubbing and tail-fanning behavior, but not ventral amplexus (unpubl. data).

Recently, several new species have been described from the *T. verrucosus* complex, including *T. himalayanus* from Nepal (Khatiwada et al. 2015) and *T. shanorum* from northern Myanmar (Nishikawa et al. 2014). Given the close geographic distance between the type localities of the two newly described species and the localities of previously identified *T.* cf. *verrucosus* populations with different courtship behaviors from India and Myanmar (Boulenger 1920; Roy and Mushahidunnabi 2001), differences in courtship behavior among these two populations may represent differential behaviors of *T. himalayanus* and *T. shanorum* respectively, and ventral amplexus may be a characteristic behavioral pattern that differentiates *T. himalayanus* and *T. shanorum* from *T. verrucosus sensu stricto*.

In contrast, Hernandez (2016) reported ventral amplexus during courtship in *T. verrucosus sensu stricto*. However, the reference Hernandez cited describes courtship behavior of *T. verrucosus* populations from Thailand (Humphrey and Bain 1990), which, based on Hernandez's book, are now considered as *T. uyenoi* Nishikawa, Khonsue, Pomchote, Matsui 2013, instead of *T. verrucosus sensu stricto*. Furthermore, the photographic evidence of ventral amplexus of *T. verrucosus sensu stricto* that Hernandez (2016) reported is of pet-trade individuals in France with no known locality information; and based on the external morphology of the individuals in the photo, these individuals should be identified as *T. shanorum*, as Hernandez suggested in his own book. Therefore, we recommend that further behavioral studies are needed to confirm the courtship behavior of *T. verrucosus sensu stricto* using topotypic individuals of the species.

Comparative reproductive biology of Tylototriton yangi

Based on our results, the reproductive biology of *Tylototriton yangi* differs substantially from what is known for other species of the *T. verrucosus* group, especially in terms of courtship behavior and egg morphology (Table 1). The courtship behavior of *T. yangi* is most similar to those of Indian populations of *T.* cf. *verrucosus*, in which they all court in water, exhibit tail-fanning movements, and display extensive nudging and rubbing behaviors

Table 1. Differential reproductive biology of members of the *Tylototriton verrucosus* group. -: absent; +: present.

Species	Source	Courtship behavior displayed by males					Characteristics of eggs/ clutches	
		Sniffing	Nose-rubbing	Tail fanning	Ventral amplexus	Courtship site	Eggs singular or forming clusters	Adhesive layer of eggs
Tylototriton yangi	Present study	+	+	+	-	Aquatic	Singular	-
Tylototriton shanjing	Ziegler et al. 2008; Li et al. 2012	+	-	+	-	Mainly Terrestrial	Singular, sometimes small clusters	+
Tylototriton cf. *verrucosus*	Boulenger 1920; Roy and Mushahidunnabi 2001; Deuti and Hedge 2007; Sparreboom 2014	+	+	+	+	Aquatic	Singular, sometimes small clusters	+
Tylototriton kweichowensis	Hu 1994; Tian et al. 1998	+	-	+	+	Aquatic	Singular	-
Tylototriton taliangensis	Fleck 1997; Fei et al. 2006; pers. comm.	+	-	+	+	Aquatic	Singular	-

(Roy and Mushahidunnabi 2001). However, the Indian population of *T.* cf. *verrucosus* displays ventral amplexus during its courtship (Roy and Mushahidunnabi 2001), which was not observed in the courtship of *T. yangi* in our study. Compared to populations of *T.* cf. *verrucosus* from the pet-trade with unknown localities, *Tylototriton yangi* displays extensive nose rubbing and nudging (sniffing?) behavior prior to tail fanning, which were not observed in pet-trade *T.* cf. *verrucosus* (Sparreboom 1999, 2014). In addition to differences in courtship behavior, *Tylototriton yangi* also differs from all populations of *T. verrucosus sensu lato* in egg morphology, in which eggs of *T. yangi* do not possess an adhesive outer layer, whereas those of the latter are adhesive and attached to aquatic vegetation (Roy and Mushahidunnabi 2001; Deuti and Hedge 2007; Wang, pers. observ.).

For other species, *Tylototriton yangi* differs from *T. shanjing* by courtship site (aquatic vs. mainly terrestrial), showing extensive nudging (sniffing?) and nose-rubbing behavior, and non-adhesive, singular eggs (vs. adhesive eggs sometimes in small clutches) (Ziegier et al. 2008; Li et al. 2012), and from *T. kweichowensis*, *T. taliangensis*, and *T. pseudoverrucosus* by showing extensive nose rubbing behavior and absence of ventral amplexus (Hu 1994; Fleck 1997; Tian et al. 1998; Fei et al. 2006; Hernandez 2016).

In contrast, recently Hernandez (2016) reported ventral amplexus during courtship in *T. yangi*, without references or photographic evidence, and he noted males of the species would develop rugose nuptial pads on their forelimbs during the breeding season, as in the amplectant salamandrid *Pleurodeles*. However, such amplexus behavior and the development of nuptial pads during breeding season were not observed during our field or captive observations. Further study is needed to confirm the presence of amplexus behavior in *T. yangi*.

Importance of chemical communication in courtship of Tylototriton

In newts and salamanders, olfactory signals are involved in intersexual recognition both within and among species (Dawley 1984, 1986). The extensive snout nudging and rubbing behavior patterns that we observed in male *T. yangi* suggests that they may obtain olfactory information from females during courtship: nudging may be sniffing. It may be that glands on the heads and in the warts of these newts show sexual dimorphism in glandular products, enabling discrimination between the sexes. On the other hand, Li et al. (2012) suggested that *T. shanjing* did not show any sniffing or nudging behavior and seemed to rely on visual cues at the beginning stage of courtship. Given these apparent differences in cues used in recognition processes among *Tylototriton* species and examples of behavioral isolation through chemical recognition in desmognathine salamanders (Tilley et al. 1990; Verrell and Mabry 2000; Mabry and Verrell 2004), it is possible that behavioral isolation also is present among species in the genus *Tylototriton*. Further work is needed to determine whether these behavioral differences, occurring before spermatophore deposition and at a time when species recognition might be expected to occur, result in decreased successes of heterospecific encounters (Verrell and Mabry 2003). Continued work on systematics and reproductive biology will surely reveal more about pattern and process in the evolutionary history of the genus *Tylototriton* generally, and the *T. verrucosus* group specifically.

Conservation of Tylototriton yangi

Our field observations indicate that scattered permanent ponds and other permanent bodies of stationary water are used for reproduction by *T. yangi*. Not all available water

Figure 6. Habitat destruction of *Tylototriton yangi* in southern Yunnan Province, China. **a)** Coal mining site at Yangjie, Mengzi, Yunnan Province, China; **b)** illegal tin mining at the type locality of *T. yangi* in Gejiu, Yunnan, China; **c)** Deforestation and infrastructure constructions at the type locality of *T. yangi* in Gejiu, Yunnan, China. *Photographs by Kai WANG.*

sources were occupied by newts during the duration of this study (e.g., the reservoir, and PBP#10, PBP#15, and PBP#16), and some pools (e.g., PBP#13 and PBP#14) were used by more newts than the others. These differences in pool use may be due to ecological factors such as nearby canopy coverage, amount of aquatic vegetation, water depth, food availability, and predation risk. We found the most newts in deep pools (30–50 cm in depth) with no large aquatic predators (e.g., large fish), some but not dense aquatic vegetation and dense surrounding terrestrial vegetation. These may be key factors for breeding site selection by *T. yangi*. Further studies are needed to determine the details of factors that affect breeding-site selection.

Having a restricted range in southern Yunnan Province of China, *Tylototriton yangi* faces a number of serious anthropogenic challenges. Habitat loss, especially of breeding habitat, is the greatest threat to the species (Hernandez 2016). Heavy tin/coal mining and accompanying deforestation were observed at our field sites during this study. This contaminated remaining potential breeding ponds and split terrestrial habitats into fragmented patches (Fig. 6). In addition to the habitat loss, illegal collections are the second most serious threats to the persistence of local populations of *T. yangi*. Local people harvest breeding adults from May to July every year, which are then dried and sold for traditional medicines. In addition, individuals are collected and sold alive as exotic pets in the illegal pet-trade. In fact, *T. yangi*, which was confused with *T. kweichowensis*, was the most common species of *Tylototriton* sold in the U.S. market before the official importation ban of Asian newts (Rowley et al. 2016), and illegally collected animals have also reached European countries such as France, Germany, and Russia (Hernandez 2016).

Because of these anthropogenic challenges, we recommend increasing attention to the conservation of the endemic species, *Tylototriton yangi*. Specifically, we recommend: 1) adding *T. yangi* to the List of Endangered Species of China as a Class II nationally protected species; 2) increasing law enforcement of the Wildlife Protection Act of China during the breeding season of the species from May to August, especially increasing patrol frequency in the pet markets and traditional medicine markets in Mengzi and Gejiu of Honghe Prefecture, Yunnan, China, 3) conserving existing adult habitats, particularly at the type locality in Gejiu, through restoration of natural plant communities and construction of artificial breeding ponds; and 4) initiating captive-breeding programs in research institutions in China, giving hope for subsequent release of newts to augment natural populations. Lastly, following the recommendation by Fei et al. (2012) and IUCN assessment criteria (extent of occurrence estimated to be < 20,000 km^2, severely fragmented, and inferred continued decline in extent of occurrence and area of occupancy), we recommend the listing of *T.*

yangi as Vulnerable under IUCN assessment criteria.

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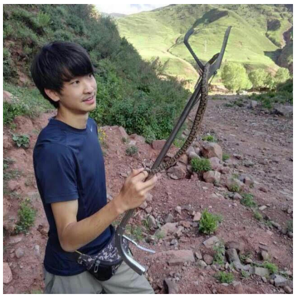

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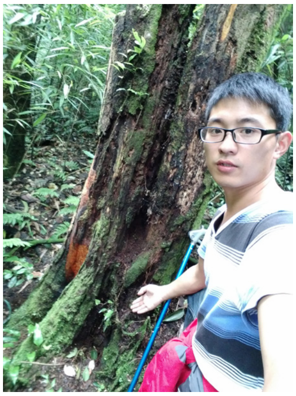

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