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Post-Ovipositional Developmental Stages of the Japanese Grass Lizard, *Takydromus tachydromoides* (Squamata: Lacertidae)

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Abstract: The Japanese grass lizard, *Takydromus tachydromoides*, is a species endemic to Japan. While several studies have investigated the reproductive ecology of *T. tachydromoides*, the embryogenesis of this species has not been reported in detail. In the current study, we observed the external morphological characteristics of 247 embryo specimens that developed under a constant temperature (28°C) throughout the duration from oviposition to hatching. We identified 17 consecutive developmental stages for the post-ovipositional developmental process of this species based on the staging criteria that have been widely used for the description of embryogenesis of lizards. The youngest embryos on the day of oviposition corresponded to stage 26, which is a relatively early period in pharyngula stages, and juveniles hatched at stage 42, approximately 30 days after oviposition. The entire developmental sequence of key morphological features was shared with other species of Lacertidae, except for the timing of the beginning of the first body pigmentation. This is the first description of the complete sequence of post-ovipositional developmental stages for the oviparous species of Lacertidae, providing valuable information for further evolutionary developmental studies.

Key words: Developmental stages; Embryo; Lacertidae; Lizard; Squamata

INTRODUCTION

Squamata emerged in the early evolutionary history of amniotes and show extreme diversity, with more than 10,000 species (Uetz et al., 2020) that show great ecological, morphological, and physiological diversity, and have adapted to a wide range of environments. However, little is known about the

mechanisms of squamate morphogenesis because not many evolutionary developmental studies have been conducted. For evolutionary developmental studies aiming to reveal these mechanisms, a description of basic embryogenesis is necessary.

In reptiles and birds embryos do not always develop at a uniform speed, even within the same species (Billett et al., 1985). For lizards in particular it is known that duration after fertilization and/or oviposition cannot be used as the index for the progress of embryogenesis even under controlled conditions,

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because the extension of egg retention within the oviduct frequently occurs (Shine, 1983). Therefore, common developmental staging criteria, defined by the development of external morphological characters, have been established for comparative embryology.

The embryonic developmental processes of external morphology in various species of lizards have been described (see Ollonen et al. [2018] and Alturk and Khannoon [2020], for a review). Many studies followed the staging criteria established by Dufaure and Hubert (1961), while Roscito and Rodrigues (2012), Werneburg et al. (2015), and Ollonen et al. (2018) followed another staging method proposed by Werneburg (2009). Further, Sanger et al. (2008) and Billy (1988) defined the original staging criteria. Noro et al. (2009) and Gregorovicova et al. (2012) described key morphological features of embryos post-oviposition.

For lacertids, Dufaure and Hubert (1961) described all the intrauterine developmental stages of viviparous *Zootoca vivipara* throughout the duration from very early embryogenesis to birth. Inukai (1927) also observed neurula stages of *Z. vivipara*. Three further studies investigated developmental processes for four oviparous lacertids, although several developmental stages of Dufaure and Hubert (1961) were not observed in them: Peter (1904) for the pre- and post-ovipositional development of *Lacerta agilis*; Dhouailly and Saxod (1974) for post-ovipositional developmental processes of *Lacerta viridis* and *Podarcis muralis*; Oka (1971) for the early period of the post-ovipositional developmental process of *Takydromus tachydromoides*. Thus, a description of the complete sequence of the developmental stages for lacertids is only available for *Z. vivipara*.

In the current study, we observed the post-ovipositional developmental process of Japanese grass lizard *T. tachydromoides* from oviposition to hatching. This species is endemic to Japan (Hokkaido, Honshu, Shikoku, Kyushu, and several neighboring

islands) and is one of the most common lizards in these areas. Several aspects of the reproductive ecology of this species have been investigated by the following studies: Inukai (1930) for egg gestation; Ishihara (1964) for oviposition and hatching; Telford (1969) for relationships between reproductive potential, and body size and age; Takenaka (1980) for body growth and sexual maturity of the female; Takenaka (1981) for relationships between body size and reproductive potential; Oka (1996) for the influence of incubation temperature on sex determination. Oka (1971) also observed the early period of post-ovipositional developmental stages (stages 25–30) of this species, particularly focusing on the morphogenesis of pharyngeal arches. However, the middle and late post-ovipositional developmental process of this species has not been characterised in detail. Thus, we described the sequence of post-ovipositional developmental stages of this species, following the widely accepted staging criteria of Dufaure and Hubert (1961), to provide basic information for further evolutionary developmental studies.

MATERIALS AND METHODS

We captured mature individuals of *T. tachydromoides* from Atsugi, Kanagawa, Japan, and bred them in captivity. One or two males and three to five females were kept in each cage. The breeding room was kept in a 12/12-h light/dark cycle at $27 \pm 3^\circ\text{C}$ throughout the breeding season. Mating was checked every morning by inspecting for the presence of a V-shaped mark on the body of females, which is left by a bite of the males during mating. Mating and egg-laying were observed from April to early August. Several females had already mated in the field at the point of capture and roughly one-fourth of the total number of embryos were obtained from eggs laid by them. Most females laid two or three clutches during the breeding season, with a maximum of four clutches. Eggs were usually laid in the morning, and the shell was leathery

and stretchable. The clutch size ranged from one to seven eggs, with a mean of 2.82 eggs. Collected eggs were incubated at 28°C in an incubator (Wakenyaku model 9200) until examined by referring to Ishihara (1964).

Embryos were examined in phosphate-buffered saline (PBS) and fixed with 10% formalin diluted with PBS. After fixation, the external morphological characteristics of embryos, which have been used for the establishment of the developmental staging criteria of various species of lizards, were observed under a dissection microscope (Leica MZ6). After fixation, morphological features of embryos at stages 26–34 were photomicrographed with a stereo microscope (Leica M205 FA) equipped with a digital camera (Leica DFC450 C). Morphological features of embryos at stages 35–38 and closer views of embryos at stages 39–42 were photomicrographed with a digital microscope (Keyence VHX-S550). Morphological features of the whole body of embryos at stages 39–42 were photographed with a single reflex digital camera (Nikon D7100).

For stage determination, we followed the staging criteria in Dufaure and Hubert (1961), referring to the comparison of developmental stages in Wise et al. (2009) and of the timing of development of key morphological features, that is characteristic for each stage in Lima et al. (2019) among species. We examined embryo specimens from at least five clutches for each developmental stage. All procedures using live animals were performed under the approval of the Animal Care and Use Committee of Tokyo University of Agriculture (approval numbers: 280121; 290137; 300104).

RESULTS

A total of 247 embryos of *T. tachydromoides* that ranged from 0 to 33 days post-oviposition were examined (Fig. 1). We identified 17 consecutive developmental stages that span stages 26 to 42 for the post-ovipositional developmental processes of this

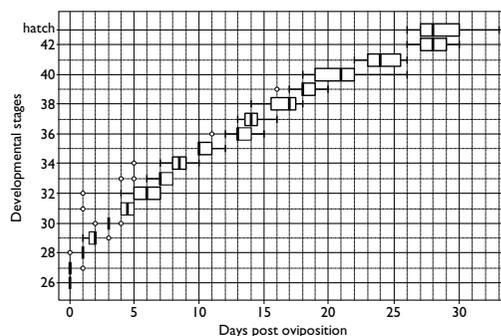


FIG. 1. Relationships between the incubation duration of eggs and developmental stages in *Takydromus tachydromoides*. Box plots show median, interquartile values, range, and outliers (white circles).

species, that are described below.

Stage 26 (Fig. 2A): The first and second pharyngeal clefts are distinct (1 and 2 in Fig. 2A). The prosencephalon, mesencephalon, and metencephalon are visible through transparency (pro, mes, and met in Fig. 2A). The neural tube is opening dorsally from the metencephalon to the level of the second pharyngeal cleft. The horseshoe-shaped optic cup (oc in Fig. 2A) and lens placode are visible. The choroid fissure is opening (cf in Fig. 2A). The otic vesicle is visible at the dorsal side to the second pharyngeal cleft (ov in Fig. 2A).

Stage 27 (Figs. 2B and C): Telencephalon and diencephalon begin to differentiate (tel and die in Fig. 2B). The primordium of the pineal gland protrudes on the dorsal side of the diencephalon (pg in Fig. 2B). The nasal pit appears (np in Fig. 2B). The primordial region of fore and hind limbs slightly protrude (fl and hl in Figs. 2B and C). The unsegmented tail bud begins to extend (tb in Fig. 2B).

Stage 28 (Fig. 2D): The first to third pharyngeal clefts are visible. Each region of the brain begins to bulge. The optic cup expands compared to the previous stage. Fore and hind limb buds laterally protrude (fl and hl in Fig. 2D). The unsegmented tail bud begins to curve.

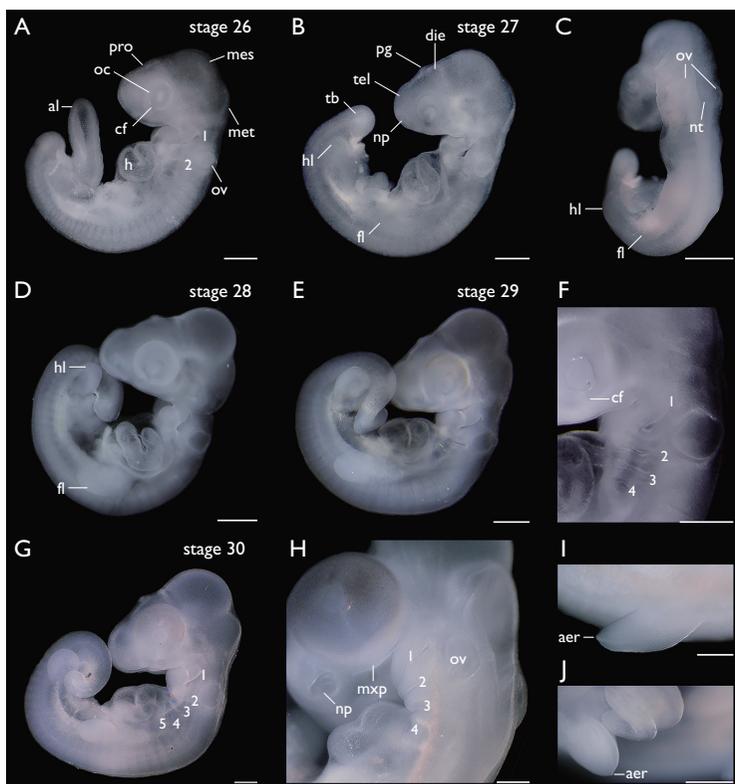


FIG. 2. Post-ovipositional development of *Takydromus tachydromoides*, at stages 26–30. Scale bars=0.5 mm. Lateral view of the whole embryo at stage 26 (A). Lateral (B) and dorsolateral (C) views of the embryo at stage 27. Lateral view of the whole embryo at stage 28 (D). Lateral view of the whole embryo (E) and a closer view of the pharyngeal arches at stage 29 (F). Lateral view of the whole embryo (G), ventrolateral view of the cranial and neck region (H), closer views of forelimb bud (I), and hindlimb bud (J) at stage 30. Abbreviations: aer, apical ectodermal ridge; al, allantois; cf, choroid fissure; die, diencephalon; fl, forelimb bud; h, heart; hl, hindlimb bud; mes, mesencephalon; met, metencephalon; mxp, maxillary process; np, nasal pit; nt, neural tube; oc, optic capsule; ov, otic vesicle; pg, pineal gland; pro, prosencephalon; tb, tail bud; tel, telencephalon; 1–5, pharyngeal clefts.

Stage 29 (Figs. 2E and F): The first to fourth pharyngeal clefts are distinct (1 to 4 in Fig. 2F). The margins of the choroid fissure contact each other, while they are not fused (cf in Fig. 2F). The dorsal region of the retina begins to pigment (Fig. 2E). The fore and hind limb buds develop into a stump shape. The tail bud elongates and begins to coil during this stage.

Stage 30 (Figs. 2G to J): First to fourth, or first to fifth pharyngeal clefts are visible (1 to 5 in Figs. 2G and H). The fifth cleft appears

and fuses during this stage. The choroid fissure closes. The location of the nasal pits moves to the ventral surface of the nasal prominences (np in Fig. 2H). The maxillary process begins to extend anteriorly (mxp in Fig. 2H). Apical ectodermal ridges are distinct on the fore and hind limb buds (aer in Figs. 2I and J).

Stage 31 (Figs. 3A and B): The fourth pharyngeal clefts fuse. The telencephalon divides into a left-and-right pair (tel in Fig. 3A). Pigmentation in the eye increases to the

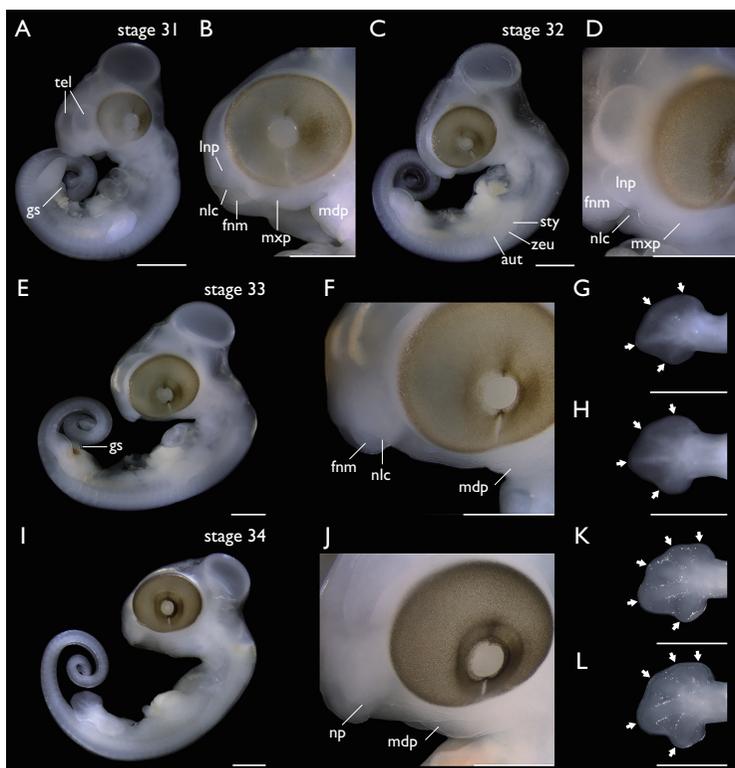


FIG. 3. Post-ovipositional development of *Takydromus tachydromoides*, at stages 31–34. Scale bars=1.0 mm. Lateral view of the whole embryo (A) and a closer view of facial primordia (B) at stage 31. Lateral view of the whole embryo (C) and a closer view of facial primordia (D) at stage 32. Lateral view of the whole embryo (E) and closer views of facial primordia (F), forelimb bud (G), and hindlimb bud (H) at stage 33. Lateral view of the whole embryo (I) and closer views of facial primordia (J), forelimb bud (K), and hindlimb bud (L) at stage 34. Arrows in G, H, K, and L indicate digital condensations. Abbreviations: aut, autopod; fnm, frontonasal mass; gs, genital swelling; lnp, lateral nasal prominence; mdp, mandibular process; mxp, maxillary process; nlc, nasolabial cleft; np, nasal pit; sty, stylopod; tel, telencephalon; zeu, zeugopod.

entire region. The nasolabial cleft is formed between the frontonasal prominence and the lateral nasal prominence (Fig. 3B). The maxillary process reaches the lateral nasal prominence. A paddle-shaped digital plate appears on the fore and hind limbs that slightly flex. A genital swelling is observed between hindlimb buds (gs in Fig. 3A).

Stage 32 (Figs. 3C and D): All pharyngeal clefts fuse. The iris begins to darkly pigment. The maxillary process extends further to the anterior level of the eye. The frontonasal mass and the lateral nasal prominence fuse at

the dorsal region of the nasolabial cleft (Fig. 3D). The mandibular process begins to extend anteriorly. The stylopod, zeugopod, and autopod are differentiated (Fig. 3C). The paddle-shaped digital plate increases in width.

Stage 33 (Figs. 3E to H): The neural tube almost closes and opens only at the most rostral opening at the level of the endolymphatic sac. The maxillary process fuses with the lateral nasal prominence (Fig. 3F). First, digital condensations appear on three to four digits (arrows in Figs. 3G and H). Paired genital swelling is distinct (gs in Fig. 3E).

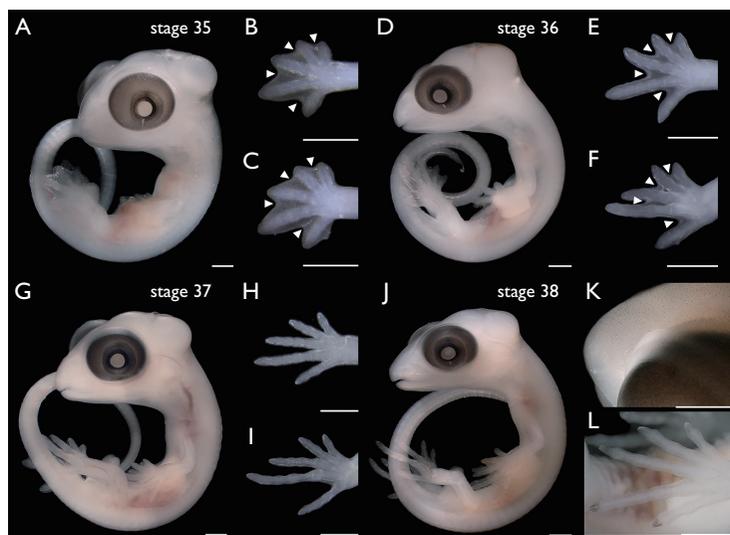


FIG. 4. Post-ovipositional development of *Takydromus tachydromoides*, at stages 35–38. Scale bars=1.0 mm. Lateral view of the whole embryo (A), closer views of the forelimb bud (B), and hindlimb bud (C) at stage 35. Lateral view of the whole embryo (D), closer views of the forelimb bud (E), and hindlimb bud (F) at stage 36. Lateral view of the whole embryo (G), closer views of the forelimb bud (H), and hindlimb bud (I) at stage 37. Lateral view of the whole embryo (J), closer views of the dorsal surface of the head (K), and forelimb bud (L) at stage 38. Arrowheads in B, C, E, and F indicate interdigital webbings.

Stage 34 (Figs. 3I to L): The neural tube closes. The outer margin of the iris is distinct (Fig. 3J). The upper eyelid begins to extend laterally. Three facial primordia fuse with each other, and only a slit of the nasal pit is left on the surface of the head (np in Fig. 3J). The mandibular process extends anteriorly to the level of the lens (mdp in Fig. 3J). Digital condensation is distinct for all five digits (arrows in Figs. 3K and L). Interdigital webbings begin to reduce.

Stage 35 (Figs. 4A to C): The circular edge of the pineal eye is pigmented. The lower eyelid appears. The lower jaw develops from the mandibular process and its anterior tip reaches the tip of the upper jaw. Digits begin to differentiate in size (Figs. 4B and C). The interdigital webbings reduce for releasing the tips of digits (arrowheads in Figs. 4B and C).

Stage 36 (Figs. 4D to F): Eyelids cover the edge of the eye. The auditory pit is observed as a slight depression. The interdigital webbings deeply reduce to approximately half

the length of the digits (arrowheads in Figs. 4E and F). Unpigmented scales appear on the tail and dorsal sides of the neck and trunk region. Ribs are faintly visible on the lateral sides of the trunk region.

Stage 37 (Figs. 4G to I): The internal edge of the eyelids becomes oval in shape. The interdigital webbings completely recede and claws are faintly visible on the tip of the digits (Figs. 4H and I). Unpigmented scales appear on the ventral side of the trunk and the dorsal side of the limbs.

Stage 38 (Figs. 4J to L): The auditory pit is distinctly depressed and the tympanic membrane is faintly visible. Tiny pigmented spots sparsely cover the dorsal surface of the head (Fig. 4K). Limbs further elongate and are clearly segmented (Fig. 4L). Pigmented spots appear on the incomplete claws (Fig. 4L).

Stage 39 (Fig. 5A): The formation of scales increases to the whole body, except for the dorsal surface of the head. The number of

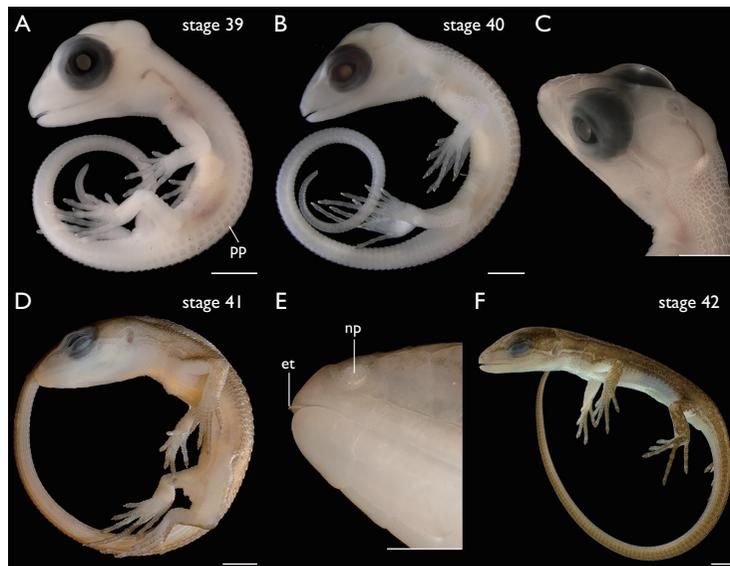


FIG. 5. Post-ovipositional development of *Takydromus tachydromoides*, at stages 39–42. Scale bars=2.0 mm. Lateral view of the whole embryo at stage 39 (A). Lateral view of the whole embryo (B) and a closer view of the dorsal surface of the head and neck region (C) at stage 40. Lateral view of the whole embryo (D) and a closer view of the snout region (E) at stage 41. Lateral view of the whole embryo at stage 42 (F). Abbreviations: et, egg tooth; np, nasal pit; pp, pigmentation pattern.

pigmented spots on the head increases. Scales on the dorsal surfaces of the neck and trunk begin to pigment to form the spotted pattern.

Stage 40 (Figs. 5B and C): Cranial large scales are formed and the pattern is observed (Fig. 5C). The pigmentation on scales of the dorsal side of the body and limbs increases.

Stage 41 (Figs. 5D and E): Eyelids are fully developed in size and can be closed. The nasal pits are still sealed (np in Fig. 5E). Scales throughout the body are deeply pigmented and now represent the pattern that corresponds to that of the neonatal animal. The hemipenis is withdrawn into the cloaca during this stage. A single egg tooth anteriorly protrudes on the tip of the upper jaw (et in Fig. 5E). The egg yolk is almost completely consumed.

Stage 42 (Fig. 5F): The nasal pit is opened. The egg yolk is completely consumed. The embryo is quite active, and no obvious external differences are observed compared to the hatched neonatal animal.

DISCUSSION

In the current study we examined embryos of *T. tachydromoides* from oviposition to hatching and identified 17 developmental stages (stages 26 to 42). There was no significant difference between developmental stages of embryos from eggs laid by females captured after mating and that from eggs laid by females captured before mating. Developmental stages among all embryos from the same clutch did not vary at any period of incubation, except for embryo specimens that represent obvious abnormal growth, similar to *Calotes versicolor* investigated in Muthukkaruppan et al. (1970). On the other hand, the developmental stages of embryos from different clutches were not always uniform, even though they were examined after the same duration of incubation (see Fig. 1).

As stated in Inukai (1930), the day of the hatching of *T. tachydromoides* differed by a

few days among neonatal individuals from the same clutch. The incubation at 28°C led to the highest embryonic viability for this species (Ishihara, 1964), and the mean duration from oviposition to hatching was 28.7 days (SD=2.36). While the optimum temperature of incubation differs among species, the average duration of hatching is approximately two months in many lizards (Werner, 1971; Dhouailly and Saxod, 1974; Flores et al., 1994; Thompson and Russell, 1999; Ji et al., 2002; Noro et al., 2009; Whiteley et al., 2017; Lima et al., 2019). On the other hand, in *Anolis* spp., the incubation duration is characteristically short among lizards and juveniles hatched after incubation of 22–27 days (Sanger et al., 2008). Thus, the incubation duration of *T. tachydromoides* is considerably shorter than that of many other lizards.

In most lizards, the embryos at oviposition correspond to stages 28–30 (Shine, 1983; Wise et al., 2009), which is characterized by the distinct protrusion of limb buds. Embryos that correspond to earlier stages are laid in *Paroedura picta* (Noro et al., 2009), *Salvator merianae* (Iungman et al., 2019), *Aspidoscelis uniparens* (Billy, 1988), *Anolis* spp. (Sanger et al., 2008), *Calotes versicolor* (Muthukkaruppan et al., 1970), *Furcifer lateralis* (Blanc, 1974), and several lacertids (see below). In the current study, we examined 26 embryos of *T. tachydromoides* from 10 clutches on the day of oviposition. Among them, 18 embryos, six embryos, and two embryos corresponded to stages 27, 26, and 28, respectively. The earliest stage was stage 26, similar to the results of observations of two lacertids, *L. viridis* and *P. muralis*, in Dhouailly and Saxod (1974). An embryo of *T. tachydromoides* examined just after the oviposition in Oka (1971) corresponded to stage 25. In *P. muralis*, *P. bocagei*, and *P. hispanica*, earlier developmental stages (stages 22–25) were observed in embryos at oviposition of wild individuals (Braña et al., 1991). On the other hand, embryos at the oviposition of the oviparous population of *Z. vivipara* correspond to stage 30 or later

(Braña et al., 1991; Rodríguez-Díaz and Braña, 2011). These results suggest that lacertids (with the possible exception of *Z. vivipara*) tend to lay embryos relatively earlier when compared to other lizards. The formation of limbs begins at stage 27, and many lacertids lay embryos at or before that stage. Therefore, during the post-ovipositional developmental process of lacertids, the morphogenesis of limbs can be observed from an early period. The earliest stages in the post-ovipositional developmental process of *T. tachydromoides* differed between the current study and Oka (1971). The duration of gestation and incubation of this species are influenced by environmental conditions, particularly temperature (Inukai, 1930). Thus, it is considered that the difference between these two observations was caused by external environmental conditions.

The developmental sequence of key morphological features for staging is basically constrained in each taxon of squamates, but is not necessarily uniform among taxa (Andrews et al., 2013). The entire developmental sequence in the embryogenesis of *T. tachydromoides* proceeded without apparent differences from other lacertids, except for the timing of the first body pigmentation. In *T. tachydromoides*, the first body pigmentation was observed at stage 38, after the complete reduction of interdigital webbings and the beginning of the formation of scales and claws. In *L. agilis*, this was observed in the embryo that corresponds to stage 36, in the period that scales begin to develop and interdigital webbings reduce (see the text and Fig. 32 in Peter [1904]). In *Z. vivipara*, this was observed at stage 35, before the beginning of the development of scales and claws (Dufaure and Hubert, 1961). It is known that the timing of the first body pigmentation is variable even among related families in Iguania (Rapp Py-Daniel et al., 2017). Meanwhile, in Lacertidae, the timing of the first body pigmentation is variable even among closely related species. In the developmental process of many lizards, the first body pigmentation occurs at around

stage 38, after the beginning of the formation of scales and claws and/or the reduction of interdigital webbings (Lima et al., 2019). Thus, it is considered that the timing of the first body pigmentation in *T. tachydromoides* is common for other lizards, while that in *Z. vivipara* is considerably earlier than in other lizards.

Our investigation presented the post-ovipositional developmental process of *T. tachydromoides* with the organization and description of the standard developmental stages of lizards. The following several characteristics of this species are considered suitable for further evolutionary developmental studies. This species is abundant in Japan and is easily bred in captivity. Multiple embryo specimens are available from a single clutch, and their developmental process proceeds at a constant speed. The post-ovipositional developmental process begins from a relatively early period of organogenesis, and the progress of the developmental process is considerably faster than that of other lizards. The basic information presented in the current study is expected to contribute to further evolutionary developmental studies using *T. tachydromoides*.

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