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Source: Current Herpetology, 41(2) : 196-204

Published By: The Herpetological Society of Japan

URL: <https://doi.org/10.5358/hsj.41.196>

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Seasonal Changes in Breeding Characters of Two Syntopic *Onychodactylus* Salamanders with Special Reference to Differentiation in Their Breeding Seasons

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Abstract: Hynobiid salamanders of the genus *Onychodactylus* are known to possess palmar and tarsal black tubercles or asperities during the breeding season, which were once considered male-specific characters used for spawning activity. However, such tubercles and asperities have also been observed in females and are not always developed in breeding individuals; thus, their functions remain unclear. In this study, we investigated the developmental process of the tubercles and asperities in two *Onychodactylus* species in the Chugoku Mountains. We found that these traits appear rapidly immediately before the start of spawning. Tubercles and asperities were also observed in females, in which they showed a similar temporal developmental pattern to that observed in males; thus, they may be adaptive traits for migration to underground spawning sites or for breeding activity at these sites. In the Chugoku Mountains, *O. kinnebur*i and *O. japonicus* are known to share breeding sites for spawning, but their reproductive isolation mechanism remains unknown. In the course of our survey, we found a temporal gap in breeding season between the two species, which may be involved in premating reproductive isolation.

Key words: *Onychodactylus*; Reproductive isolation; Secondary sexual characters; Sympatry

INTRODUCTION

The genus *Onychodactylus* is the most basal lineage within the family Hynobiidae (Pyron and Wiens, 2011); it includes ten nominal spe-

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cies that are endemic to Northeast Asia (Frost, 2021). Various studies, including osteology, anatomy, and developmental studies, have been conducted on *Onychodactylus* salamanders (e.g., Okajima, 1908, 1922; Iwasawa and Kera, 1980). Although the life history of *Onychodactylus* has been reported, our knowledge of its reproductive ecology remains limited because it spawns in subterranean waters near headstreams where observing the salamanders is difficult (Kudo, 1934; Okada, 1937; Sato, 1943; Akita, 1982; Totsune et al., 1995; Nanbu, 1996; Park, 2005; Yoshikawa et al., 2013; Yoshikawa and Matsui, 2014).

During the breeding season, horny black claws on the tips of digits are prominent in both sexes of adult *Onychodactylus* individuals; in addition, secondary sexual characters appear (i.e., in males, dermal skin folds appear on the posterior edge of hindlimbs and tail-fins develop); in some species, black asperities appear on the palms and soles (Sato, 1943; Yoshikawa et al., 2013; Yoshikawa and Matsui, 2014). The palmar and plantar black asperities were once considered unique characters of males that were used for clasping egg sacs during the fertilization process (Okada, 1937; Nakamura, 1941a; Sato, 1943). However, they have since been reported in females of an *O. japonicus* population in Ishikawa Prefecture (Akita, 1985) and of *O. kinneburi* (Yoshikawa et al., 2013). The black asperities are also known to appear with gonad maturation and disappear with its atrophy (Nakamura, 1941a), but they do not always appear in individuals gathering for breeding (Totsune et al., 1995; Akita, 2013). Furthermore, Yoshikawa et al. (2013) described the black asperities in terms of two different components: tubercles (wart-like structures) and asperities (uniform, thin, and slightly rough sheet-like structure that covers the areas of palm and sole where there are no tubercles). Documentation of the developmental process of tubercles and asperities from the field is currently lacking, and the functions of these characters remain unclear. In this study, we investigated the seasonal developmental pattern of the tubercles and asperities

of *Onychodactylus* salamanders by conducting surveys at the breeding sites used by *O. kinneburi* and *O. japonicus*.

Currently, *O. kinneburi* is known to be distributed in the Shikoku and the Chugoku Mountains, and it occurs sympatrically with *O. japonicus* in the latter area (Yoshikawa et al., 2013; Naito and Kambayashi, In press). This sympatry is suggested to have been caused by secondary contact; i.e., the two lineages separated 5.4–4.3 million years ago, and *O. kinneburi* on Shikoku island dispersed to Honshu via the landed Seto Inland Sea in the last glacial age (Yoshikawa et al., 2008). Although the two species are known to share breeding sites for spawning (Yoshikawa et al., 2013), they are reproductively isolated and genetically distinct in terms of both mitochondrial DNA (Yoshikawa et al., 2008) and nuclear markers (Yoshikawa et al., 2010). However, no significant differences in body size, breeding location, and breeding season have been detected between the two species (Yoshikawa et al., 2013); hence, the mechanism of their reproductive isolation in sympatry remains unknown. Based on our observations, we also describe differentiation in the breeding seasons of *O. kinneburi* and *O. japonicus*, which may be involved in their premating reproductive isolation.

MATERIALS AND METHODS

We conducted surveys at the two breeding sites of *Onychodactylus* salamanders located 1,180–1,220 m asl on Mt. Kanmuri (34°28' N, 132°04' E, 1,339 m asl) in the Western Chugoku Mountains (Totsune et al., 1995). At the breeding sites, subterranean water flowed from the crevices of the andesite bedrock, and the water temperature was 6.7–10°C. Larvae at stage 66–67 (Iwasawa and Kera, 1980), which seemed to be hatchlings from the previous year, were outflowing from the spring. The surrounding area was dominated by deciduous broad-leaved trees including *Fagus crenata*, with *Rodgersia podophylla* and *Eutrema japonica* thriving at the water's edge on the

TABLE 1. Number of breeding individuals of *Onychodactylus* collected in each survey. M: male, EBF: egg-bearing female, PSF: postspawning female.

		4/11	4/21	4/27	5/4	5/9	5/14	5/23	5/30	6/6	6/14	6/21	6/30
<i>O. kinneburgi</i>	M	6	5	1	1	0	1	0	3	0	0	0	0
	EBF	1	5	0	1	0	0	0	1	0	0	0	0
	PSF	0	0	0	1	0	1	0	0	0	0	0	0
<i>O. japonicus</i>	M	0	1	1	5	2	10	2	0	0	3	1	0
	EBF	0	0	0	1	4	11	7	1	0	0	0	0
	PSF	0	0	0	0	0	0	0	1	2	1	0	0

forest floor. It is known that *O. kinneburgi* and *O. japonicus* occur sympatrically on Mt. Kanmuri (Yoshikawa et al., 2013) and have been observed to gather at these breeding sites (Naito, 2017). In this system, where the *Onychodactylus* salamanders spawn in the subterranean headwaters, the occurrence and breeding of other hynobiid salamanders, such as *Hynobius kimurae* and *H. sematonotos*, have also been confirmed downstream (Naito, 2018, 2019).

Twelve surveys were conducted approximately every seven days from April 11, 2021, until June 30, when no adults were observed. We collected adult *Onychodactylus* salamanders at the spring outlets and from under stones and fallen trees within 20-m upstream and downstream of the springs between 0900 and 1200 h. Spawning was thought to take place at the back of the springs, but for the sake of continuous observation, we did not excavate the bedrock. Eighty obtained individuals were immediately anesthetized in 0.1% ethyl p-aminobenzoate solution on site. We identified the sexes of these individuals based on their secondary sexual characters (i.e., the shape of the tail, hindlimbs, and cloaca), which are prominent during the breeding season; for female individuals, the presence of ovarian eggs was also determined by looking through the ventral side. Snout-vent length (SVL; from the tip of snout to the anterior end of cloaca) was measured to the nearest 0.1 mm with a digital caliper (BLD-100, Niigata Seiki), and the presence of the tubercles, asperities, and claws on their limbs were recorded. The sala-

manders were then labeled by inserting Visible Implant Elastomer Tags and Coded Wire Tags (Northwest Marine Technology), after which they were awakened and released at the collection sites. The *Onychodactylus* species were identified based on the color of the dorsal stripe and the presence of dark markings on the chest (Yoshikawa et al., 2013). Ten individuals that did not show any secondary sexual characters were determined as nonbreeding individuals and their data were not used in this study.

We used Mann–Whitney U tests for SVL comparisons between the two species. Statistical tests were conducted using R version 3.6.1 (R Core Team, 2019) with a significance level of $P=0.05$.

RESULTS

A total of 17 males and 10 females of *O. kinneburgi* and 25 males and 28 females of *O. japonicus* were collected during the survey (Table 1). Only one individual (an *O. japonicus* male) was recaptured on another day. SVLs (means $\pm$ 1SD followed by ranges in parenthesis) of *O. kinneburgi* were 72.6 ± 3.1 mm (69.0–79.7 mm) in males and 77.9 ± 4.2 mm (73.5–87.4 mm) in females, and those of *O. japonicus* were 70.5 ± 2.9 mm (63.4–76.4 mm) in males and 74.0 ± 2.9 mm (69.4–80.7 mm) in females. The *O. kinneburgi* specimens were significantly larger than the *O. japonicus* specimens in terms of SVL (Mann–Whitney U test: male, $U=294$, $P=0.036$; female, $U=234$, $P=0.0011$).

The maximum numbers of *O. kinneburgi* and

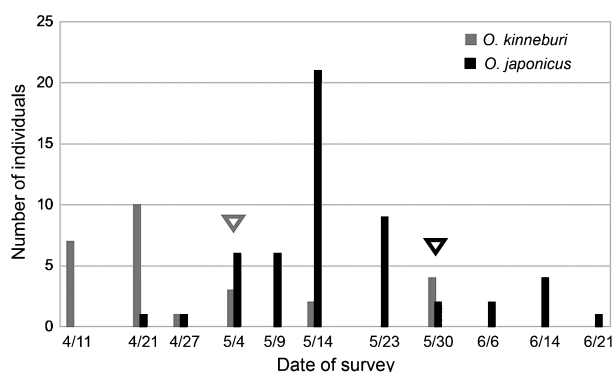


FIG. 1. Number of *Onychodactylus* salamanders collected on each survey day. The gray and black inverse triangles indicate the dates of the first appearance of postspawning females of *O. kinnebuli* and *O. japonicus*, respectively.

O. japonicus were collected on April 21 and May 14, respectively; there was a 23-day gap between these two peaks (Fig. 1). In *O. kinnebuli*, all females carried ovarian eggs until April 21, but spent individuals were collected from May 4, suggesting that spawning began on this date (Table 1). In contrast, postspawning female *O. japonicus* individuals were first obtained on May 30, which was 26 days after the first observation of spent *O. kinnebuli* females were sampled (Table 1).

We confirmed the development of black claws on the digits of all obtained breeding individuals (showing secondary sexual characters). Black claws were present on all digits of *O. kinnebuli* individuals throughout the survey period. In contrast, claws were found on all digits of all 14 *O. japonicus* individuals until May 9, whereas one claw was missing from one of the 21 individuals collected on May 14, one to 18 claws were missing from three of the four individuals collected on June 14, and eight claws were missing from one individual collected on June 21.

We observed tubercles and asperities on the hindlimbs of not only males but also females of both species (Fig. 2). Tubercles and asperities were observed in 19 and 17 of the 27 *O. kinnebuli* individuals and in 15 and 11 of the 53 *O. japonicus* individuals, respectively. Some of these individuals had only tubercles

(Fig. 2B), but there were no individuals that had only asperities. For males of *O. kinnebuli*, 100% of individuals had hindlimb tubercles in the first survey (April 11), and this condition persisted until May 30 when they were last collected (Fig. 3A). For females of *O. kinnebuli*, tubercles were not observed on April 11; however, the percentage of individuals with tubercles increased to 40% after 10 days, and then it again fell to 0% after May 4 (Fig. 3A). For males of *O. japonicus*, there were no individuals with hindlimb tubercles until April 27, but the percentage rapidly increased from May 4 and remained at 100% from May 23 (Fig. 3A). The percentage of *O. japonicus* females with tubercles rapidly increased from May 23 to reach 100% on June 6, after which no individuals with tubercles were found (Fig. 3A). The asperities on hindlimbs showed a similar temporal developmental pattern to that of the hindlimb tubercles (Fig. 3B). At the end of the breeding seasons, some individuals were found to have partially shed tubercles and asperities on their hindlimbs.

On the forelimbs, only one individual (an *O. kinnebuli* male) showed both tubercles and asperities, whereas tubercles were not found on any other individuals. Forelimb asperities were also observed regardless of sex; they occurred in 12 *O. kinnebuli* individuals and nine *O. japonicus* individuals. For *O. kinnebuli*, the

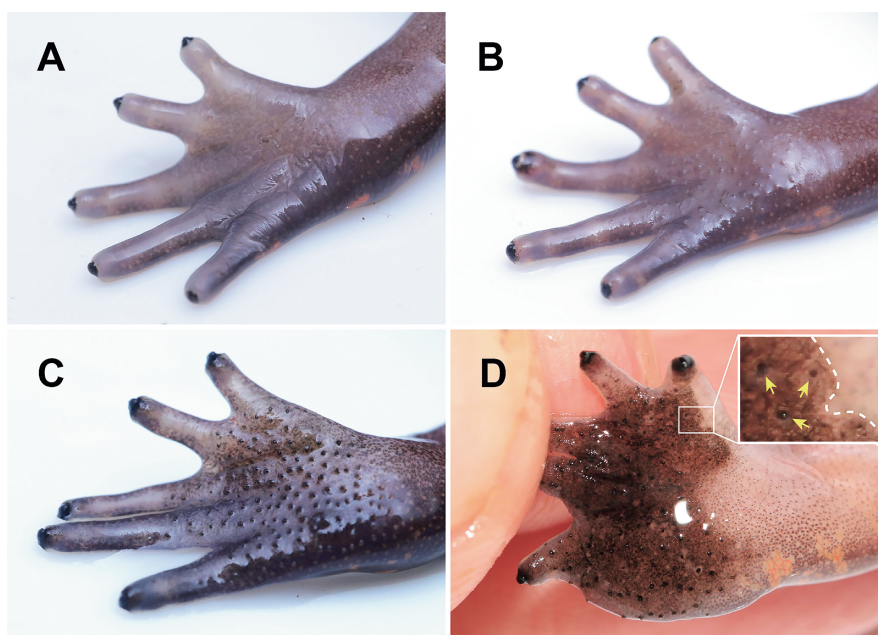


FIG. 2. Sole of the right hindlimb of *Onychodactylus japonicus*. A) Female collected on May 23. No structures on the sole can be observed. B) Female collected on May 30. Tubercles have begun to develop. C) Female collected on May 30. Tubercles have turned black and asperities have appeared. D) Male collected on May 4. Dermal skin fold has developed on the posterior edge of the hindlimb, and tubercles (yellow arrows) and asperities (left side of the white dashed line) have appeared all over the sole.

percentage of males with asperities fluctuated between dates, but it tended to be higher for both sexes between April 21 and 27, i.e., just prior to the appearance of postspawning females (Fig. 3C). In contrast, for *O. japonicus* males, 0% of individuals showed forelimb asperities until May 14, but the percentage rapidly increased after May 23 to reach 100% after June 14 (Fig. 3C). The percentage of *O. japonicus* females with asperities sharply increased up until May 30 when postspawning females were obtained and declined thereafter (Fig. 3C).

DISCUSSION

This study, conducted at Mt. Kanmuri, revealed that there is a temporal gap in breeding seasons between *O. kinneburi* and *O. japonicus*, which we postulate serves as a premating isolation period that reduces possi-

ble hybridization when the two species spawn in the same habitat. On Mt. Ishizuchi and Mt. Kurokasa in the Shikoku Mountains, breeding individuals of *O. kinneburi* can be first observed in early May in the vicinity of the breeding sites, and the number of such individuals peaks in mid-to-late May (Sato, 1943; Tamura, 2013). Even though the altitudes of the study sites on Mt. Kanmuri are lower than those on the Shikoku Mountains (1,250 m asl at Mt. Ishizuchi [Sato, 1943] and 1,400 m asl at Mt. Kurokasa [Yoshikawa et al., 2013]), the breeding season of *O. kinneburi* at the Chugoku Mountains (which peaks in mid-April) seems to be earlier than that of the Shikoku populations. However, the breeding phenology of amphibians is known to vary among years (Loman, 2016), and more surveys conducted in the same year would be necessary to verify the shift in breeding seasons among these *O. kinneburi* populations. Since premating isolation

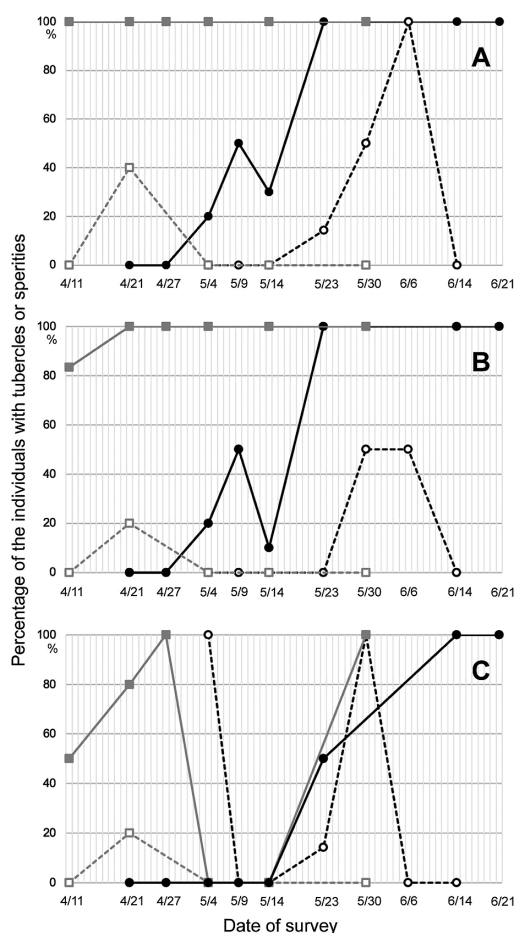


FIG. 3. Percentage of *Onychodactylus* salamanders with tubercles or asperities on each survey day. A) Hindlimb tubercles; B) hindlimb asperities; C) forelimb asperities. Gray solid line and filled squares represent males of *O. kinnebury*; gray dashed line and open squares represent females of *O. kinnebury*; black solid line and filled circles represent males of *O. japonicus*; black dashed line and open circles represent females of *O. japonicus*.

between any two populations is known to be reinforced by selection against hybrid individuals when their fitness decreases (Dobzhansky, 1940), a similar mechanism may have caused the allochronic isolation between *O. kinnebury* and *O. japonicus*, which are thought to have made secondary contact in the Chugoku Mountains after the last glacial period (Yoshikawa et

al., 2008). Artificial fertilization methods have been established for *O. japonicus* (Nakamura, 1939; Iizuka et al., 2010); therefore, using such methods, future experimental evaluations of the fitness of hybrid individuals would be desirable.

For *O. koreanus*, the duration of the actual spawning period is estimated to be less than a month (Park, 2005); hence, it is probable that the 23–26-day temporal gap in breeding season between *O. kinnebury* and *O. japonicus* detected here contributes to the low hybridization rate between the two species (Yoshikawa et al., 2010). However, there was some temporal overlap in the occurrence of the species at the breeding sites (Fig. 1), and in one case, a pre-spawning female of *O. kinnebury* and post-spawning female of *O. japonicus* were obtained on the same date (May 30; Table 1); thus, we cannot rule out other unknown mechanisms being involved in premating isolation. In amphibians, there are closely related species occurring in sympatry and reproductive isolation mechanisms including differences in body size and spawning sites prevent their hybridization (e.g., Matsui, 1994; Tominaga et al., 2005). In this study, SVL significantly differed between the two *Onychodactylus* species. Although Yoshikawa et al. (2013) compared other morphological characters of these species, the sample size of *O. kinnebury* from the Chugoku Mountains was insufficient and further observations are required. It is also possible that the microenvironments at which the two species spawn are different or that they use pheromones, like other hynobiid salamanders (Eom et al., 2009), to identify their own species. Indeed, it has been suggested that chemicals that excite males are released during breeding because males react vigorously to eggs immediately after spawning but show no interest in eggs that have been laid for a long time (Nakamura, 1941b).

According to Sato (1943), *Onychodactylus* salamanders begin to show black claws two to three months before spawning, and closer to the breeding season, they migrate upstream to their breeding sites, suggesting that the black

claws are potentially used for migration in streams. In this study, the hypothesis that black claws are an adaptive trait used to move through running water was supported by our observation that they were found on the digits of both males and females at least 23 days (*O. kinnebury*) and 39 days (*O. japonicus*) before the appearance of postspawning females.

We confirmed that not all breeding individuals showed tubercles and asperities on their hindlimbs, as previously reported by other researchers (Totsune et al., 1995; Akita, 2013). There were no individuals that bore only asperities (Fig. 2), suggesting that the development of tubercles may precede that of asperities on the hindlimbs of *Onychodactylus* salamanders. For females of *O. kinnebury* and both males and females of *O. japonicus*, the percentage of individuals with hindlimb tubercles rapidly increased before the appearance of postspawning females (Fig. 3A). Conversely, all male individuals of *O. kinnebury* had tubercles, which may have developed before the survey, as the tubercles began to develop in *O. japonicus* males about 3 weeks earlier than they developed in females (Fig. 3A). Additional surveys of tubercles from an earlier season are needed to further examine this finding. We also found that the tubercles and asperities on hindlimbs appeared in females of both *Onychodactylus* species and developed over quite a short period during their breeding seasons. It has been suggested that Japanese *Onychodactylus* salamanders enter the subterranean water from narrow spring outlets for breeding, after which they move through the rock crevices in all directions (Totsune et al., 1995). Therefore, the tubercles and asperities on hindlimbs are considered adaptive traits for ascending in the groundwater or operating at the spawning site. The observation that these traits appeared later and disappeared earlier in females relative to males may be attributable to the shorter stay at the spawning site of females compared with that of males (Kudo, 1934).

According to field observations and rearing experiments, males of *O. kinnebury* and *O. japonicus* use their enlarged hindlimbs to assist

in egg-laying behavior (Kudo, 1934; Nakamura, 1941b; Sato, 1943) and to grasp female individuals (Naito et al., 2022). The development of the dermal skin fold on the hindlimbs of males was a prominent observation in our survey, and the area where the tubercles and asperities appeared was obviously larger in males than that in females (Fig. 2C, D). Thus, it is likely that hindlimb tubercles and asperities also serve as cleats when grasping the egg sacs or female individuals.

Although the seasonal change of the forelimb asperities is not clear due to the paucity of observations, the percentage of individuals with forelimb asperities fluctuated more than the percentage with hindlimb tubercles and asperities, suggesting that their appearance may not be regulated on a strictly seasonal basis and that substantial differences may exist among individuals (Fig. 3C). Nonetheless, rapid development of asperities on forelimbs was observed in *O. japonicus* immediately before the start of spawning, and asperities tended to appear in many individuals of *O. kinnebury* during the peak of the breeding season. Given that forelimb asperities developed in both males and females, they may be adaptive traits for ascending or operating in the subterranean water, similar to the function of the tubercles and asperities on hindlimbs.

ACKNOWLEDGMENTS

We would like to thank Hatsukaichi City Office, Yoshiwa Branch for allowing us to use the Kogawa forest road on Mt. Kanmuri, Enago (www.enago.jp) for the English language review, and three anonymous reviewers for valuable comments on this manuscript.

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Accepted: 27 May 2022