

## Effects of Body Size, Operational Sex Ratio, and Age on Pairing by the Asian Toad, *Bufo stejnegeri*

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**Jung-Hyun Lee and Daesik Park (2009)** Effects of body size, operational sex ratio, and age on pairing by the Asian toad, *Bufo stejnegeri*. *Zoological Studies* 48(3): 334-342. Male and female *Bufo stejnegeri* pair within a stream and overwinter together in the stream for 3 mo before spawning in early spring. To investigate whether body size, the operational sex ratio (OSR; the ratio of sexually active males to females), or age affects pairing of *B. stejnegeri* during the long pairing period, we surveyed 2 study sites on 2 separate occasions between 2005 and 2007. In total, 684 toads were sampled, consisting of single males, amplexant males, and amplexed females. No single females were found. When the OSR was slightly male-biased (e.g., 1.85 or 1.96), amplexant males were larger than single males, but when the OSR was highly male-biased (e.g. 2.75 or 4.1), size-assortative pairing occurred. The forelimbs and hindlimbs of amplexant males were thicker than those of single males. At a study site, the frequency distributions of amplexant and single males across different ages were significantly biased, such that most of the single males were 1 (16.7%) and 2 yr old (76.3%). These results suggest that competition occurs between males during the long pairing period of *B. stejnegeri*, and that body size and age affect pairing of male toads; these effects may differ with variable OSRs.  
<http://zoolstud.sinica.edu.tw/Journals/48.3/334.pdf>

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Most frog and toad males pair with females when they are ready to breed and maintain their relationship until spawning (Duellman and Trueb 1986). In general, a pairing period is short, ranging in most anurans from several minutes to a few hours (Duellman and Trueb 1986, Tsuji and Lue 1998). However, pairing in several species such as *Atelopus oxyrhynchus* (Dole and Durant 1974), *Myobatrachus gouldii* (Roberts 1981), and *Bufo stejnegeri* (Yang et al. 2001) lasts for several months. In these species, the breeding season is over 3 mo long and includes both a long pairing period and a relatively short spawning period of 1-2 wk. Several previous studies on how pairing competition progresses during a breeding period suggest that the length of a breeding (pairing) period and the operational sex ratio (OSR; the ratio

of sexually active males to females) (Emlen and Oring 1977) affect pairing competition (Davies and Halliday 1977, Tejedo 1988).

Variations in the length of a breeding period may be an important factor in shaping the pairing patterns in toads. In species with short breeding periods, the time window available for large males to encounter all possible female mates is relatively limited. Also, because a short breeding period allows small males find single females in places where large males are not present, size-assortative pairing occurs (Davies and Halliday 1977, Arntzen 1999). In species with a long breeding period, the large male advantage is prominent, and size-assortative pairing is rare. Here, sufficient time is available for competition to occur, and large males consistently out-compete small males for access to

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females (Wilbur et al. 1978, Hillis et al. 1984, Olson et al. 1986). Despite this association, pairing patterns have not been studied in a toad species for which the pairing period is extraordinarily long.

The OSR can also affect pairing patterns. In toad species, the OSR is usually male-biased (Arak 1983). In many animal species, a male-biased OSR increases the intensity of male mating competition and can change the mating tactics of breeding males (Kvarnemo and Ahnesjö 1996, Jirotkul 1999, Hasumi 2001, Rohr et al. 2005). Highly male-biased OSRs result in increased levels of male-male fighting in some toads and have often been associated with males attempting to dislodge already paired males (Tejedo 1988). Although the reported success of single males replacing amplexant males varies across the literature (Reading and Clarke 1983, Lamb 1984, Tejedo 1988), it could be an important factor in successful pairing in situations where the pairing period is exceptionally prolonged.

The Asian toad, *B. stejnegeri* (Schmidt 1931, Matsui 1980, Zhao and Adler 1993), has a very long pairing period that lasts for 3 mo. Male and female *B. stejnegeri* are unpaired when they enter a stream to breed between late Dec. and early Jan., before the stream freezes (Kang and Yoon 1975). Within the stream, males and females pair and overwinter together in the stream for about 3 mo. As the ice melts in late Mar., females of paired toads begin to spawn over a period of 1-2 wk, attaching their egg strings to water plants (Kang and Yoon 1975, Yang et al. 2001). *Bufo stejnegeri* does not make complex advertising calls (Matsui 1980), but they often produce release calls. However, this is all that is currently known because behavioral and ecological studies of Asian toads are scarce.

To investigate whether body size, OSR, or age affects pairing by male *B. stejnegeri* during the long pairing period, we surveyed toads inhabiting 2 study sites on 2 occasions between 2005 and 2007.

## MATERIALS AND METHODS

### Study sites and field surveys

Our study sites were specific spans of small streams. The stream area for the Chuncheon study site measured approximately 20 m long x 2.5 m wide x 0.4m deep (37°51'N, 127°39'E; elevation 80 m), and that for the Woraksan study

site was 10 m long x 2 m wide x 0.2 m deep (37°50'N, 127°47'E; elevation 240 m). We selected study sites at which shore areas were shallow, center areas were deep, and water velocities were slow. These factors are characteristic of known major breeding sites of a stream-breeding toad (*B. torrenticola*, Tsuji and Kawamichi 1996). One side of the stream at the Chuncheon study site was connected to the banks of harvested rice fields, and the other side was blocked by an artificially constructed stream rock wall (approximately 1.5 m high). The stream at the Woraksan study site was bordered by dry stream bed (> 10 m wide), lined with pebbles and rocks of various sizes.

We surveyed both study sites on 2 occasions when the streams were not frozen. Because the Woraksan study site was at a relatively low latitude, the breeding stream froze later and melted earlier than the stream at the Chuncheon study site. We surveyed the Chuncheon study site on 10 Dec. 2005 and 30 Mar. 2006 and the Woraksan study site on 7 Mar. 2006 and 9 Jan. 2007. We observed 3 egg strings deposited between water plants at the Chuncheon study site on 30 Mar. 2006. At the Woraksan study site, more than 20 egg strings were found among pebbles on 27 Apr. 2007.

During each survey, we searched under rocks and collected as many toads as possible from each study site. Single males, amplexant males, and amplexed females were kept separately in plastic boxes (25 cm long x 8 cm wide). For each toad, we measured the snout-vent length (SVL) to the nearest 0.01 cm using digital Vernier calipers and body mass to the nearest 0.01 g using a digital field balance (Kern TMB 120-1, Wildeck, Germany). We did not use anesthesia during these measurements because most of the toads were inactive, given the low water temperature (< 10°C). At the Chuncheon study site in Mar. 2006 and at the Woraksan study site in Jan. 2007, we measured the diameter of the right forelimb (humerus) and the right hindlimb (tibia) of amplexant and single males to the nearest 0.01 cm using digital Vernier calipers. We clipped the outermost 2 digits of the central toe from the right hindlimb of toads captured at the Chuncheon study site in Dec. 2005 and at the Woraksan study site in Jan. 2007 and individually preserved each one in 10% neutral-buffered formalin for a skeletochronological study to determine their ages. We then released each toad at the site of capture. Males and females that were paired when they were caught were released in the state

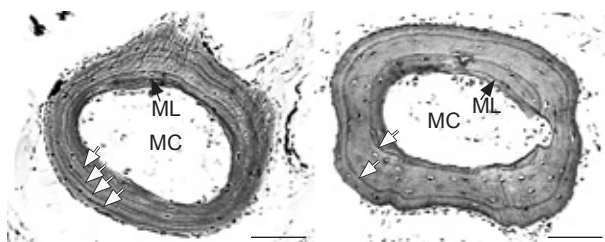
of separation.

### Skeletochronological study

To prepare the clipped toes for skeletochronology, we followed the procedures described by Hemelaar and van Gelder (1980). In brief, we first rinsed the clipped toes in running tap water for 24 h to clean them and then decalcified the toes by submerging them in 5% nitric acid for 20-30 min. Dehydrated toes were embedded in paraffin, and a rotary microtome (Erma, Tokyo, Japan) was used to cut 10  $\mu\text{m}$  thick sections. Sections were stained with Harris' hematoxylin-eosin and observed under a microscope (x400). Growth zones and lines of arrested growth (LAGs) were clearly visible in the cross-sections of the phalanges (Fig. 1). The number of LAGs was counted, as in previous studies (Leary et al. 2005, Cheong et al. 2007, Lee et al. 2008), and individual ages were independently determined by both authors. When there was a discrepancy between the 2 estimations, LAG scores were corroborated, and a consensus conclusion was drawn.

### Statistical analysis

We used the independent-sample *t*-test to compare SVL, body mass, and limb diameter differences between amplexant and single males because the data passed the normality test (Kolmogorov-Smirnov,  $p > 0.05$ ). The forelimb and hindlimb diameters were log-transformed, divided by log-transformed SVLs, and then compared. We applied the Mann-Whitney U-test to compare differences in ages between males and females and between amplexant and single males because the data did not pass the normality test



**Fig. 1.** Phalangeal cross-sections of a 4 yr old male (left) and a 2 yr old female (right) Asian toad *Bufo stejnegeri* from the Chuncheon study site. Each white arrow indicates a line of arrested growth. Black arrows indicate the metamorphosis line (ML), and MC represents the medullary cavity. Scale bars = 50  $\mu\text{m}$ .

(Kolmogorov-Smirnov,  $p < 0.05$ ).

For the correlation analysis, we used Pearson's correlation coefficients to determine relationships among long-transformed values of the forelimb diameter, hindlimb diameter, and SVL of males, as the data passed the normality test (Kolmogorov-Smirnov,  $p > 0.05$ ). We used Spearman's rank correlation coefficients to determine the correlation of ages between amplexant males and amplexed females because the data did not pass the normality test (Kolmogorov-Smirnov,  $p < 0.05$ ). In addition, we used a Chi-squared test to determine whether the frequency distribution of amplexant and single males differed across ages. The data were analyzed using SPSS vers. 11.0 (Chicago IL, USA). All significance levels were tested at alpha = 0.05 (2-tailed).

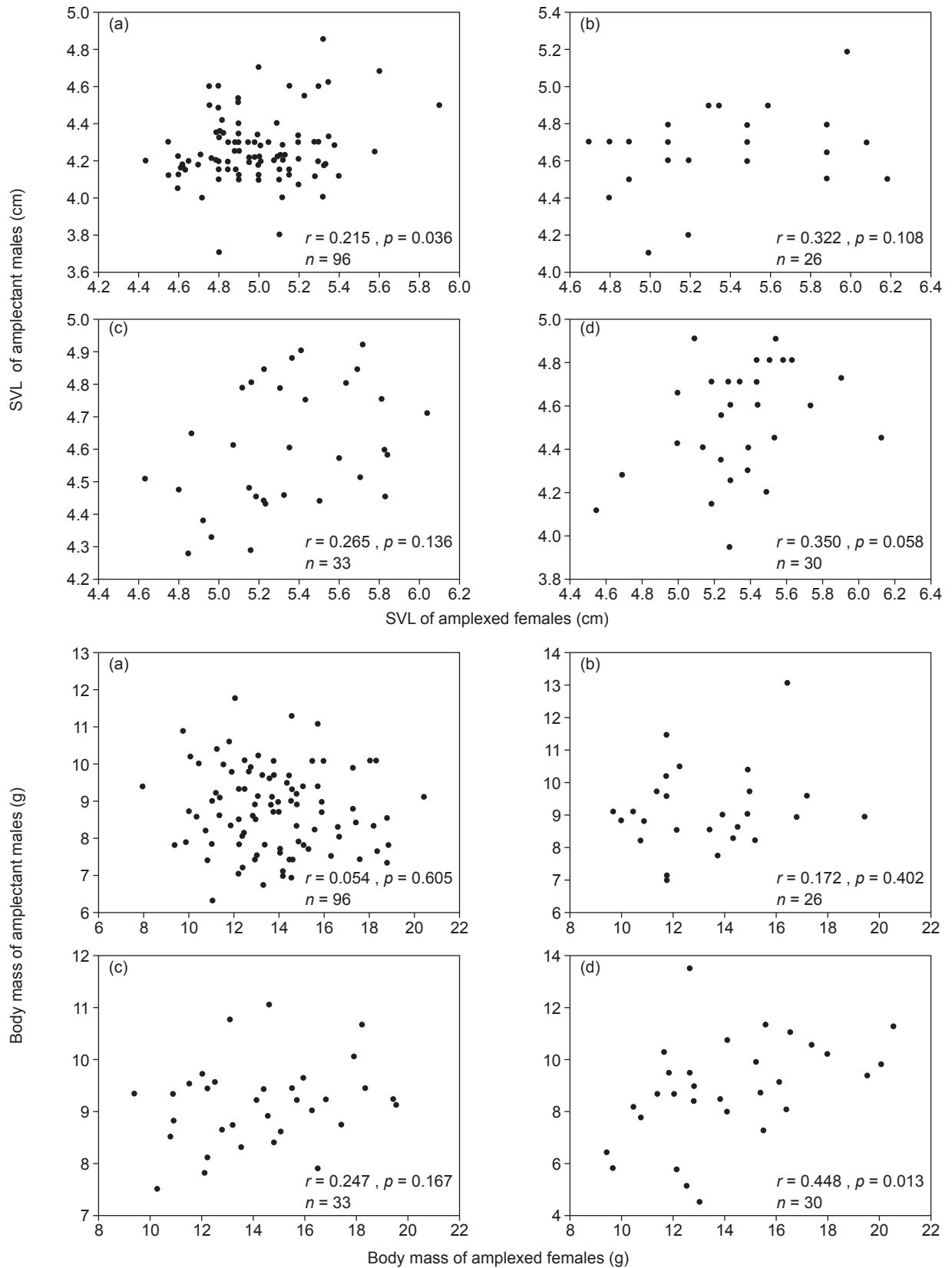
## RESULTS

### Body size and OSR effects on pairing

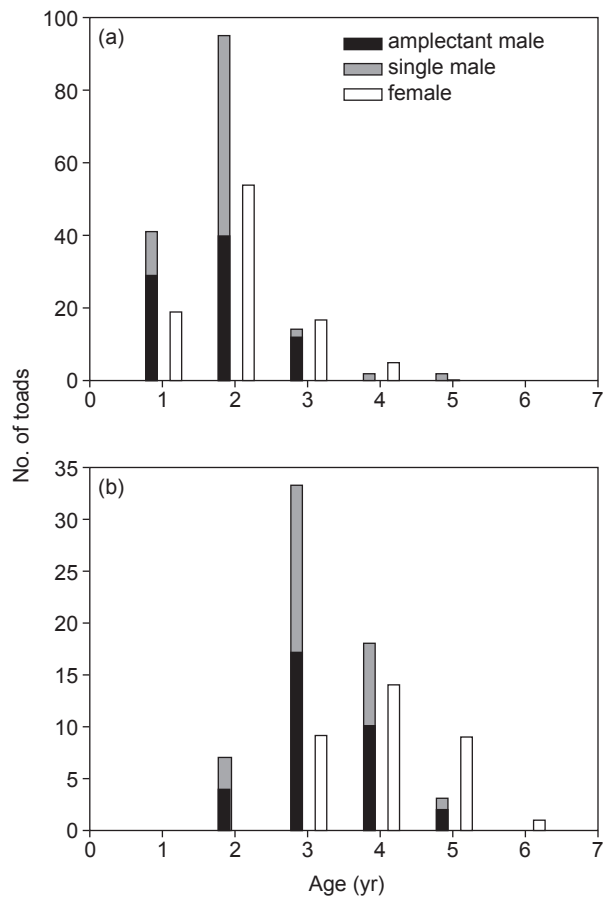
In total, 684 *B. stejnegeri* were sampled over 4 field surveys at the 2 study sites; this collection consisted of single males, amplexant males, and amplexed females (Table 1). No single females were found. The OSR was male-biased at both study sites, and it varied with the survey date, ranging 1.85-4.10 (Table 1).

At the Chuncheon study site in Mar. 2006, the OSR was 1.96, and amplexant males had significantly greater SVLs ( $t = 2.54$ ,  $d.f. = 49$ ,  $p = 0.014$ , Table 1), but not body masses ( $t = 1.04$ ,  $d.f. = 49$ ,  $p = 0.302$ , Table 1), than single males. This difference was not found in Dec. 2005, when the OSR was 2.75 ( $p > 0.05$  for both the SVL and body mass, Table 1). At the Woraksan study site in Jan. 2007, amplexant males were significantly larger than single males when the OSR was 1.85 ( $t = 2.65$ ,  $d.f. = 59$ ,  $p = 0.010$  for SVL;  $t = 3.08$ ,  $d.f. = 59$ ,  $p = 0.003$  for body mass, Table 1), while no size difference was found in Mar. 2006, when the OSR was 4.1, ( $p > 0.05$  for both SVL and body mass, Table 1). Significant size-assortative pairing was observed at the Chuncheon study site in Dec. 2005, when the OSR was 2.75 ( $r = 0.215$ ,  $p = 0.036$  for SVL; but  $r = 0.054$ ,  $p = 0.602$  for body mass, Fig. 2a), and at the Woraksan study site in Mar. 2006, when the OSR was 4.1 ( $r = 0.350$ ,  $p = 0.058$  for SVL;  $r = 0.448$ ,  $p = 0.013$  for body mass, Fig. 2d).

The forelimb and hindlimbs of amplexant



**Fig. 2.** Relationship between the snout-vent length (SVL, upper panel) and body mass (bottom panel) of paired male and female Asian toad *Bufo stejnegeri* in Dec. (a) and Mar. (b) at the Chuncheon study site and in Jan. (c) and Mar. (d) at the Woraksan study site. Assortative-pairing was found in the Chuncheon study site in Dec. 2005 (a, upper panel) and at the Woraksan study site (d, upper and bottom panel) in Mar. 2006.



**Fig. 3.** Age distribution of amplexant males, single males, and amplexed females of the Asian toad *Bufo stejnegeri* at the Chuncheon (a) and Woraksan (b) study sites.

males were thicker than those of single males at the Chuncheon study site in Mar. 2006 ( $t = 7.07$ ,  $d.f. = 48$ ,  $p < 0.001$  for forelimb;  $t = 2.45$ ,  $d.f. = 48$ ,  $p = 0.018$  for hindlimb). At the Woraksan study site in Jan. 2007, such a difference was seen in hindlimb diameter ( $t = 2.66$ ,  $d.f. = 55$ ,  $p = 0.010$ ) but not forelimb diameter ( $t = 0.66$ ,  $d.f. = 55$ ,  $p = 0.515$ ). Male forelimb and hindlimb diameters were significantly correlated with the SVL at both study sites ( $p < 0.05$  for all comparisons).

**Age structure and its effects on pairing**

We determined the ages of 343 toads (Figs. 1, 3), including 72 single males, 82 amplexant males, and 95 amplexed females from the Chuncheon study site in Dec. 2005, and 28 single males, 33 amplexant males, and 33 amplexed females from the Woraksan study site in Jan. 2007.

At the Chuncheon study site, male ages ranged 1-5 yr (mean  $\pm$  SD =  $1.96 \pm 0.74$ ,  $n = 154$ ), and female ages ranged 1-4 yr (mean  $\pm$  SD =  $2.08 \pm 0.77$ ,  $n = 95$ , Fig. 3a). The difference between the ages of males and females was significant (U-test,  $z = 2.15$ ,  $n = 249$ ,  $p = 0.032$ ). There was no significant difference between the ages of amplexant and single males (U-test,  $z = 1.302$ ,  $n = 154$ ,  $p = 0.193$ ), but the frequency distributions of amplexant and single males differed across ages ( $X^2 = 7.22$ ,  $d.f. = 1$ ,  $p = 0.007$ , Fig. 3a). At the Chuncheon study site, the percentages of 1-,

**Table 1.** Snout-vent length (SVL, mm) and body mass (g) of amplexed females, amplexant males, and single males of the Asian toad *Bufo stejnegeri* at the study sites during each survey. The operational sex ratio (OSR) is the ratio of amplexant and single males to amplexed females captured at the study site during each survey. An independent-sample *t*-test was applied to compare the SVL and body mass of amplexant and single males. Data are presented as the mean  $\pm$  standard error

Study site	Time of year of survey (OSR)		Amplexed females	Amplexant males	Single males	<i>t</i> -test ( <i>t</i> -value)	<i>p</i>
Chuncheon	Dec. (2.75)	SVL	49.80 $\pm$ 0.30	42.60 $\pm$ 0.20	42.80 $\pm$ 0.20	0.90	0.369
		Mass	13.81 $\pm$ 0.25	8.74 $\pm$ 0.90	8.73 $\pm$ 0.90	0.05	0.961
		<i>n</i>	96	96	168		
	Mar. (1.96)	SVL	53.40 $\pm$ 0.90	46.50 $\pm$ 0.40	44.80 $\pm$ 0.50	2.54	0.014
		Mass	13.38 $\pm$ 0.49	9.15 $\pm$ 0.25	8.73 $\pm$ 0.32	1.04	0.302
		<i>n</i>	26	26	25		
Woraksan	Jan. (1.85)	SVL	53.70 $\pm$ 0.60	45.90 $\pm$ 0.40	44.60 $\pm$ 0.30	2.65	0.010
		Mass	14.32 $\pm$ 0.48	9.11 $\pm$ 0.14	8.38 $\pm$ 0.20	3.08	0.003
		<i>n</i>	33	33	28		
	Mar. (4.1)	SVL	53.60 $\pm$ 0.60	45.30 $\pm$ 0.50	45.10 $\pm$ 0.20	0.45	0.651
		Mass	14.22 $\pm$ 0.55	8.74 $\pm$ 0.36	8.68 $\pm$ 0.12	0.22	0.824
		<i>n</i>	30	30	93		



2-, and 3 yr old single males were 16.7%, 76.4%, and 2.7%, and in the same 3 age groups, those of corresponding amplexant males were 35.4%, 48.8%, and 14.6%, respectively (Fig. 3a). A less age-biased distribution was found in amplexant males than in single males. No significant correlation between the ages of amplexant males and amplexed females was found (Spearman correlation test,  $r_s = 0.06$ ,  $n = 81$ ,  $p = 0.600$ ).

At the Woraksan study site, male and female ages ranged 2-5 (mean  $\pm$  SD =  $3.28 \pm 0.73$ ,  $n = 61$ ) and 3-6 yr (mean  $\pm$  SD =  $4.06 \pm 0.83$ ,  $n = 33$ , Fig. 3b), respectively. There was a significant difference between male and female ages (U-test,  $z = 4.143$ ,  $n = 94$ ,  $p < 0.001$ ). Frequency distributions of amplexant and single males across ages did not differ ( $X^2 = 0.009$ ,  $d.f. = 1$ ,  $p = 0.764$ ), nor was there a significant difference between the ages of amplexant and single males (U-test,  $z = 0.26$ ,  $n = 61$ ,  $p = 0.798$ , Fig. 3b). At the Woraksan study site, the percentages of 1-, 2-, and 3 yr old single and amplexant males were similar (10.7%, 57.1%, and 28.6%, for single males and 12.1%, 51.6%, and 30.3%, for amplexant males, respectively; Fig. 3b). No significant correlation between the ages of amplexant males and amplexed females was found (Spearman correlation test,  $r_s = 0.281$ ,  $n = 33$ ,  $p = 0.113$ ).

## DISCUSSION

In this study, we found that large *B. stejnegeri* males were more successful in pairing than small males when the OSR was slightly male-biased, such as at 1.85 or 1.96, but size-assortative pairing occurred when the OSR was highly male-biased, such as at 2.75 or 4.10. In addition, age and the sizes of the forelimbs and hindlimbs of males impacted their pairing success. These results suggest that pairing competition occurs during *B. stejnegeri*'s long pairing period, that body size and age affect successful pairing of male toads, and that these effects depend on the OSR of a given pairing site.

Because male anurans pair when they are ready to breed (Duellman and Trueb 1986), our finding of paired *B. stejnegeri* in Dec. and Mar. and of spawning in Mar. and Apr. suggests that the breeding season of this species is between Dec. and Apr., and that its spawning period may be from late Mar. to mid-Apr. In addition, our survey revealed that some males from all age classes paired with females, and that all females captured

were in a paired state; this observation suggests that only breeding males and females participate in pairing competition within an overwintering stream. Such a long breeding period, including a long pairing period, has only been reported for Andean bufonids and desert-dwelling myobatrachids (Dole and Durant 1974, Roberts 1981 1984). One pair of *Ate. oxyrhynchus* maintained its pairing for more than 125 d before spawning within a stream (Dole and Durant 1974), and *Arenophryne rotunda* and *M. gouldii* pair and remain underground for 5-6 mo before spawning (Roberts 1981 1984). It is unknown whether pairing competition occurs in these species during such a long pairing period. Although we do not know whether a given male and female of *B. stejnegeri* remain together throughout the entire pairing period, our observation that pairing patterns change during a pairing period suggests that pairing competition occurs during the period.

During a prolonged period of pairing, various factors may result in temporal changes in the OSR of *B. stejnegeri*. In frogs, toads, and salamanders, local movements of individuals within a breeding site, different time schedules for migration of males and females to a breeding site, and different stopover durations of males and females within a breeding site are known to affect local OSRs (Reading and Clarke 1983, Tejedo 1988, Park and Park 2000, Rohr et al. 2005). At the Chuncheon study site, we found many fewer toads in Mar. 2006 compared to 10 Dec. 2005, even though the same sampling method was used. Our discovery of 3 egg strings in Mar. 2006 implies that the decreased OSR may have resulted from males and females leaving the breeding site after spawning. At the Woraksan study site, a high OSR in Mar. 2006 probably resulted from males moving locally within the stream rather than the arrival of new toads from outside the stream, because the stream was still frozen (Kang and Yoon 1975). Given that we surveyed only particular parts of the streams, it is possible that additional toads may have moved into our study site from other parts of the stream where toads were overwintering. Movement patterns of toads under the ice have not been studied.

Our results have 2 important implications for the study of pairing competition in toads. First, we determined whether body size, OSR, and age affect successful pairing of male toads in the context of an extremely long pairing period. Several studies have investigated pairing competition in prolonged-breeding toad species,

such as *B. houstonensis* (Hillis et al. 1984), *B. quercicus* (Wilbur et al. 1978), and *B. boreas* (Olson et al. 1986), but the actual male pairing competition period was as short as several hours to mere days in those species (Tsuji and Lue 1998). Although previous studies did not find size-assortative pairing in prolonged-breeding toads (Wilbur et al. 1978, Hillis et al. 1984), we found size-dependent or size-assortative pairing in *B. stejnegeri*. Second, our results showed that pairing patterns may vary depending on the OSR within a pairing site, similar to previous reports (Tejedo 1988, Rohr et al. 2005). In our study, slightly male-biased OSRs induced size-dependent pairing, but highly male-biased OSRs resulted in size-assortative pairing. These results suggest that *B. stejnegeri* would be a good model to study male-male competition during a long pairing period.

How do different OSRs induce dissimilar pairing patterns in *B. stejnegeri*? Our results showed that slightly male-biased OSRs resulted in size-dependent pairing, such that large males were more successful in pairing than small males. This might have been caused by the fact that in slightly male-biased OSRs, large males can out-compete small males (Höglund 1989, Arntzen 1999). In addition, large males might successfully dislodge small males from amplexed females and more easily endure such attempts from the relatively few smaller males, resulting in more successful pairings by large males. Such a large male advantage in pairing has frequently been reported for several *Bufo* species (Davies and Halliday 1977, Olson et al. 1986, Böll and Linsenmair 1998, Arntzen 1999). However, in a highly male-biased OSR, size-assortatively paired amplexant males (whether small, medium, or large) might possess advantages for enduring frequent replacement attempts by single males (small, medium, or large). Size-assortatively paired males and females of *B. bufo* are more tightly paired and thus less likely to be dislodged (Arak 1983, Höglund 1989). In this case, males that encountered similar-sized females had a better chance of maintaining the subsequent pairing, resulting in a size-assortative pairing pattern. In *B. stejnegeri*, it is not known whether size-assortative pairs are more resistant to dislodging attempts than randomly sized pairs.

The diameter of the forelimb and hindlimb of male toads may affect pairing competition. Like previous findings (Lee 2001, Lee and Corrales 2002), amplexant males had thicker hindlimbs than single males at both study sites. At the Chuncheon study site, amplexant males also

had thicker forelimbs than single males. Because recent studies have shown that the muscles of the forelimbs and hindlimbs of male toads are functionally adapted for long-term maintenance of pairing (Wilson et al. 2004, Clark and Peters 2006), thicker forelimbs and hindlimbs in amplexant male toads should provide an advantage for pairing maintenance.

Age factors may affect the pairing of male *B. stejnegeri* during the long pairing period. Most previous studies failed to find any significant correlation between age and pairing success of male toads (Hemelaar 1983, Leary et al. 2005). However, 1 previous study reported that inexperienced young males paired with inexperienced young females more often than with experienced old females because of behavioral differences (Reading 2001). In the present study, the ages of amplexant and single males did not differ, and there was no relationship between the ages of amplexant males and amplexed females at either the Chuncheon or Woraksan study site. However, the frequency distribution of amplexant and single males across different ages was significantly biased at the Chuncheon study site, although not at the Woraksan study site. At the Chuncheon study site, most single males consisted of 1- (16.7%,  $n = 12$  of 72) and 2 yr old (76.4%,  $n = 55$  of 72) males, with the remainder comprising 6.8% of the sample ( $n = 5$  of 72). Amplexant males were less age-biased, with 29 1 yr olds (35.4%), 40 2 yr olds (48.8%), and 13 of the remaining males (15.8%) out of a total of 82. At the Woraksan study site, the percentages of 1-, 2-, and 3 yr old single and amplexant males were similar. Considering the different survey times, Dec. for the Chuncheon study site and Jan. for the Woraksan study site, it is possible that the age distribution of successfully amplexant males may change during a pairing period within a site. Further studies are needed to clarify advantages of age in the pairing of *B. stejnegeri*.

In agreement with previous age studies on *B. bufo* (Hemelaar 1983), the mean age of *B. stejnegeri* females was higher than that of males at both the Chuncheon and Woraksan study sites. Unlike *B. bufo*, which breeds for the first time around age 4-5 yr (Hemelaar 1983), *B. stejnegeri* participated in pairing at relatively young ages, probably because of earlier maturation and a shorter lifespan. At the Chuncheon study site, 1 yr old males were observed in successfully paired relationships. At the Woraksan study site, 2 yr old males and 3 yr old females participated in their first

mating.

In conclusion, our results demonstrate that *B. stejnegeri* males compete for pairing during a long pairing period, and that body size, OSR, and age affect their pairing success rate. Because very few toad species have such a long pairing period, *B. stejnegeri* can serve as a useful model to study mating competition in this context.

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