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Comparison of reproductive characteristics and host use of the Korean bitterling, *Acheilognathus signifer* (Cyprinidae, Pisces), between two populations in the wild environment

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Abstract. Reproductive characteristics and host use of two populations of the Korean bitterling, *Acheilognathus signifer*, which uses mussels as a host, were investigated from April to July in 2011 and 2013. The study sites in Korea were Goesan, where *A. signifer* prefers *Lamprotula leai* to three other mussels as a host and Danyang, with a single mussel species, *Unio douglasiae* and therefore no host choice. The two populations of *A. signifer* did not show any differences in the appearance such as standard length of females during spawning season. However, the Goesan population had longer ovipositors (mean \pm SD, 21.68 ± 2.84 vs. 19.33 ± 1.85) and a higher egg number (22.46 ± 12.31 vs. 17.16 ± 7.23) than the Danyang population. With regard to the host mussels that had *A. signifer* eggs, *L. leai* in Goesan had longer shells (57.24 ± 6.84 vs. 41.66 ± 4.88) and hosted more *A. signifer* eggs (11.10 ± 8.35 vs. 4.40 ± 5.30). We assume that the difference in ovipositor length and egg number of *A. signifer* between the two populations may be related to phenotypic plasticity required for oviposition to minimize egg ejection and maximize embryo survival.

Key words: ovipositor, freshwater mussel, egg position, coevolution

Introduction

Approximately 40 species of bitterlings (Cyprinidae, Pisces) have been described from south-eastern Asia, Chinese mainland, and Japan, of which 14 species are found in Korea (Kim & Park 2002). During the spawning season, females develop a long ovipositor that they use to lay eggs in the gill cavities of mussels through the mussel's exhalant siphon. Males have bright nuptial colouration and defend territories around living freshwater mussels. Males eject their sperm into the inhalant siphon of the mussels, so that fertilization occurs within the gill cavities of mussels as a host. The embryos develop inside the mussels for 2-4 weeks until the yolk sac is absorbed and they enter the free-swimming stage (Smith et al. 2004).

Freshwater mussels have larvae termed glochidia that are obligate ectoparasites of fish (Dillon 2000). Glochidia are usually brooded in the outer demibranch of the mussels (some species use inner and outer

demibranches). After attachment to the gill and fins of fish, the glochidia are encysted by fish tissue and survive for several days. This development strategy enables glochidia to disperse. The bitterling-mussels relationship is thought of a mutualistic relationship: bitterlings use mussels as spawning hosts while mussels take advantage of bitterlings to disperse their glochidia (Wheeler 1978). However, there has been increasing evidence that bitterling-mussels relationship, at least in some species, may not be mutually beneficial and possess cost for both partners (Smith et al. 2004, Reichard et al. 2007).

Bitterlings have morphological, physiological and behavioural adaptations for using host mussels, thereby serving as a valuable evolutionary ecology model system (Oshiumi & Kitamura 2009). The fitness of female bitterlings in terms of survival of embryos in their host differs not only among mussel species, but also mussel size, reproductive state, and

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sex (Reynolds et al. 1997, Smith et al. 2000, Mills & Reynolds 2002a). A longer ovipositor allows eggs to be deposited deeper within the gill cavities of mussels, reducing competition for space and oxygen between deposited eggs (Kitamura 2006a). Mussels have evolved counter-adaptations to bitterling embryo development; they can eject bitterling embryos from their gills by contracting their valves (Reichard et al. 2010). Bitterlings make sophisticated oviposition decisions to prevent ejection (Smith et al. 2000). Bitterling embryos vary with regard to shape, number and size, and four egg types have been recognized in Korea: bulb like, pear shape, spindly and ovoid (Kim et al. 2011).

Acheilognathus signifer is an endemic Korean bitterling designated as an endangered species in the Red Data Book of endangered fishes of Korea (NIBR 2011). This species is distributed in the River Hangang, Namhangang, Bukhangang and in North Korea. *A. signifer* was thought to spawn from April to June and use only one species (subspecies) of mussel, *Unio douglasiae sinuolatus*, as a host (Back & Song 2005). However, Kim et al. (2013) recently documented that this species prefers the host mussel *Lamprotula leai* to *U. d. sinuolatus*.

Recent studies of the European bitterling, *Rhodeus amarus*, confirmed several oviposition decision factors: mussel species, sex, ventilation rate, reproductive state and the presence of bitterling embryos (Smith et al. 2001, Mills & Reynolds 2002a, b, Reichard et al. 2010). The reproductive ecology of the Japan bitterlings (e.g. *A. rhombeus*, *A. tabira tabira*, *A. t. jordani*, *Tanakia lanceolata*, *T. limbata*, *R. atremius suigensis* and *R. a. atremius*) has been studied and adaptations for utilizing mussels during the spawning season such as seasonal spawning activity, changes in body size, egg size, egg proportion, ovipositor length and size-specific fecundity have been discussed (Kitamura 2007, Kitamura et al. 2009a, Oshiumi & Kitamura 2009). The previous investigations of the bitterling-mussels relationship have focused on *R. amarus* populations and several species of mussels of recent and ancient sympatry or on contrast between invasive and native mussel species (Reichard et al. 2010, 2012, 2015). However, few studies have compared reproductive ecology and host use between bitterling populations that prefer different host mussels which have lived in ancient sympatry.

Phenotypic plasticity, the ability of an organism to change its phenotypes in different environments, is common and often highly adaptive (Price et al.

2003). Although *A. signifer* populations do not differ genotypically (unpublished data), we have predicted that there are several characters that enable them to adaptively use different host species. The aims of the present study were to describe and compare some of the reproductive characteristics of females and use of different host mussels between two populations in the wild environment. We selected two sites that *A. signifer* inhabits and where different species of mussels coexist: Goesan, Dalcheon (stream), where *A. signifer* mainly uses *L. leai* as a host mussel among four coexistent mussels, and Danyang, Gadaecheon (stream), where *A. signifer* spawns using *U. d. sinuolatus*, and no other species of mussel are present. We predicted that different use of host mussel species by the two *A. signifer* populations will result in differences in their reproductive characteristics. We surveyed body size, ovipositor length, egg size, egg volume, number of mature eggs, and number of eggs deposited in mussels to assess the reproductive characteristics and host use of *A. signifer* between two populations.

Material and Methods

Study sites and populations

Samples of two bitterling populations were collected from the Dalcheon (stream), Goesan-gun (36°40'37.24" N, 127°46'39.70" E) ("Goesan population") and the Gadaecheon (stream), Danyang-gun (37°03'24.81" N, 128°23'21.44" E) ("Danyang population") in Korea from April to July in 2011 and 2013. Populations were selected based on the abundance of *A. signifer* and the presence of different species of unionid mussels. At Goesan, three bitterling species, namely *A. signifer* (relative abundance 48.7 %, standard length 33.7-64.0 mm, n = 112; Fig. 1A), *A. yamatsutae* (37.8 %, 46.1-84.3 mm, n = 87) and *A. lanceolatus* (7.8 %, 70.3-82.9 mm, n = 18), coexist together with the oily shinner, *Sarcocheilichthys variegatus wakiyae* (5.7 %, 71.2-74.9 mm, n = 13). These bitterlings have four potential host mussel species: *L. leai* (relative abundance 64.9 %, shell length 35.3-75.1 mm, n = 122; Fig. 1C), *U. d. sinuolatus* (24.5 %, 29.2-49.9 mm, n = 46; Fig. 1D), *Anodonta arcaiformis flavotincta* (8.0 %, 37.2-59.4 mm, n = 15), and *Lanceolaria grayana* (2.7 %, 40.2-73.9 mm, n = 5). At Danyang, *A. signifer* (standard length 37.7-68.5 mm) is the only bitterling species and *U. d. sinuolatus* (shell length 13.1-52.7 mm, n = 295) the only mussel species (Kim et al. 2013). Although the two study sites belong to the River Namhangang, Goesan was about 150 km away from Danyang along the waterway and two sites separated by Chungju dam constructed in 1985. Therefore the study populations

were separated for at least 30 years. Because *A. signifer* is designated as an endangered species by the Ministry of the Environment of Korea, we received permission from the Ministry of Environment of Korea for this study from 2011 to 2013.

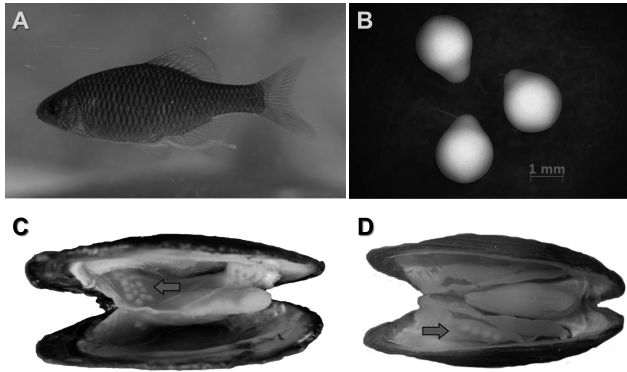


Fig. 1. Photographs of (A) a female *Acheilognathus signifer* (62.3 mm, standard length) in aquarium, (B) eggs of *A. signifer*, (C) *Lamprotula leai* (61.2 mm, shell length) and (D) *Unio douglasiae sinuolatus* (42.3 mm, shell length) containing eggs of *A. signifer*. Arrows indicate the eggs of *A. signifer* in the mussels.

Reproductive characteristics of females

To compare the reproductive characteristics of female *A. signifer* between the two populations, we collected bitterling females using fish traps with paste bait (which attract the bitterling to the traps) every 2 or 3 weeks from 12 April to 12 July in 2013 between 07:00 and 10:00 a.m., corresponding to spawning season (Fig. 1A). After confirming egg maturity by gently squeezing the females' abdomen, we measured the standard length (SL) and ovipositor length (OPL) to the nearest 0.01 mm for all females with at least five mature eggs. Mature eggs were counted and fixed in 5 % formaldehyde solution (Fig. 1B). After all data were recorded, the captured bitterlings were immediately released back into the stream.

Mature eggs preserved in 5 % formaldehyde solution were measured to the nearest 0.01 mm under a stereoscopic microscope (Nikon, SMZ-10, Japan) using the AxioVision LE program (version 4.5, Carl Zeiss, Germany). Egg volumes were estimated as follows: egg volume (mm^3) = $(4 \times 3^{-1}) \pi \times (1 \times 2^{-1}) a \times [(1 \times 2^{-1}) b]^2$, where a and b are the length of the major and minor axes, respectively (Coleman 1991). Egg proportion was estimated as the proportion of the major axis to the minor axis. Ten females with over five mature eggs from each 2- or 3-weekly survey were randomly selected for analyses (exception for 12 July for the Goesan population and 12 May for the Danyang population because of failure to capture 10 females with over five eggs).

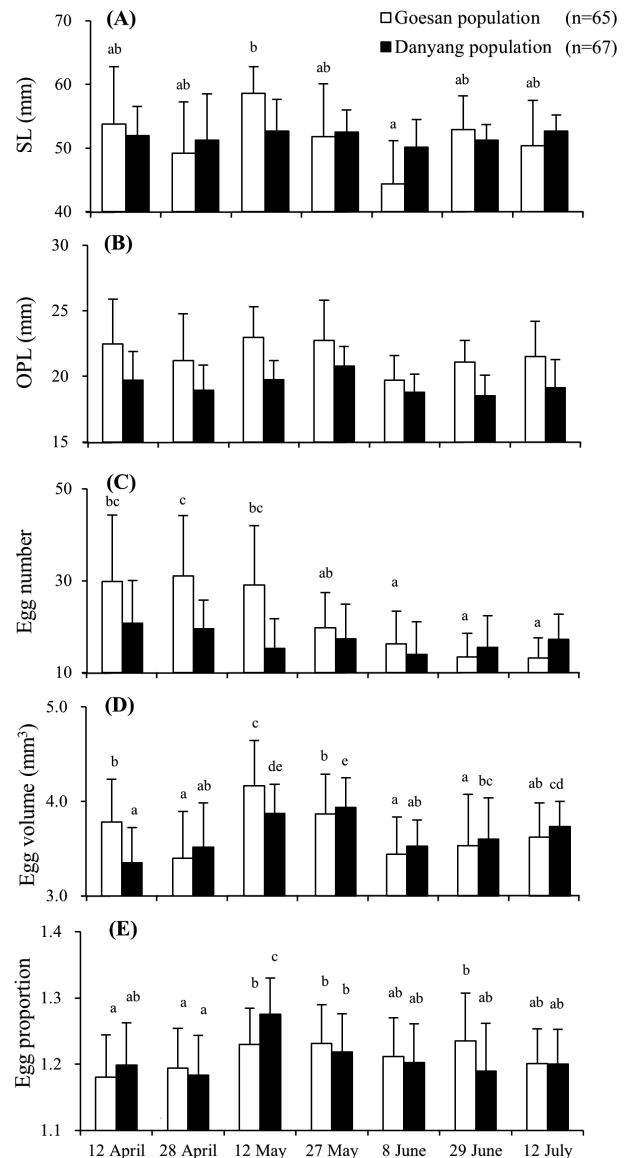


Fig. 2. Seasonal change (mean \pm SD) in (A) standard length (SL), (B) ovipositor length (OPL), (C) egg number, (D) egg volume and (E) egg proportion of female *Acheilognathus signifer* with mature eggs in the two study populations. Letters above bars denote significantly different groups as determined by Scheffé test ($P < 0.05$) during the study period; values with the same letter did not differ significantly.

Host use

To determine host use to mussels in the wild environment, mussels were collected by hand along the bank where mussel density was relatively high between April and July in 2011. The presence of *A. signifer* eggs within the mussels was checked by using a mussel-opening device that enables mussels to be opened to about 1 cm. Shell length to the nearest 0.01 mm were then measured. After checking and measuring, the mussels were returned to their collection sites. The egg shape among *A. signifer* (egg size, long \times short axis; 2.20×1.81 mm), *A. lanceolata*

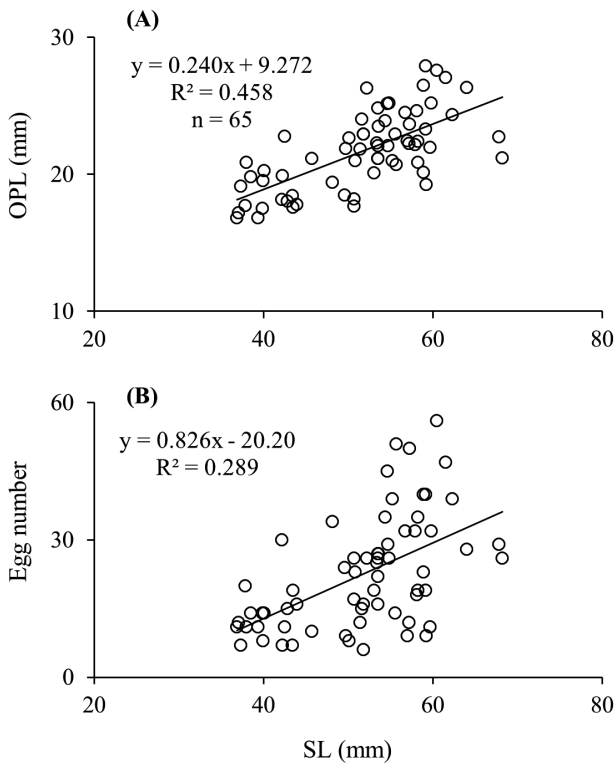


Fig. 3. Relationship between standard length (SL) and (A) ovipositor length (OPL) and (B) egg number of female *Acheilognathus signifer* with mature eggs in the Goesan population during spawning season.

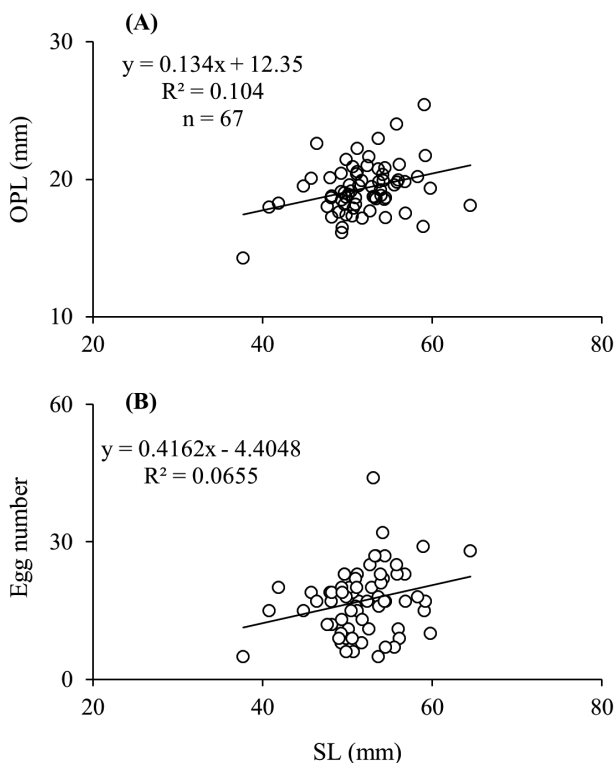


Fig. 4. Relationship between standard length (SL) and (A) ovipositor length (OPL) and (B) egg number of female *Acheilognathus signifer* with mature eggs in the Danyang population during spawning season.

(4.58×1.49 mm) and *A. yamatsutae* (1.91×1.57 mm) within the mussels were identified by the egg type and shape (Kim 1997, Kim et al. 2011). Additionally we have directly observed the development stage of three bitterling's eggs from eggs to larvae in the Petri dish.

Scientific name

Arai & Akai (1988) and Chang et al. (2014) proposed the generic name of *Acheilognathus signifer* should be *Tanakia*, based on morphology of larvae, juveniles and adults, karyotypes and phylogenetic analysis. Given a lack of clear definitions, we restrain from using *Tanakia* for *A. signifer* in this paper.

Statistical analyses

Statistical analyses were conducted using SPSS (SPSS version 18.0, IBM, U.S.A.). A one-way ANOVA and Scheffé test were carried out on study period and SL, OPL, egg number, egg volume, and egg proportion during the spawning season in each of the two populations. Two-way ANOVA and ANCOVA were used to test the OPL and egg number among study period and between two populations on SL as a covariate for confirming the effect of SL (because OPL and egg number on SL are inherently positively correlated). To examine for an interaction of the mussel size with eggs or without eggs and study period on mussel size and between two populations, two-way ANOVA was carried out.

Results

Reproductive characteristics of females in the two populations

The reproductive characteristics of females with mature eggs in the two populations are shown in Table 1. Pear-shaped mature eggs from females with a SL of at least 36 mm were obtained in both populations. In the Goesan population, the SL of females collected on 12 May 2013 was longer than that on 8 June 2013 (Scheffé test, SL, $F_{6, 58} = 3.7$, $P = 0.003$; Fig. 2A). There was no difference in the OPL of females during the study period (one-way ANOVA, $F_{6, 58} = 1.7$, $P = 0.131$; Fig. 2B). The egg numbers of females collected on 28 April were larger than those collected on 8 June, 29 June and 12 July (Scheffé test, $F_{6, 58} = 5.3$, $P < 0.001$; Fig. 2C). The egg volume of females collected on 12 May 2013 was the largest (Scheffé test, $F_{6, 622} = 33.9$, $P < 0.001$; Fig. 2D). The egg proportions of females collected on 12 May and 27 May and 29 June 2013 were higher than those collected on 12 April and 28 April 2013 (Scheffé test, $F_{6, 622} = 10.3$, $P < 0.001$; Fig. 2E).

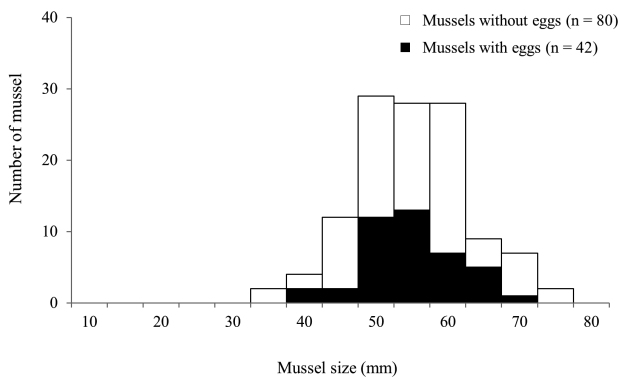


Fig. 5. Mussel size-frequency distribution of *Lamprotula leai* in the Goesan population. White and black bars indicate mussels without and with eggs of *A. signifer*, respectively.

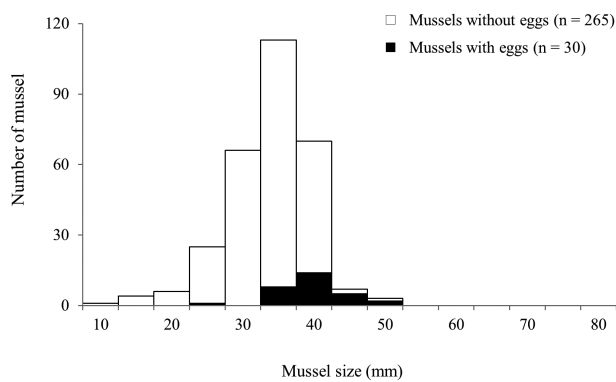


Fig. 6. Mussel size-frequency distribution of *Unio douglasiae sinuolatus* in the Danyang population. White and black bars indicate mussels without and with eggs of *A. signifer*, respectively.

In the Danyang population, there was no significant difference in SL, OPL or egg number of females among the study period (one-way ANOVA, SL, $F_{6,60} = 0.5$, $P = 0.797$; OPL, $F_{6,60} = 1.8$, $P = 0.114$; egg number, $F_{6,60} = 1.1$, $P = 0.370$; Fig. 2A, B, C). The egg volumes of females collected on 12 May and 27 May 2013 were larger than those measured for females collected on 12 April and 28 April 2013 (Scheffé test, $F_{6,630} = 30.7$, $P < 0.001$; Fig. 2D). The egg proportion of females collected on 12 May 2013 was the highest (Scheffé test, $F_{6,630} = 19.4$, $P < 0.001$; Fig. 2E).

In the two populations, OPL and egg number were significant positively correlated with the SL of females (linear regression, both $P < 0.001$; Figs. 3 and 4). There were significant differences in OPL of

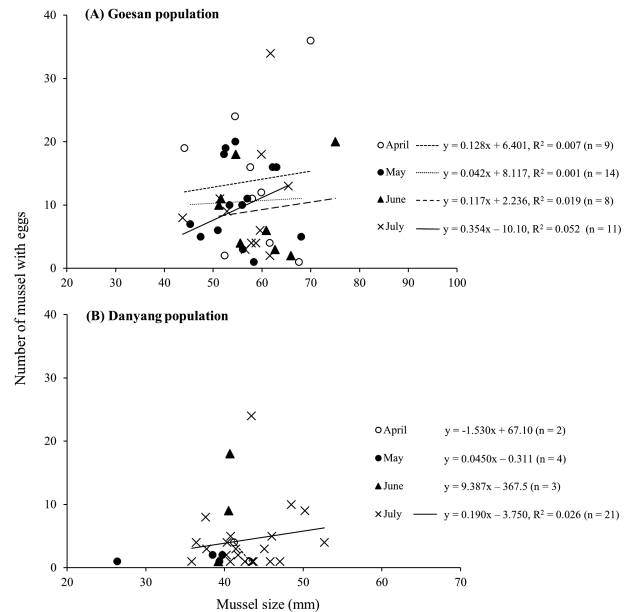


Fig. 7. Relationship between mussel size and number of mussels with eggs of *Acheilognathus signifer* in the Goesan population (A) and Danyang population (B) during spawning season.

females among study period and the two populations, and there was no interaction (two-way ANOVA, study period, $F_{6,117} = 2.8$, $P = 0.012$; two populations, $F_{1,117} = 32.5$, $P < 0.001$; interaction, $F_{6,117} = 0.5$, $P = 0.755$). The OPL of females did not differ within study period, but large difference between the two populations was found, and there was no interaction and a strong effect of SL as a covariate (ANCOVA, study period, $F_{6,117} = 1.9$, $P = 0.081$; two populations, $F_{1,117} = 48.1$, $P < 0.001$; interaction, $F_{6,117} = 0.1$, $P = 0.997$; SL, $F_{1,117} = 48.0$, $P < 0.001$). Although the SL was analyzed as a covariate the SL did not make the different result for two populations. There were significant differences in the egg number of females among study period and two populations, and there was interaction (two-way ANOVA, study period, $F_{6,117} = 5.5$, $P < 0.001$; two populations, $F_{1,117} = 8.9$, $P = 0.003$; interaction, $F_{6,117} = 2.6$, $P = 0.023$). The egg number of females significantly differed among study periods and the two populations, and there was interaction and a strong effect of SL as a covariate (ANCOVA, study period, $F_{6,117} = 6.3$, $P < 0.001$; two populations, $F_{1,117} = 12.9$, $P < 0.001$; interaction, $F_{6,117} = 2.8$, $P = 0.013$; SL, $F_{1,117} = 48.0$, $P < 0.001$). Although the SL was analyzed

Table 1. Reproductive features of female *Acheilognathus signifer* with mature eggs in the two populations.

Populations	Number	Standard length (mm)		Ovipositor length (mm)		Egg number		Egg volume (mm ³)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
Goesan	65	51.65 \pm 8.02	36.87-68.17	21.68 \pm 2.84	16.81-27.90	22.46 \pm 12.31	6-56	3.69 \pm 0.53	2.15-5.24
Danyang	67	51.82 \pm 4.45	37.72-64.46	19.33 \pm 1.85	14.28-25.58	17.16 \pm 7.23	5-44	3.64 \pm 0.41	2.13-4.67

as a covariate the SL did not make the different result for study periods and the two populations.

Host use in the two populations

In the Goesan population, the total number of *L. leai* having *A. signifer* eggs was 42 (prevalence 34.4 %, $n = 122$). Only an individual of *U. d. sinuolatus* had *A. signifer* eggs (2.1 %) and there were no *A. signifer* eggs in *A. a. flavotincta* and *L. grayana*. There was no significant difference in the mussel size that had *A. signifer* eggs (57.24 ± 6.84 mm, range; 43.71-75.07, $n = 42$) versus those that did not (57.39 ± 7.98 mm, range; 35.73-75.14, $n = 80$) and the study period (two-way ANOVA, mussel size with eggs or without eggs, $F_{1,113} = 0.1$, $P = 0.906$; study period, $F_{3,113} = 0.6$, $P = 0.603$; Fig. 5). The number of *A. signifer* eggs within *L. leai* was 11.10 ± 8.35 (range, 1-36, $n = 455$).

In the Danyang population, 30 *U. d. sinuolatus* had *A. signifer* eggs (prevalence 10.1 %, $n = 295$). There was significant difference in the mussel size in having *A. signifer* eggs (41.66 ± 4.88 mm, range; 26.39-52.67, $n = 30$) versus those that did not have eggs (35.59 ± 5.53 mm, range; 13.11-50.09, $n = 265$) and the study period (two-way ANOVA, mussel size with eggs or without eggs, $F_{1,287} = 15.8$, $P < 0.001$; study period, $F_{3,287} = 5.1$, $P = 0.002$; Fig. 6). The number of *A. signifer* eggs within *U. d. sinuolatus* was 4.40 ± 5.30 (range, 1-24, $n = 132$).

The mussel size and egg number of *A. signifer* within the mussels significantly differ between the two populations, but not within study period, and there was no interaction between the two populations and the study period (two-way ANOVA, mussel size, two population, $F_{1,64} = 87.3$, $P < 0.001$; study period, $F_{3,64} = 1.7$, $P = 0.184$; interaction, $F_{3,64} = 0.8$, $P = 0.516$; egg number, two populations, $F_{1,64} = 8.3$, $P = 0.005$; study period, $F_{3,64} = 0.4$, $P = 0.773$; interaction, $F_{3,64} = 1.0$, $P = 0.413$, Figs. 5, 6, 7).

Discussion

The present study was designed to analyze specific characteristics and host use of *A. signifer* in two bitterling populations that use different host mussels: *L. leai* (prevalence: 63.1 %) and *U. d. sinuolatus* (2.1 %) (Kim et al. 2013). Although the SL of females in the two populations was very similar, OPL and egg number were significantly different between them (Table 1, Fig. 2). Kitamura (2006a) and Kitamura et al. (2012) suggested that the primary importance of OPL is to reach the appropriate part of the gill cavity in the mussel. In the present study, we confirmed that females had longer ovipositors in the Goesan

population than females in the Danyang population. We assume that the difference in ovipositor length of *A. signifer* between the two populations may be adaptive for females to deposit their eggs deeper or lower inside the mussel cavity and the survival of embryos is likely to be higher and the risk of premature embryo ejection lower. However, further research is required to clarify the relationship between ovipositor length of bitterling and egg position length within the mussels at individual level in rearing experiment. We note that our contrast is composed of two populations only; therefore the differences in the reproductive characteristics may arise from other sources, such as predation pressure or environmental conditions.

The SL and OPL of female *A. signifer* from both populations did not change during the study period except for one case (Fig. 2). These results are similar to Kitamura's report (2006b) but differ from those in previous studies (Kitamura 2006a, b, 2007, Kitamura et al. 2009a) where the SL and OPL of bitterling females were observed to change seasonally. Kitamura (2006a) pointed out that the relationship between bitterlings and mussels can be investigated further by studying fluctuations in ovipositor length and mechanisms for laying eggs using the ovipositor. The egg volume and egg proportion of the females changed during the study period (Fig. 2). *A. signifer* eggs were small and thin earlier in the spawning period (April), and then subsequently became larger. Egg number of females started to increase from April to May and was highest in May, but decreased in June and July. These findings are consistent with those reported in several studies of other bitterlings, *R. ocellatus kurumeus*, *A. cyanostigma* and *R. atremius*, which suggest that embryo survival may be related to egg size and shape (Kamler 1992, Kitamura 2005, 2006b, Kitamura et al. 2009a). *A. signifer* may have small and thin eggs due to the low water temperature and low feeding activity of females earlier in the spawning season (April). In contrast, during the peak season (May), eggs may become larger because of optimal water temperatures and an improvement in the condition of the females, resulting in enhanced embryo survival and development. Later in the spawning season (June and July), eggs may become smaller and thinner again because of high temperatures and because females were in decreased body condition due to several rounds of spawning.

L. leai in Goesan had a larger shell length and more eggs than *U. d. sinuolatus* in Danyang (Figs. 5 and 6). Moreover, the Goesan population of *A. signifer* had a longer ovipositor than *A. signifer* in Danyang.

Based on previous reports (Mills & Reynolds 2002a, Kitamura et al. 2012), the reason that *A. signifer* had more eggs in the Goesan population may be due to the larger shell length of *L. leai*, which is associated with a larger gill area to deposit eggs in comparison with *U. d. sinuolatus* (Kim 2014). However, there was no positive relationship between mussel size and the egg number of *A. signifer* in either population. These findings are consistent with those found in an earlier study of host mussel utilization of *A. longipinnis* (Kitamura et al. 2009b). In the current study, most *A. signifer* eggs were observed in *L. leai* that had a shell length of 50–60 mm in Goesan and *U. d. sinuolatus*, which had a shell length of 35–45 mm in the Danyang population (Figs. 5 and 6). Mills et al. (2005) suggested that the width and length of the interlamellar spaces plays very important role for the bitterling embryo survival. It is possible that the preferred shell size categories of the respective host mussels are superior in terms of the favourable width

and length of the interlamellar space and minimize the premature ejections of the *A. signifer* eggs and embryos.

For bigger mussels, bitterling embryos are more likely to be deposited nearer the exhalant siphon in the gill cavity and may face a greater risk of ejection (Kitamura 2006a). In the present study, the shell length of *U. d. sinuolatus* and the OPL of *A. signifer* from the Danyang population were smaller and shorter, respectively, than those of *L. leai* and *A. signifer* from the Goesan population. The dissimilar OPL of *A. signifer* between the two populations may be related to phenotypic plasticity to use of different host mussel species and sizes to minimize egg ejection and maximize embryo survival (Mills et al. 2005, Kitamura et al. 2012).

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