Title: Breeding ecology and seasonal abundance of the giant water bug *Appasus japonicus* (Heteroptera, Belostomatidae)

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Abstract

Males of the giant water bug *Appasus (= Diplonychus) japonicus* Vuillefroy (Belostomatidae: Heteroptera) care egg masses on their back, but little is known about the relationship between seasonal abundance and breeding ecology of the species. In the present study, therefore, a field survey based on a mark-and-recapture census was carried out at three survey points within a rice paddy area (0.3 km^2) where *A. japonicus* forms a meta-population in northern Okayama, Japan. We investigated the body size, seasonal abundance, dispersion, egg mass size (the number of eggs within one egg mass), number of egg masses, and the total eggs cared on the back of each male as the fundamental parameters of the population and breeding. Significant differences in egg mass size, number of egg masses, and total number of eggs that males cared was found among the survey points. The present results suggested the possibility that the differences in breeding parameters of *A. japonicus* were influenced by differences in environmental factors among the microhabitats. These results are discussed in conjunction with previous reports on seasonal abundance and breeding systems in Belostomatidae bugs.

INTRODUCTION

Males of the giant water bug (Belostomatidae: Heteroptera) are unique in that they perform exclusive post-zygotic paternal care for the eggs that females lay on the male's dorsum from oviposition until hatching (e.g., Lauck & Menke 1961; Smith 1997; Munguia-Steyer & Macias-Ordonez 2007). The belostomatids are predacious water bugs and include more than 140 species belonging to seven genera (Menke 1979; Smith 1997). Two Belostomatinaes *Appasus japonicus* Vuillefroy, 1864 and *A. major* (Esaki, 1934) are widely distributed in Japan, excluding the Ryukyu Islands. The morphological and distributional differences between *A. japonicus* and *A. major* have been identified (Hori 2001; Tsunoda 2002). Much research on *A. major* in the field has been conducted on the breeding strategy (Ichikawa 1989; Kawano & Hoshikawa 2003; Ohba & Perez Goodwyn 2009), diet, seasonal occurrence (Okada & Nakasuji 1993b), and habitat utilization (Saijo 2001, 2002; Mukai & Ishii 2007). For *A. japonicus*, the seasonal occurrence and diet have been examined in the field (Okada & Nakasuji 1993a, b; Ohba & Nakasuji 2006). However, the life history and breeding characteristics, such as the number of eggs cared by an *A. japonicus* male, have not been well investigated.

Appasus japonicus is declining in number in some regions of Japan, and it is designated as a *Red Data List* species in 31 of 47 prefectures (Association of Wildlife

Research & EnVision 2007). Therefore, it is important to study the ecology of this species in order to obtain fundamental information for more effective management of the *A. japonicus* population. Especially, the information of breeding ecology in the field is important because of key of population dynamics.

In the present study, a field survey based on a mark-and-recapture census was carried out at three survey points within a rice paddy area in northern Okayama, Japan, where *A. japonicus* forms a meta-population. The body size, seasonal occurrence, dispersion, egg mass size (the number of eggs within an egg mass), number of egg masses, and total number of eggs cared on the back of each male were investigated over one year as parameters of fundamental population and the breeding. The size of egg mass cared by one male differed at the three survey points from June to July, the middle of the breeding season. To investigate the factors inducing the difference in the egg mass size in mid breeding season, four items were compared among the survey points: body size, sex ratio, proportion of egg-carrying males, and water temperature.

MATERIALS AND METHODS

Study sites

Field surveys were conducted at three survey points (A, B, and C) within 0.3 km^2 at Misaki Town, Okayama Prefecture, Japan in 2006 (Fig. 1). We conducted censuses weekly from April to September and biweekly from October to November. The survey points A, B, and C were a ditch connected to a fallow field (24.5 m^2), a ditch connected to a rice field (12.1 km^2), and a wetland and ditch connected to rice field (137.2 km^2), respectively. The water level at survey point A fluctuated a little, with the water depth at 0.05-0.15 m. Survey point B was filled with water to depths of about 0.5 m during the mid-May to the end of June (irrigation period for rice cultivation). In early July, the water was drained from the abutting rice field, and the rice field continued to drain for a few weeks, eventually becoming fully drained, with the ground exposed to the sun (drainage period for rice cultivation). Nevertheless, the water at survey point B remained at 0.03 m deep, during the drainage period for rice cultivation. At survey point C, the water in the waterway was 0.4 m depth during the irrigation period for rice cultivation, and 0.03 m depth during the drainage period and after harvesting. The water level in the wetland also varied with that of the abutting rice field, like survey point B. Water in the wetland was up to 0.05 m deep, but often completely dried up in places after mid-July. The survey points A-B, A-C, and B-C were 10 m, 300 m, and 290 m apart, respectively, as the crow flies (see Fig. 1).

Seasonal abundance

To investigate the seasonal abundance of *A. japonicus* adults at each study point, a sweeping census using a 3-mm mesh D-frame dipnet (0.28 m wide) was done for 30 minutes. The width of the prothorax of newly captured adults was measured using calipers (CNO-15, TJM Design, Tokyo) as an index of body size, and then each was numbered individually on the thorax using paint markers (Paint Marker®, Mitsubishi Pencil Co. Ltd., Tokyo). The individual number, age (overwintered or new adult) and sex, and the number of eggs on the backs of males were recorded. We recognized new adults by the intact wings and/or soft body. After recording, the insects were immediately released at their point of capture. The Jolly-Seber method (Jolly, 1965; Seber, 1965) was applied in order to estimate the number of adults. The Mann-Whitney *U* test was used to compare the number of times each sex was captured.

To evaluate the relative abundance of nymphs of *A. japonicus*, the above-mentioned D-frame dipnet was pulled 0.5 m along the water surface 20 times at each survey point. This procedure was repeated 3 times in one day. The number of nymphs in each instar was counted.

Egg mass cared on back of each male

When we found egg-carrying males during the investigation of seasonal abundance, we took a picture of the egg mass on the back of each male using a digital camera (CAMEDIA X-2 and μ810, Olympus, Tokyo) in order to count the number of eggs. In the pictures, each egg mass was evaluated by the number of eggs, shape, color, and embryonic developmental stage. In Belostomatidae species, the volume of the egg increases as the embryo develops (Madhavan 1974). If eggs had been added to an egg mass before all eggs hatched, then the total number of eggs at recapture was recorded. The number of egg masses and the total number of eggs carried by each male was examined by one-way analysis of variance (ANOVA) and Scheffe's post hoc test. The egg mass size (the number of eggs within one egg mass) was analyzed using two-way ANOVA with month (May, June, July, and August) and survey point (A, B, and C) as main factors. Separate one-way ANOVAs were applied to the differences among the survey points in each month when a significant "month-by-survey-point interaction" effect was encountered. Scheffe's tests were performed to assess the differences among the survey points when significant effects were detected by one-way ANOVA.

Body size, sex ratio, proportion of egg-carrying males, and water temperature

The prothorax width of adults was analyzed using two-way ANOVA with sex and survey point (A, B, and C) as main factors. Temperatures at 0.02-0.03 m depth from water surface were recorded at intervals of 2 hours for 24 hours from 00:00 on 5 July 2007 using a digital water thermometer (CT-450WR, Custom Co. Ltd., Tokyo). The day was sunny occasionally cloudy. To examine the differences in sex ratios (relative female-to-male ratio) and proportion of egg-carrying males among the survey points in June and July (middle breeding season), $R \times C$ tests of independence were applied. Numbers estimated by the Jolly-Seber method was used in the analysis of sex ratio. If significant effects were found, then pairwise comparisons by χ^2 test were performed among each survey point correcting the significance level for multiple comparisons by the sequential Bonferroni method (Rice 1989).

RESULTS

Seasonal abundance

Because no noteworthy differences were observed in the seasonal abundances of *A. japonicus* among the three survey points, we show the total number from all three survey points in Figure 2. Overwintered adults were abundant from mid-June to early July. Egg-carrying males were observed from 23 April to 27 August. First instar nymphs were seen from late May. From early June, the second and third instar nymphs were observed. The fourth and fifth instar nymphs appeared in late June and early July, respectively. Newly emerged adults were seen mid-July. We did not find new adults caring eggs within the survey year.

Males were captured 2.9 ± 2.5 (mean ± S.D.) times and females 2.8 ± 2.3 times. The maximum number of captures was 13 times for males and 16 times for females; no significant difference was found between sexes in the median numbers of captures (Mann-Whitney U test, $P = 0.47$). We detected four cases of migration among the three survey points by females only (Figure 3). One female migrated from survey point A to B, two females migrated from survey point A to C, and one female migrated from C to A.

Egg mass cared on backs of males

The number of eggs per mass, number of egg masses, and total number of eggs cared on the back of each male were 72.4 ± 33.2 (max. 155), 1.9 ± 1.2 (max. 8), and 123.0 ± 92.2 (max. 531) (mean ± S.D., n = 148) at all survey points. There were significant differences in the number of egg masses and the total number of eggs among the three survey points (one-way ANOVA, number of egg masses, $F_{2, 158} = 4.56$, $P = 0.02$; total number of eggs, $F_{2, 145} = 22.56$, $P < 0.001$ for log-transformed data). The number of egg masses and the total number of eggs at survey point C were significantly higher than those in either survey point A or B (Scheffe's test, $P < 0.05$, Figure 4-I, II). A two-way ANOVA on the egg mass size revealed that the effects of month and survey point were significant but the month-by-survey point interaction effect was not (month: $F_{3, 242} = 10.19$, $P < 0.001$, survey point: $F_{2, 242} = 32.55$, $P < 0.001$, month-by-survey point: $F_{6, 242} = 1.45$, $P = 0.20$ for log-transformed data). From June to July, the egg mass size at survey point C was the highest among the three survey points (Figure 4-III). No differences were found in the egg mass size among the survey points in May and August. In this study, a total of 702 adults (333 males and 369 females) were examined, but no egg-carrying females were found.

Body size, sex ratio, proportion of egg-carrying males, and water temperature

Two-way ANOVA on the prothorax width revealed that sex had a significant effect, but survey point and sex-by-survey point interaction effects were not significant (sex: $F_{1, 629}$ = 16.03, P < 0.001, survey point: $F_{1, 629}$ = 0.59, P = 0.55, sex-by-survey point: $F_{1, 629}$ = 1.26, P = 0.28). Females had a significantly wider prothorax than males; female: n = 329, 7.30 ± 0.42, and male: n = 298, 7.12 ± 0.40 (mean ± S.D.). The sex ratio (the relative female-to-male ratio) was significantly different among the three survey points ($R \times C$ test: df = 2, χ^2 = 18.6, P < 0.0001, Figure 5-I). The sex ratio at survey point A was significantly higher than at survey points B and C, and the sex ratio at survey point B was significantly lower than at survey point C (χ^2 test: A vs. B; χ^2 = 11.7, P < 0.001: A vs. C; χ^2 = 9.4, P = 0.003: B vs. C; χ^2 = 5.1, P = 0.03). The proportion of egg-carrying males was significantly different among the three survey points ($R \times C$ test: df = 2, χ^2 = 25.8, P < 0.0001, Figure 5-II). At survey point C, the proportion of egg-carrying males was significantly higher than that at both survey points A and B (χ^2test: A vs. B; χ^2 = 2.4, P = 0.09: A vs. C; χ^2 = 11.1, P = 0.002: B vs. C; χ^2 = 24.8, P < 0.001). During the day, the water temperature at survey point C was higher than that at both survey points A and B regardless of the fluctuation (Figure 6).

DISCUSSION

Seasonal occurrence, migration and breeding ecology

The seasonal abundances of *A. japonicus* revealed by the present study were about 1 month later than that revealed by a previous study (Okada & Nakasuji 1993b). The numbers of overwintered and newly emerged adults peaked in June and mid September, respectively (Figure 2). The nymphs appeared from late May. Okada and Nakasuji (1993b) suggested that *A. japonicus* produces 1-2 generations within a year in the plains in Okayama. We did not find breeding by the new adults in the present study. The reason may be that our study site is in the cool mountain region in northern Okayama. This suggests variations in the life-history strategy of *A. japonicus* even within western Japan that depend on altitude or the difference in the temperature of the study sites.

Because some migration among the three survey points was observed (Figure 3), the population of *A. japonicus* may form a meta-population within the entire study site. All migrating individuals were females. This may be the first report concerning sexual differences in the migration of belostomatids, although the sample size is too small to be definitive. This may be explained by the fact that the males carrying eggs on their back are not able to migrate by flying, while females can.

The seasonal abundances of *A. japonicus* revealed by the present study and of

A. major revealed by Okada & Nakasuji (1993b) were almost identical. However, it is interesting that the egg mass size cared on the male's dorsum is different between the two species: *A. japonicus* with a smaller body size cared more eggs than *A. major* with a larger body size. An *A. major* male cared up to four egg masses during one breeding season, and the maximum number of egg mass size was 127 (Ichikawa 1989). In contrast, the maximum numbers of egg masses and egg mass size carried by *A. japonicus* during one season were 8 and 155, respectively. This may be due to the difference in egg size between the two species; egg widths were 0.97-1.38 mm (range) in *A. japonicus* and 1.45-1.94 mm in *A. major* (S. Ohba, unpubl. data, 2008).

Detecting egg-carrying females in the fields is one of key issues in the breeding systems of Belostomatidae species. In six Columbian Belostomatinae species, *Belostoma flumineum* Say, 1832 (Kruse & Leffler, 1984), *Abedus indentatus* (Haldeman, 1854) (Kraus, 1985), *B. oxyurum* (Dofour, 1863) (Schnack & Domizi 1985), *B. elongatum* Montandon, 1908, *B. elegans* (Mayr, 1871), and *B. micantulum* (Stål, 1860) (Estévez et al. 2006), a few egg-carrying females were found in the fields when "free" males were probably scarce. We did not find egg-carrying *A. japonicus* females in the field, but we have observed that females laid eggs on the back of other females and substrata (aquatic plants *Egeria* spp. and metal gauze) when only females were reared in a cup in the laboratory (K. Kato, unpubl. data, 2007). Although the eggs did not hatch, it suggested the possibility of brood parasitism by females in *A. japonicus*.

Differences in the egg mass size among microhabitats

We found a difference in egg mass size among the three survey points especially in mid breeding season (June and July); males at survey point C had larger egg masses compared to points A and B (Figure 4-III). The difference might be explained by 1) the larger male body size at survey point C compared to survey points A and B, 2) the highest skew of the sex ratio at point C, 3) the shortage of egg-carrying males at point C; if most males have not matured, females will intensively lay eggs on the backs of the limited number of propagable males, or 4) a difference in environmental factors such as water temperature among the three survey points. Prothorax width as an index of body size was almost the same at all the survey points (see results). The sex ratio at survey point A, rather than at survey point C, was the highest of the three survey points (Figure 5-I). In addition, contrary to our expectation, the proportion of egg-carrying males was the highest at survey point C (Figure 5-II). Thus, the first three above-mentioned possibilities do not explain the difference in the egg mass size.

Because there are gene flows among these survey points (Figure 3), this difference in the egg mass size may depend on environmental factors, rather than genetic differences. A likely explanation is the difference in water temperature among the survey points (see Figure 6). Because survey points A and B were filled with water trickling down from the adjacent mountain, the water temperature at survey points A and B was lower than that at survey point C (Figure 6). In contrast, survey point C was sunny, and hence irrigated with warm water. It is known that the egg production rate in females of *Appasus major*, a species closely related to *A. japonicus*, accelerates as the water temperature increases (Ichikawa 1993). Consequently, the egg production rate of females at survey point C might be the highest of the three survey points.

Although the water temperature at survey point B was higher than that at survey point A (Figure 6), the number of egg masses, total number of eggs, and egg mass size at survey point B were not higher than that at survey point A (Figure 4). The sex ratio at survey point A was higher than at survey point B (Figure 5-I). As a result, females may intensively lay eggs on the backs of the limited number of propagable males in survey point A. Thus, both water temperature and sex ratio will determine the egg mass size. Moreover, the reproduction of *A. japonicus* will be limited by its food resources within microhabitat, as for many other predatory insects (e.g., Lenki, 1984;

Pearson & Knisley, 1985; Juliano, 1986). The interaction of these factors (water temperature, sex ratio and food resource) on the egg mass size might be an interesting subject for future study.

Although the factor that induced the difference in egg mass size among the survey points remains to be determined, the present results showed the possibility that the breeding and seasonal abundance of *A. japonicus* were influenced by the difference in microhabitats. Water temperature of breeding site of *A. japonicus* may be a key point from the view point of conservation ecology, because warm habitat might enhance the breeding capacity of this species.

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Figures legends

Figure 1. Study site in Obara, Misaki Town, northern Okayama Prefecture, Japan.

Figure 2. Seasonal occurrence of *A. japonicus* adults and nymphs in all study sites in 2006. Vertical lines in adults and nymphs show variance calculated by Jolly-Seber method and standard error of the mean, respectively.

Figure 3. Migration among survey points in *A. japonicus*. Numbers in parenthesis are the total numbers of marked insects. Arrows indicate the direction they were going. The numbers beside the arrows indicate the number of moving adult insects. Roman and italic letters show females and males, respectively.

Figure 4. Number of egg masses (I), total number of eggs (II), and egg mass size (number of eggs per mass) (III) at the three survey points (A, B, and C). Vertical lines indicate the standard error. Same letters denote no significant differences among the survey points ($P < 0.05$, Scheffe's test).

Figure 5. Sex ratio (I) and the proportion of egg-carrying males (II) in June and July at the three survey points (A, B and C). Numbers estimated by Jolly-Seber method was used in the sex ratio (I). Same letters denote no significant differences among the survey points ($P < 0.05$, χ^2 with sequential Bonferroni).

Figure 6. Water temperatures at the three survey points on 5 July 2007.

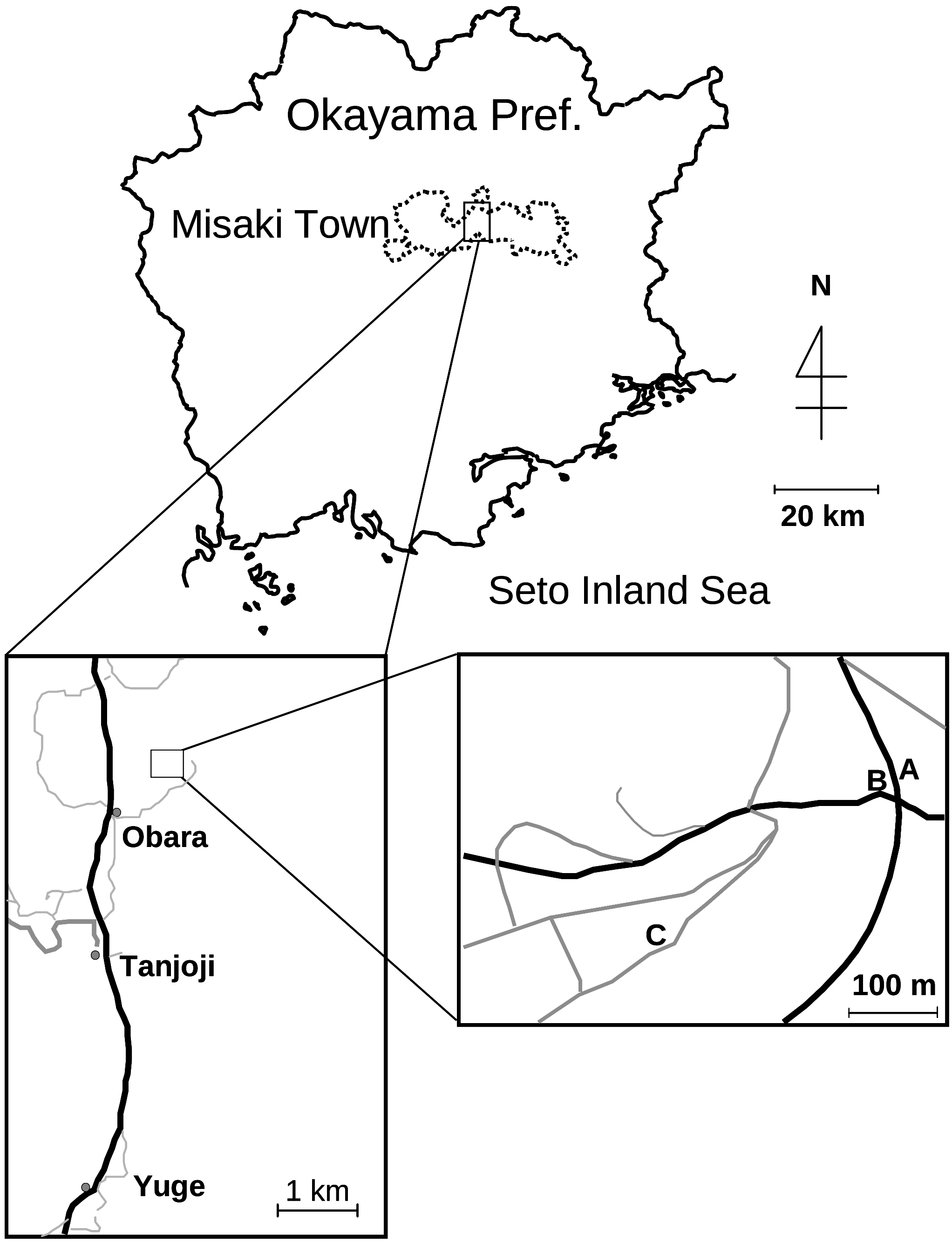
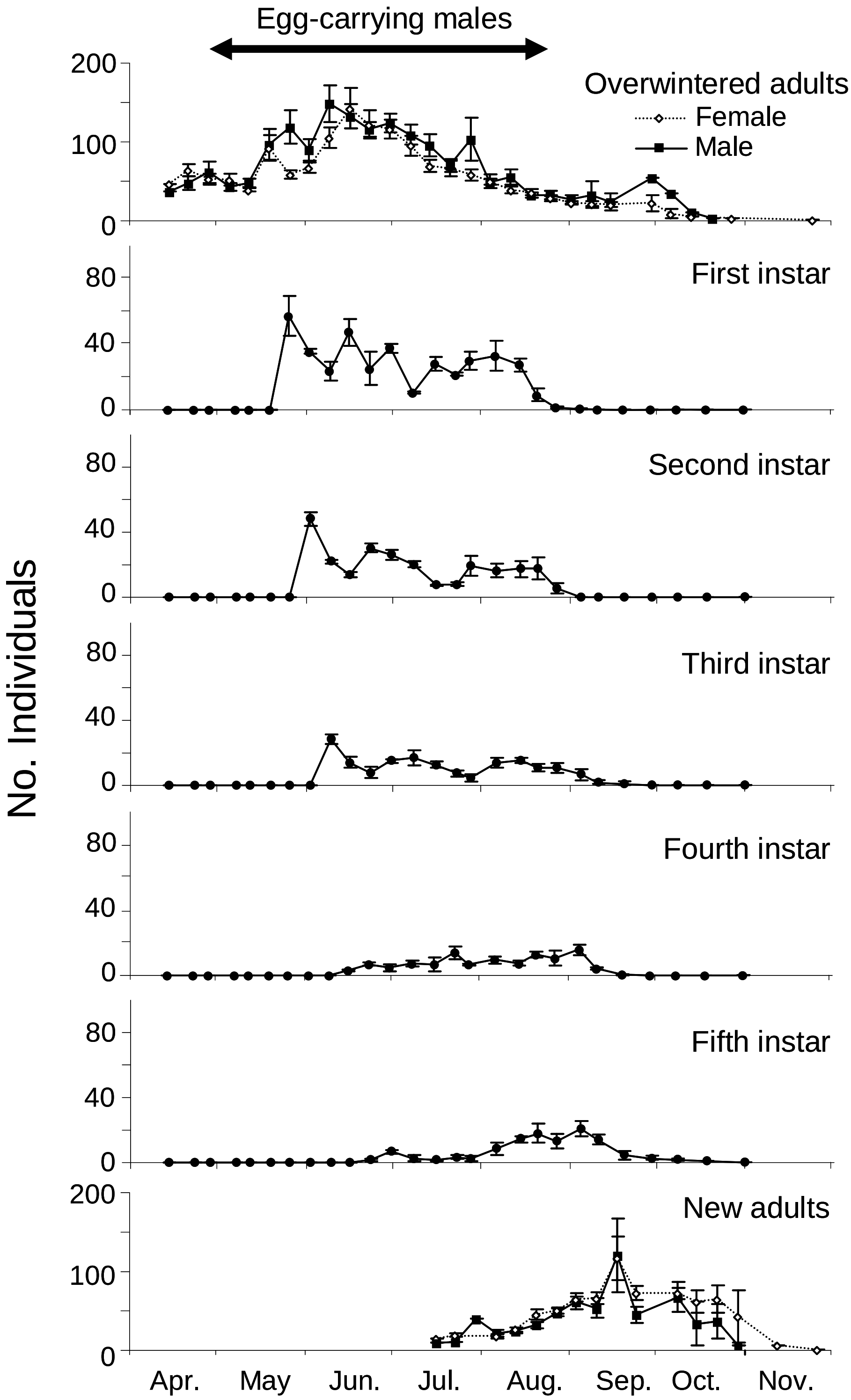

Okayama Pref.
Misaki Town
N
20 km
Seto Inland Sea
Obara
Tanjoji
Yuge
1 km
A
B
C
100 m

Figure 1



Egg-carrying males
Overwintered adults
Female
Male
First instar
Second instar
Third instar
Fourth instar
Fifth instar
New adults
No. Individuals
200
100
0
80
40
Apr.
May
Jun.
Jul.
Aug.
Sep.
Oct.
Nov.

Figure 2

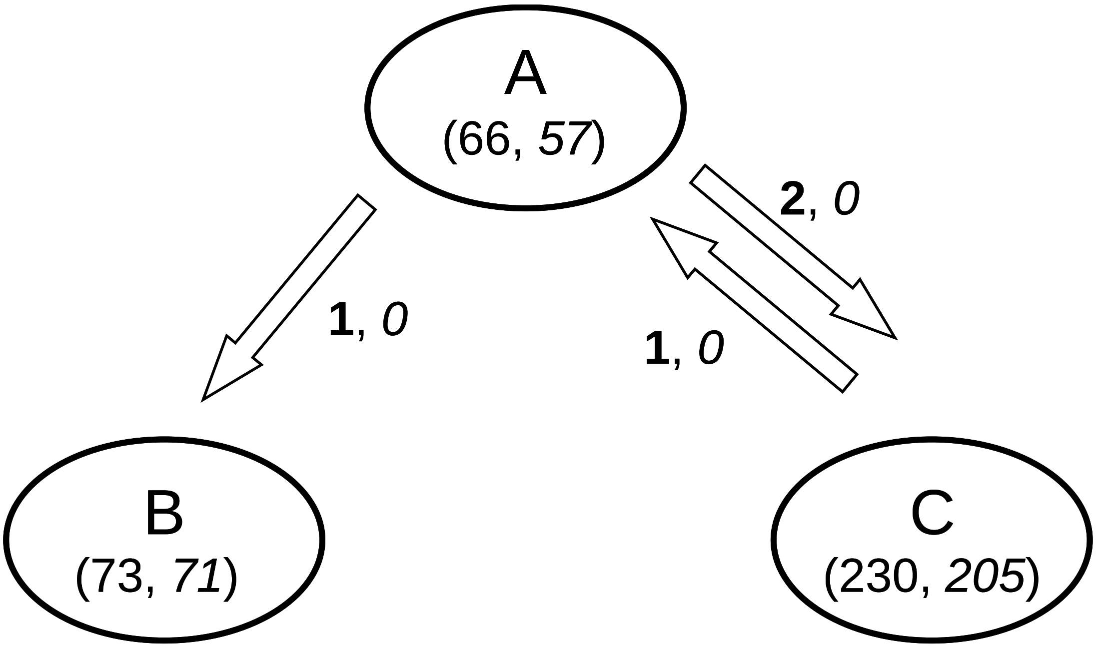
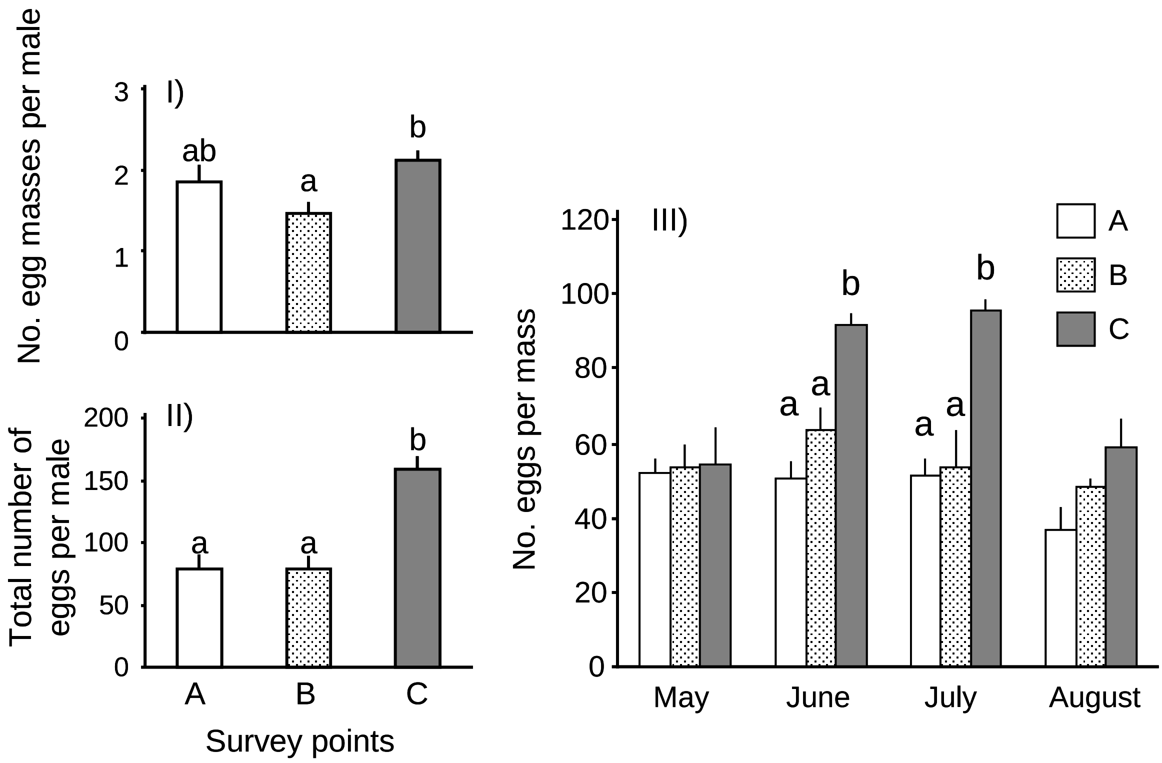


Figure 3



No. egg masses per male
I)
3
2
1
0
ab
a
b
Total number of eggs per male
II)
200
150
100
50
0
a
a
b
A
B
C
Survey points
No. eggs per mass
III)
120
100
80
60
40
20
0
a
a
b
a
a
b
May
June
July
August
A
B
C

Figure 4

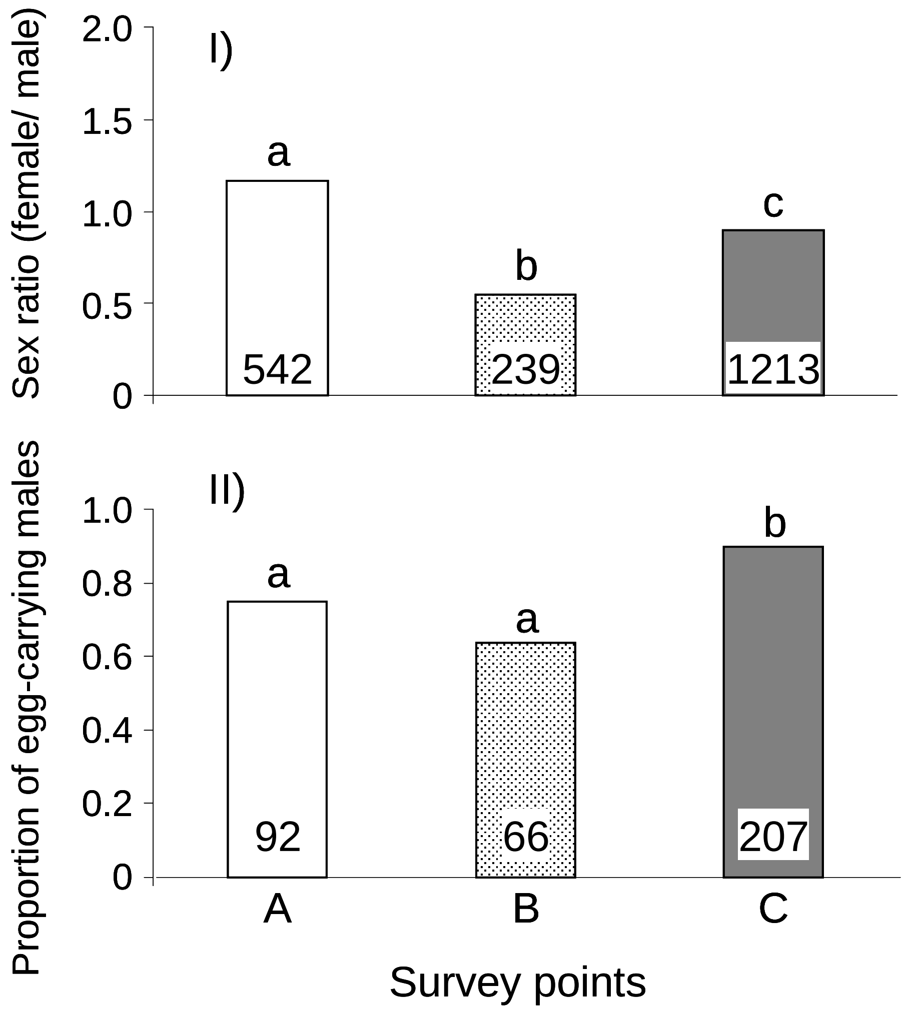

I)
Sex ratio (female/ male)
2.0
1.5
1.0
0.5
0
a
b
c
542
239
1213
II)
Proportion of egg-carrying males
1.0
0.8
0.6
0.4
0.2
0
a
a
b
92
66
207
A
B
C
Survey points

Figure 5

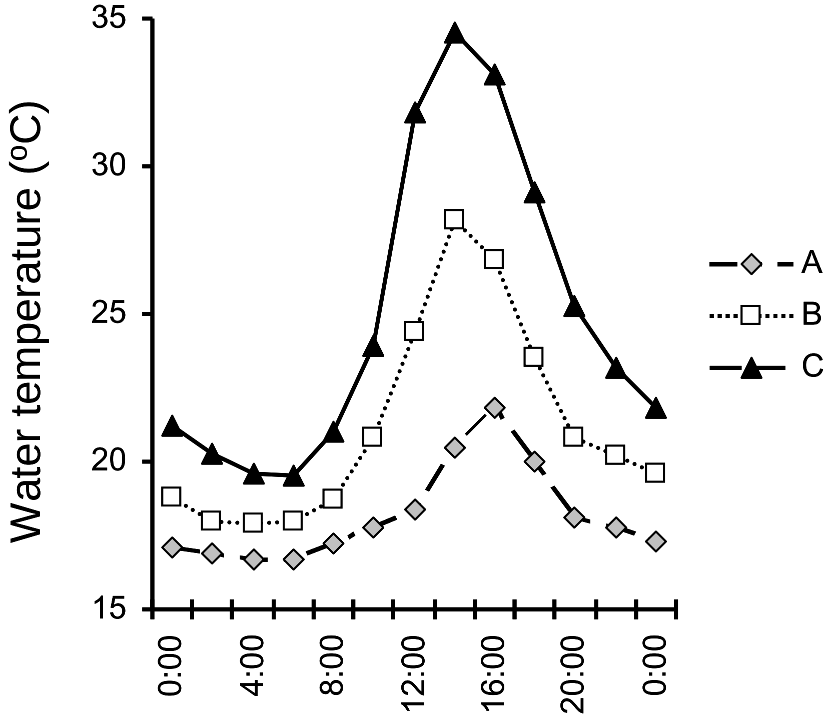

35
30
25
20
15
Water temperature (ºC)
0:00
4:00
8:00
12:00
16:00
20:00
0:00
A
B
C

Figure 6