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Why Are Larval *Fejervarya kawamurai* Scarce in the Paddies Where Larvae of Other Frogs Are Abundant? Experimental Insights

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Abstract: Although *Fejervarya kawamurai* is a common frog in the paddies of western Japan, their larvae seem to be scarce in the paddies where larvae of *Hyla japonica* and *Pelophylax nigromaculatus* are abundant. To examine the cause of this phenomenon, we surveyed the reproductive season of these three species at two sites with different irrigation schedules using an automatic recording system. In addition, we conducted several experiments to test three possible explanations; (1) maternal avoidance from *P. nigromaculatus* larvae in the selection of oviposition site, (2) predation on *F. kawamurai* eggs by *P. nigromaculatus* and the larvae of other species, and (3) negative interactions of *P. nigromaculatus* larvae on the larvae of *F. kawamurai*. In paddies flooded throughout the year, calling of *H. japonica* and *P. nigromaculatus* started far earlier than in *F. kawamurai*, whereas in paddies irrigated in mid-May, these three species started calling simultaneously. We found significant predation pressures on *F. kawamurai* eggs from larvae of *H. japonica*, *Rana japonica*, *P. nigromaculatus*, *P. porosus brevipodus*, *Glandirana rugosa*, *F. kawamurai*, and *Rhacophorus schlegelii*, whereas no significant predation pressures from larvae of *B. japonicus* was detected. We found no evidence of maternal choice by *F. kawamurai* for oviposition sites and no effects of *P. nigromaculatus* larvae on sympatric larvae of *F. kawamurai*. Our results suggest the possibility that the mortality rate of *F. kawamurai* eggs might increase through predation at paddies where *H. japonica* and *P. nigromaculatus* start oviposition earlier than does *F. kawamurai*.

Key words: *Fejervarya kawamurai*; Reproductive season; Maternal choice; Larval predation; Larval coexistence

INTRODUCTION

Wet paddy fields are suitable reproductive sites for many anuran species, and it is com-

mon for larvae of several species to coexist in these habitats. Under such coexistence, some interspecific relationships are expected, but little research has been conducted on this topic in Japan. Noha and Shimada (2017) studied larval assemblages in 92 paddies in west Mikawa district in Aichi Prefecture, and found apparent negative correlations in larval

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abundance between *Fejervarya kawamurai* and other species (*Hyla japonica* and *Pelophylax nigromaculatus*). To understand this relationship, both biotic (e.g., interspecific interactions) and/or abiotic factors (e.g., differences in preference of oviposition sites) should be studied. However, based on an assumption that *H. japonica* and *P. nigromaculatus* start calling earlier than in *F. kawamurai* in Aichi Prefecture, Noha and Shimada (2017) pointed out the possibility of a negative effect from pre-existing larvae of *H. japonica* and *P. nigromaculatus* to larval *F. kawamurai*. To explain this phenomenon, Noha and Shimada (2017) suggested three possibilities; (1) maternal choice (females tend to lay eggs in the paddies with low larval density), (2) larval predation (pre-existing larvae consume eggs of *F. kawamurai*), and (3) larval competition (coexistence of larger larvae increases the mortality rate of larval *F. kawamurai*). However, because their study was based on the data taken in the field, the mechanism of these issues remained unclear. In order to test these possibilities, we conducted three rearing experiments mainly focusing on the effect of *P. nigromaculatus* larvae. We also studied the reproductive season of *H. japonica*, *P. nigromaculatus*, and *F. kawamurai* using automatic call recording systems at two sites with different irrigation

schedules. From these data, we discuss the possibility of interspecific relationships surrounding *F. kawamurai* in paddy fields in Aichi Prefecture.

MATERIALS AND METHODS

Survey of calling season

We set two voice recorders (YAMAZEN YVR-R302) to record surrounding sounds for five minutes every evening (20:00–20:05). One recorder was set at a paddy at the Aichi University of Education (site A: Igaya, Kariya city, Aichi Pref.; alt. 20 m) from 20 April to 10 July, 2013. The other was set at a paddy about 10 km north of site A (site B: Kurozasa, Miyoshi city, Aichi Pref.; alt. 100 m) from 20 April to 10 July, 2016. Both sites were inhabited by *H. japonica*, *P. nigromaculatus*, and *F. kawamurai*. In addition, *Rhacophorus schlegelii* was observed at site B. At site A, paddies were irrigated on 14 May 2013, whereas paddies at site B were flooded throughout the year. We identified anuran species from their mating calls through hearing all parts of the records.

Experiment 1: Examination on maternal choice for oviposition sites

We used an experimental pool (570 cm×280 cm in inside dimension, surrounded by concrete walls) at site A. At the top of the wall, we

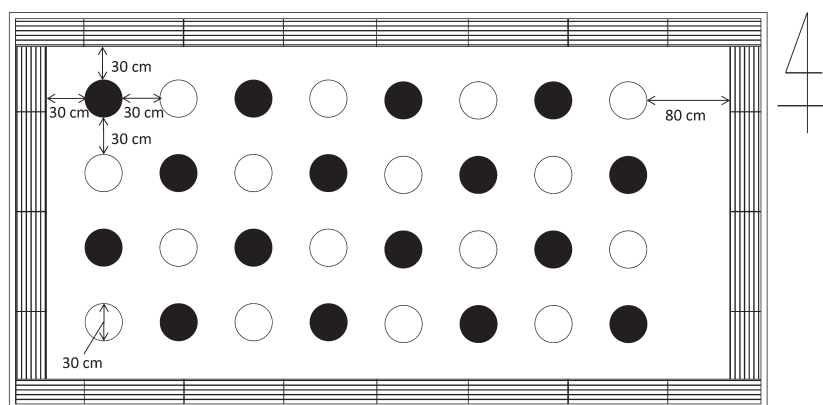


FIG. 1. A schematic picture of the experimental pool used in experiment 1. Closed and open circles indicate the tubs with and without larval *Pelophylax nigromaculatus*, respectively.

set overhanging plastic boards to prevent frogs from escaping (Fig. 1). We covered the bottom of the pool with a layer of soil. The height of wall from the soil was 25 cm. In the pool, we put 32 tubs (30 cm in diameter) with soil (3 cm in depth) and water from a nearby well. In each half of the tubs (16 tubs), we placed three larvae of *P. nigromaculatus* at stages 26–31 of Gosner's (1960) table, while we put nothing in the other half (16 tubs). The density of larvae (42.4 individuals/m²) was greater than the maximum mean larval density of *P. nigromaculatus* calculated in each paddy based on the original data of Noha and Shimada (2017: 17.5 individuals/m²; the maximum value among averages of five quadrats [40 cm×20 cm] from each paddy; data from 92 paddies). Larvae were collected from paddy fields around sites A and B. The location of tubs with and without larvae is shown in Fig. 1. We established the tubs in the daytime (12:00–14:00 h), and released 16 males and 16 females of *F. kawamurai* at night (21:00–24:00 h). The frogs were collected from paddies around site A. The next morning, we counted the number of eggs in each tub. We repeated this experiment five times (15–16, 23–24, and 30–31 May and 7–8 and 23–24 June 2017). All adult *F. kawamurai* and larval *P. nigromaculatus* used in each experiment were replaced by new individuals.

We designed generalized linear mixed models (GLMM) to examine the relationship between the number of eggs and several environmental factors following the principle of model selection using AIC (Akaike's Information Criteria). We used the *glmmADMB* package in R. ver. 3.4.1 (R Core Team, 2017). The number of eggs collected from each tub was used as a response variable for GLMM. The negative binomial distribution was used as the probability distribution. The explanatory variables for model selection were (1) PNI: presence/absence of larval *P. nigromaculatus*, (2) DATE: date of experiment (number of days from 15 May 2017), (3) WEATHER: weather on the day of the experiment (clear/cloudy), (4) TEMP: average air

temperature during the night (0:00–6:00) (calculated from meteorological data measured every 10 min at Nagoya by the Japan Meteorological Agency [http://www.data.jma.go.jp]). In addition to these four fixed effect variables, two additional variables, TUB (the variation among tubs) and EXPERIMENT (the variation among experiments) were added as random effects to consider the variability of tubs and experiments. We did not consider the effects of interactions between explanatory variables during model selection.

Experiment 2: Examination of predation on eggs

We collected larvae of *P. nigromaculatus* in stages 34–38 of Gosner's (1960) table, and kept them together with eggs of *F. kawamurai* for two days. We also conducted similar experiments for other seven species (*Bufo japonicus formosus*, *H. japonica*, *Rana japonica*, *P. porosus brevipodus*, *Glandirana rugosa*, *F. kawamurai*, and *Rh. schlegelii*). The numbers of larvae, collection dates, and collection sites are shown in Table 1. Among the eight species used in this experiment, *H. japonica*, *P. nigromaculatus*, and *P. porosus brevipodus* were collected with coexisting *F. kawamurai*. In the collection sites of other four species, we observed no *F. kawamurai*, although they sometimes reproduce with *F. kawamurai* in Aichi Prefecture (e.g., *B. japonicus formosus* and *R. japonica*: Takatsu, 1998; *G. rugosa* and *Rh. schlegelii*: Shimada et al., 2015). We followed Matsui and Seki (2008) for species identification. Eggs of *F. kawamurai* were obtained from experiment 1. We started each experiment on the next day following egg-laying.

The experiment was conducted in a semi-natural rearing house at Aichi University of Education in five periods (Table 1). The dimensions of the rearing tanks were 36 cm×52 cm×18 cm. Each tank was filled with circulated water to a depth of 3 cm. Water was constantly supplied to each tank through a tube from an uppermost tank at the rate of 250 ml/min. The overflowed water

TABLE 1. The date, temperatures (average \pm 2SE, ranges in parenthesis), and the information of examined larvae in experiment 2 (Species, numbers, collection dates, and collection sites).

No.	Dates of experiment	Temperatures	Species	N	Collection dates	Collection sites
1	31 May–2 June 2017	23.6 \pm 0.36 (21.5–26.0)	Control <i>Rana japonica</i>	12 12	— 19 May 2017	— Yanami, Toyota, Aichi Pref.
2	7–9 June 2017	21.3 \pm 0.34 (19.5–24.0)	Control <i>Bufo japonicus formosus</i> <i>Hyla japonica</i> <i>R. japonica</i> <i>Pelophylax nigromaculatus</i> <i>Rhacophorus schlegelii</i>	20 20 11 8 10 20	— 4 June 2017 7 June 2017 19 May 2017 7 June 2017 19 May 2017	— Damine, Shitara, Aichi Pref. Ikoma, Toyota, Aichi Pref. Yanami, Toyota, Aichi Pref. Ikoma, Toyota, Aichi Pref. Yanami, Toyota, Aichi Pref.
3	14–16 June 2017	23.0 \pm 0.35 (20.5–25.0)	Control <i>H. japonica</i> <i>P. nigromaculatus</i> <i>Fejervarya kawamurai</i>	10 10 10 10	— 13 June 2017 12 June 2017 13 June 2017	— Sato, Anjo, Aichi Pref. Ikoma, Toyota, Aichi Pref. Sato, Anjo, Aichi Pref.
4	20–22 June 2017	23.7 \pm 0.32 (22.0–26.0)	Control <i>P. porosus brevipodus</i> <i>F. kawamurai</i>	20 20 10	— 17 June 2017 19 June 2017	— Takaoka, Toyota, Aichi Pref. Igaya, Kariya, Aichi Pref.
5	29–31 July 2017	28.1 \pm 0.18 (27.5–29.5)	Control <i>Glandirana rugosa</i>	20 20	— 17 July 2017	— Odo, Toyota, Aichi Pref.

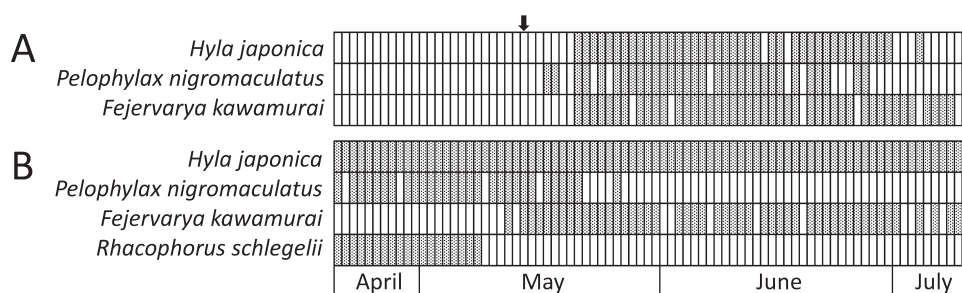


FIG. 2. Calling of frogs observed at site A, where paddies were irrigated in mid-May (black arrow), and site B, where paddies were flooded throughout the year. Closed and open boxes indicate the day on which calling of each species were observed or not observed, respectively.

from each tank was filtered and pumped up to the uppermost tank. About half of the water was automatically replaced twice a day. Six enclosures (14 cm×14 cm×6 cm plastic quadrats) were placed in the tank. A single larva was kept in each enclosure with ten eggs of *F. kawamurai*. Because we have no exact data about the numbers of embryos in an egg mass of *F. kawamurai*, and the densities of larvae of each species in wild, we are not certain if this number (ten eggs for a larva) is a natural density or not. Larvae were fed with boiled spinach before the experiments, but not during the experiment. We counted the number of eggs or hatchlings every 24 hours. For every experiment, control examinations were conducted. Water temperature was recorded every two hours with a data logger (Thirmochron G from KN Laboratories). We applied ANOVA with Dunnett's tests for multiple comparison for the number of living *F. kawamurai*, using the multcomp package in R. When we counted living individuals, we did not distinguish between embryos and hatchlings.

Experiment 3: Examination of larval coexistence

We kept a cohort of larval *F. kawamurai* (stage 28–32) with and without a larva of *P. nigromaculatus* for two weeks (21 July to 4 August, 2017) and compared the mortality and the growth between controls and experiments. We used the rearing equipment used in experiment 2. The density of larvae (51.0

individuals/m² in *P. nigromaculatus* and 153.0 individuals/m² in *F. kawamurai*) were larger than the maximum of mean larval density calculated in a paddy based on the original data of Noha and Shimada (2017) (17.5 and 135.0 individuals/m², respectively; See Experiment 1 for detail). Larvae of *F. kawamurai* used in this experiment were reared from eggs obtained in experiment 1. On the first day of experiment, 20 larvae of an identical cohort were fixed to record initial size and developmental stages. Larvae of *P. nigromaculatus* were collected from paddy field around site A on 17 July 2017. Only larvae in Gosner's stages 34–38 were used.

Boiled spinach was fed every day, and old spinach was removed before feeding. The average water temperature during the experiment period was 28.0C±0.10 (average±2SE), ranging from 23.5C to 29.5C (measured by the same equipment used in experiment 2). We fixed larvae after the experiment with 5% formalin. A few days after fixation, all samples were washed in water and wet masses were weighed with a digital balance with an accuracy of 0.1 g after wiping away water droplets with a paper towel. We also determined the developmental stages following Gosner's (1960) table using a stereoscopic microscope (Carton MSC-LED). For the analysis of mortality, we compared the numbers of alive larvae in each enclosure with Student's *t*-test. We also compared developmental stages and wet masses with Mann-Whitney's U-test and

Student's *t*-test, respectively.

RESULTS

Survey of calling season

The results of automatic recording data are shown in Fig. 2. At site A, where the irrigation was conducted on 14 May, no calls were recorded before irrigation, but *H. japonica*, *P. nigromaculatus*, and *F. kawamurai* started calling a week following the day of irrigation. These three species started calling almost simultaneously.

At site B, where paddies were flooded throughout the year, calls of *H. japonica*, *P. nigromaculatus*, and *Rh. schlegelii* were heard the first day of recording (20 April), so we could not determine when they started calling. However, the start of *F. kawamurai* calling was later than in the other three species, because we did not detect any *F. kawamurai* calling during first three weeks of monitoring.

Experiment 1: Examination of female choice for oviposition site

In total, 9477 eggs were deposited in 14 tubs out of 80 tubs with larval *P. nigromaculatus* (17.5% of tubs were used), while 10335 eggs were deposited in 11 tubs out of 80 tubs without larvae (13.8% of tubs were used). Detailed results of each experiment are shown in Table 2. The best model selected in the model selection procedure was the one including none of

TABLE 3. Model selection statistics for ten models of factors affecting the number of eggs in experiment 1. The models are ranked by AIC. Δ AIC indicates the difference of AIC from the best model.

Rank	Models	AIC	Δ AIC
1	Null model	571.55	best
2	WEATHER	573.42	1.87
3	DATE	573.45	1.90
4	PNI	573.54	1.99
5	TEMP	573.54	1.99
6	WEATHER+TEMP	574.03	2.48
7	WEATHER+DATE	575.36	3.81
8	PNI+WEATHER	575.41	3.86
9	TEMP+DATE	575.45	3.90
10	PNI+DATE	575.45	3.90

our potential explanatory variables (null model; AIC=571.55) (Table 3). The coefficient, SE and p-value of intercept of this model were 4.82, 0.55 and <0.01, respectively. The variance and SE of random effects of this model were 0.002 and 0.042 for TUB, and <0.001 and 0.011 for EXPERIMENT, respectively. From the best model we conclude that the number of eggs laid was not affected by any factors considered in the model selection.

Experiment 2: Examination of predation on eggs

Numbers of living eggs or hatchlings after the experiments are shown in Fig. 3. A sig-

TABLE 2. The number of spawned eggs and tubs with eggs of *Fejervarya kawamurai* under presence/absence of larval *Pelophylax nigromaculatus* in experiment 1. R indicated the rates of tubs activated by *F. kawamurai*.

No.	Date	Tubs with larval <i>P. nigromaculatus</i>			Tubs without larval <i>P. nigromaculatus</i>		
		Number of eggs	Number of tubs with eggs	R	Number of eggs	Number of tubs with eggs	R
1	15–16 May 2017	1680	5	31.3%	437	1	6.3%
2	23–24 May 2017	62	1	6.3%	421	1	6.3%
3	30–31 May 2017	3520	4	25.0%	4183	5	31.3%
4	7–8 Jun 2017	2967	2	12.5%	4357	2	12.5%
5	23–24 Jun 2017	1248	2	12.5%	937	2	12.5%

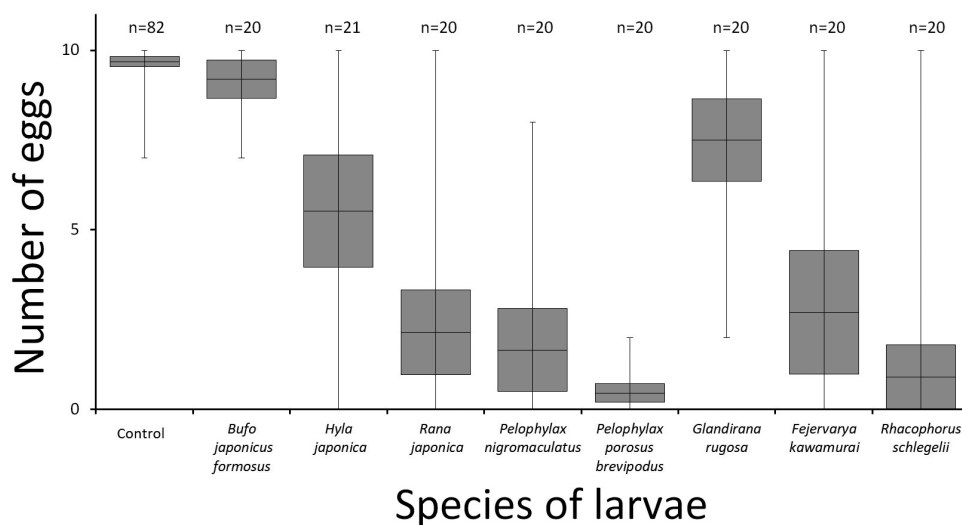


FIG. 3. Numbers of surviving *Fejervarya kawamurai* eggs and hatchlings after two days with the presence of an anuran larva (*Bufo japonicus formosus*, *Hyla japonica*, *Rana japonica*, *Pelophylax nigromaculatus*, *P. porosus brevipodus*, *Glandirana rugosa*, *F. kawamurai*, and *Rhacophorus schlegelii*). A horizontal bar, closed box, and a vertical bar indicate mean, 2SE, and range, respectively.

TABLE 4. Summary of parameter estimates in the Dunnett's test concerning experiment 2.

	Coefficient	SE	Z Value	P Value
Intercept	9.68	0.2479	39.06	1.00×10^{-5}
<i>Bufo japonicus formosus</i>	-0.48	0.5599	-0.86	9.78×10^{-1}
<i>Hyla japonica</i>	-4.16	0.5490	-7.58	1.00×10^{-5}
<i>Rana japonica</i>	-7.53	0.5599	-13.46	1.00×10^{-5}
<i>Pelophylax nigromaculatus</i>	-8.03	0.5599	-14.35	1.00×10^{-5}
<i>P. porosus brevipodus</i>	-9.23	0.5599	-16.49	1.00×10^{-5}
<i>Glandirana rugosa</i>	-2.18	0.5599	-3.90	1.12×10^{-3}
<i>Fejervarya kawamurai</i>	-6.98	0.5599	-12.47	1.00×10^{-5}
<i>Rhacophorus schlegelii</i>	-8.78	0.5599	-15.69	1.00×10^{-5}

nificant difference was detected with ANOVA ($p < 0.01$). The result of Dunnett's test is shown in Table 4. Compared with the control (survival rate $96.8 \pm 1.3\%$; average $\pm 2SE$; $N = 82$), all species except for *B. japonicus formosus* (survival rate $92.0 \pm 5.3\%$; average $\pm 2SE$; $N = 20$) exhibited significant differences. The average survival rates were lowest in *P. porosus brevipodus* ($4.5 \pm 2.6\%$; average $\pm 2SE$; $N = 20$), followed by *Rh. schlegelii* ($9.0 \pm 9.8\%$;

$N = 20$), *P. nigromaculatus* ($16.5 \pm 11.5\%$; $N = 20$), *R. japonica* ($21.5 \pm 11.8\%$; $N = 20$), *F. kawamurai* ($27.0 \pm 17.3\%$; $N = 20$), *H. japonica* ($55.2 \pm 15.7\%$; $N = 21$), and *G. rugosa* ($75.0 \pm 11.5\%$; $N = 20$). We observed predation on eggs by larvae of *R. japonica* (Fig. 4), *P. nigromaculatus*, and *F. kawamurai*. We also observed several larvae of *B. japonicus formosus* trying to eat eggs, but they were unable to break the jelly layer of eggs.

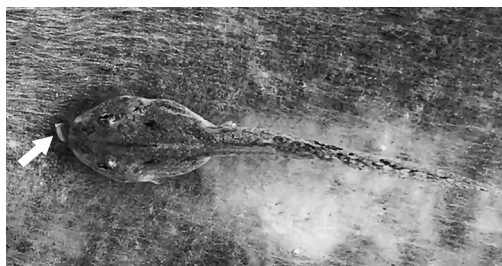


FIG. 4. A photo of larval *Rana japonica* foraging on an egg of *Fejervarya kawamurai*. The white arrow indicates the egg.

Experiment 3: Examination of larval coexistence

Because larval *P. nigromaculatus* died in seven enclosures, probably because of high water temperatures or larval disease, we excluded them from the analyses. In the remaining 14 enclosures, 34 individuals out of 42 larval *F. kawamurai* survived, whereas in 21 enclosures without *P. nigromaculatus*, 57 out of 63 individuals survived. The survival rate in the enclosures with *P. nigromaculatus* (81.0%) was slightly lower than that in the control (90.5%), but no significant difference was detected between them (*t*-test; $p=0.27$).

The developmental stage and the wet mass of larval *F. kawamurai* before and after the rearing experiment are shown in Fig. 5. Judging from apparent differences between the specimens fixed before and after the experiment, it seems that all examined larvae developed and grew during the experiment to some extent (Fig. 5). For *F. kawamurai* larvae with and without larval *P. nigromaculatus*, we detected no significant differences in developmental stages (U-test, $p=0.97$) and wet mass (*t*-test, $p=0.92$).

DISCUSSION

Our surveys of reproductive seasons of *F. kawamurai* and other coexisting species at two sites in west Mikawa exhibited contrasting patterns. In the paddies irrigated mid-May (site A), the time lag from the start of calling among *H. japonica*, *P. nigromaculatus*, and *F. kawamurai* was small (Fig. 2A). On the

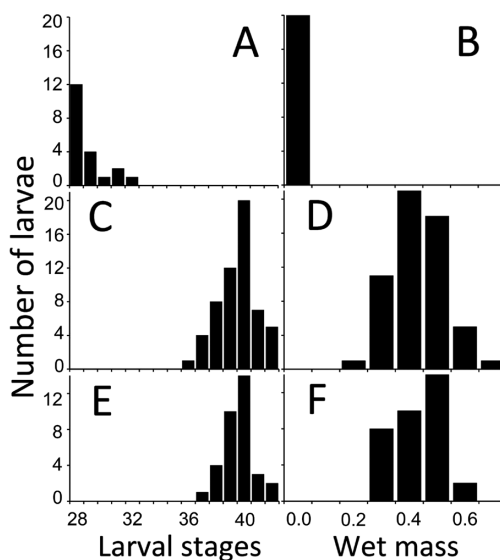


FIG. 5. The larval stages (A, C, E) and wet mass (B, D, F) of *Fejervarya kawamurai* before and after experiment 3. A and B indicate the status at the start of experiment, while C–F indicate the status after two weeks. In each enclosure, three larval *F. kawamurai* were reared with (E–F) or without (C–D) a larva of *Pelophylax nigromaculatus*.

other hand, in the paddies flooded throughout the year (site B), the start of calling for *H. japonica* and *P. nigromaculatus* was apparently earlier than in *F. kawamurai* (Fig. 2B). Time lags for initiation of calling were also observed in a similar study at a paddy in the east Mikawa area, irrigated in late April (Yamamoto, 2012). The difference between sites A and B might be caused by the difference in their irrigation schedules or some other environmental factors. It should be noted that calling of *F. kawamurai* starts later than in *H. japonica* and *P. nigromaculatus* in some paddies, but such a time lag does not always exist.

In the situation like at site B, where other species start oviposition earlier than does *F. kawamurai*, predation from pre-existing larvae might increase the mortality of *F. kawamurai* embryos. In our experiment 2, larvae of *H. japonica* and *P. nigromaculatus* ate eggs of *F. kawamurai* (Fig. 3). Although

larvae of *F. kawamurai* also foraged on conspecific eggs, such an activity would be weak until larval *F. kawamurai* grew to be large enough to eat eggs. In contrast, the effect of foraging from pre-existing larvae of other species in the situation like at site B would be more harmful because it can damage all embryos of *F. kawamurai* from the beginning. Therefore, in such situations, the high density of larval *H. japonica* and *P. nigromaculatus* might result in the low density of larval *F. kawamurai*. This might well explain the negative correlation between larval density of *F. kawamurai* and other species (*H. japonica* and *P. nigromaculatus*) observed by Noha and Shimada (2017).

Such phenomena would also be expected in some other species with earlier breeding seasons, such as *R. japonica* (late January to late March in Aichi Pref.; Aichi Prefectural Government, 1996) and *Rh. schlegelii* (April to June in Aichi Pref.; Aichi Prefectural Government, 1996), because they also ate embryos of *F. kawamurai* (Fig. 3). *Bufo japonicus formosus* also breeds earlier than *F. kawamurai* (March to April in the lowland area of Aichi Pref.; Aichi Prefectural Government, 1996), but even when it breeds sympatrically with *F. kawamurai*, it might not be an effective predator judging from the results of experiment 2 (Fig. 3).

On the contrary, in the situation observed at site A, where all anurans including *F. kawamurai* start reproduction simultaneously, the disadvantage of late breeding of *F. kawamurai* might not exist. The larval predation on anuran eggs itself would also occur in such a paddy, but contrary to the situation observed at site B, eggs laid relatively early in the season would be free from larval predation. Accordingly, whether or not eggs of *F. kawamurai* suffer from predation by pre-existing larvae would vary according to the irrigation schedule of each paddy.

Matsui (1996) remarked that anuran larvae are omnivorous, although they tend to be carnivorous when population density becomes higher or food becomes scarce. However, as

far as we know, no significant predation on anuran eggs by pre-existing anuran larvae has been reported in Japan, except for the maternal feeding behavior of *Kurixalus eiffingeri* (Maeda and Matsui, 1999). In Japanese paddy fields, it is usual that several anuran species with different breeding seasons to reproduce sympatrically (e.g. Hasegawa, 1998). Although we examined only predation on *F. kawamurai* eggs in this study, similar relationships might be observed in other pairs of species. If so, such effects would be an important factor to consider in the ecology and conservation of anurans of paddy fields in Japan. Experimentation on other species is needed.

In our experiments, we could not detect any maternal avoidance to pre-existing larvae (experiment 1) and any negative effects to larval *F. kawamurai* from larval *P. nigromaculatus* (experiment 3). However, we admit that these experiments are not enough to reject these two possibilities. First, in this study, we examined only *P. nigromaculatus*, and another candidate, *H. japonica*, should also be examined. Second, the interspecific competition among larvae has not fully been examined yet. Our experiment 3 only examined the effect of coexistence, and the real competition in wild was not tested. Actually, the presence of pre-existing larvae might cause several problems, such as shortage of food resource or deterioration of water quality, which might increase mortality of *F. kawamurai* larvae. To examine these factors more precisely, we need to explore rearing conditions that are more like the natural environment.

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