Limnology (2006) 7:31–39
DOI 10.1007/s10201-006-0163-3

NOTE

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Reproductive mode of the geographic parthenogenetic mayfly *Ephoron shigae*, with findings from some new localities (Insecta: Ephemeroptera, Polymitarcyidae)

Received: March 22, 2005 / Accepted: February 13, 2006 / Published online: April 7, 2006

Abstract The burrowing polymitarcyid mayfly *Ephoron shigae* is distributed widely in Japan. Some populations are bisexual, others are unisexual, and the distributions of the two types overlap broadly. Experimental evidence of parthenogenetic reproduction, long suspected in unisexual populations, is presented here, based on a comparative analysis of the developmental rate of fertilized and unfertilized eggs. The developmental rate of fertilized eggs from 20 mated females in a bisexual population was 98.4% ± 0.73% (mean ± SD), and no unfertilized eggs from 20 virgin females in that population developed. The developmental rates of unfertilized eggs in two unisexual populations were 89.0% ± 4.59% and 84.2% ± 1.96%, respectively. This article presents experimental evidence of geographic parthenogenesis in *E. shigae* and provides support for the previous interpretation. In addition, we discuss the relationship between the sex ratio of each population and the developmental rate of fertilized versus unfertilized eggs from the females in those populations.

Key words Reproduction · Aquatic insects · Mayfly · Parthenogenesis · Geographic parthenogenesis

Introduction

Japanese polymitarcyid mayflies have been classified into three species: *Ephoron eophilum* Ishiwata, *Ephoron limnobium* Ishiwata, and *Ephoron shigae* (Takahashi) (Takahashi 1924; Ishiwata 1996). The distribution of *E. eophilum* is restricted to certain areas in the Kanto plain, especially in the basin of the Tone-gawa River, while *E. limnobium* is endemic to Lake Biwa (Ishiwata 1996). *E. shigae* is distributed in wide areas of the islands of Honshu (northern limit: Kitakami-gawa River, Iwate Prefecture) and Shikoku, and the northern half of Kyushu Island (southern limit: Midori-gawa River, Kumamoto Prefecture) (Fig. 1; cf. Ishiwata 1996), and the mass emergence of this species has often been reported (e.g., Watanabe and Ishiwata 1997). This mayfly exhibits a strong phototaxis, and huge numbers of mayflies are attracted by street lights and accumulate on riverside roads (Fig. 2), interfering with traffic, and sometimes causing car accidents.

Watanabe and Ishiwata (1997) made efforts to obtain information on the geographic distribution and sex ratio of each population, based on questionnaires and detailed surveys of the information from publications and collections, as well as their own field research. All known *E. eophilum* and *E. limnobium* populations consisted apparently equally of males and females, indicating bisexual reproduction. However, some populations of *E. shigae* were bisexual and others were unisexual (only females) or mostly unisexual (the percentage of males were extremely low; e.g., approximately 0.2% in a population from the Shonai-gawa River in our field research and Dr. Hatta's personal communication). Consequently, the unisexual populations of *E. shigae* were thought to exhibit thelytokous parthenogenetic reproduction (i.e., parthenogenesis in which only females are produced). However, there was no previous direct experimental evidence of parthenogenetic reproduction in the unisexual populations of this species. We have started experimental studies on the parthenogenetic abilities of this mayfly at the population level. In this article, the geographic distribution of *E. shigae* is updated with some new localities having been surveyed, and new findings on the parthenogenetic abilities of *E. shigae* are added to our previous study (Tojo et al. 2002). The reproductive mode (e.g., parthenogenesis or not, and presence or absence of parthenogenetic ability) of this mayfly is reviewed and discussed, based on a comparative analysis of the developmental rate of fertilized and unfertilized eggs from different populations.

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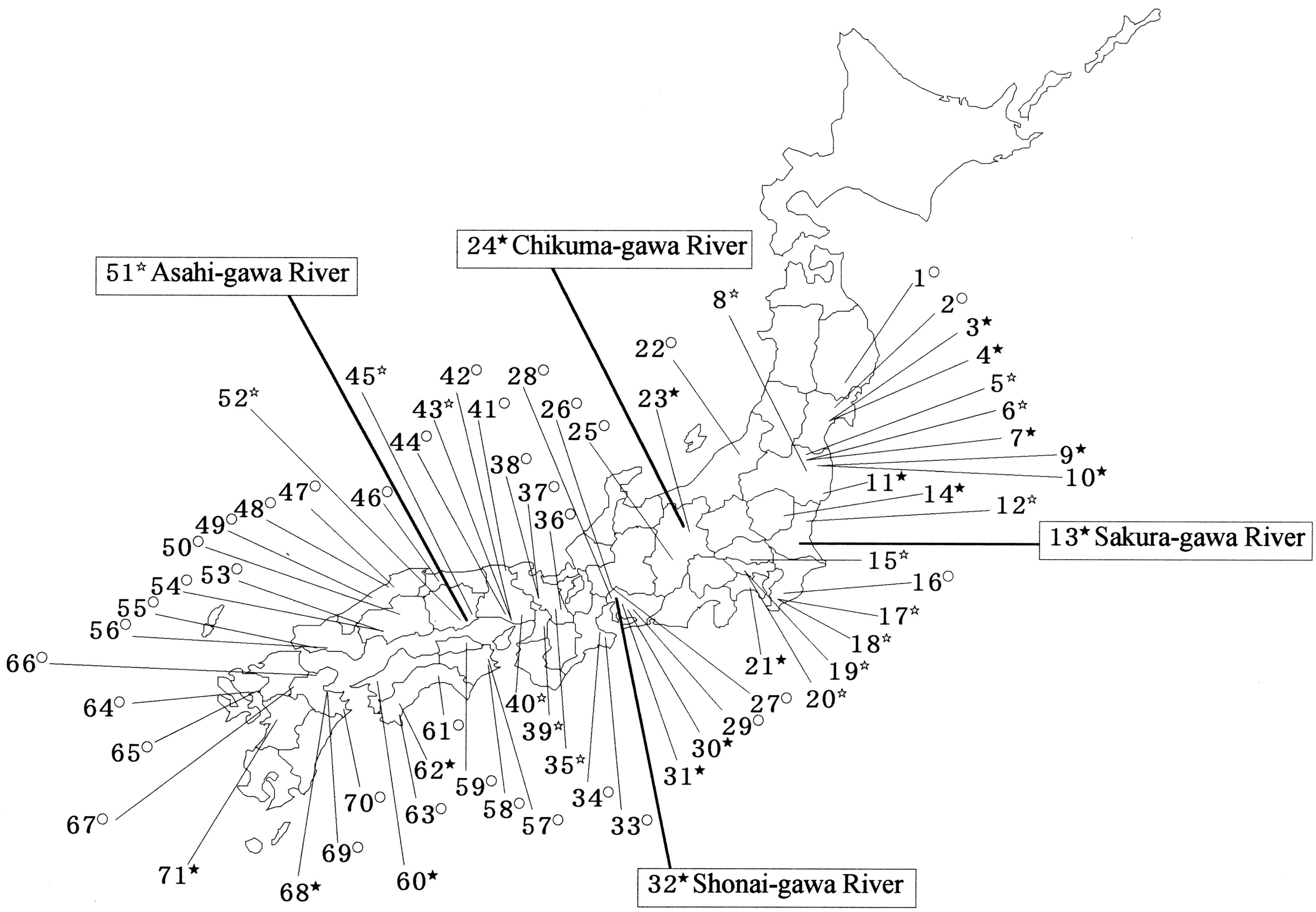

Fig. 1. Distribution of *Ephoron shigae*. Data from the present study as added to that from Watanabe and Ishiwata (1997). *Solid and open stars* indicate unisexual (including the populations with males found at very low frequency) and bisexual populations, respectively; *open circles* indicate the populations in which the sex ratio was not determined. Refer to Appendix 1 for details of the localities (*numbers*). In this study, four populations (*boxed*) were examined

Materials and methods

Distribution and sex ratio of *Ephoron shigae*

We gathered data on distribution of the mayfly *Ephoron shigae* based on our own field research and on surveys of newly published literature and records. The sex ratio was examined with the old or the last instar nymphs in each population.

Developmental rate of fertilized or unfertilized eggs of *Ephoron shigae*

A population of *Ephoron shigae* from the Asahi-gawa River (Okayama City, Okayama Prefecture: 34°40′49″ N, 133°56′03″ E) was examined as a bisexual population. In the Asahi-gawa River population, many males as well as females were observed: the ratio of males to females was almost one to one. Three populations (Sakura-gawa River in Tsukuba City, Ibaraki Prefecture: 36°07′47″ N, 140°07′35″ E; Chikuma-gawa River in Chikuma City, Nagano Prefecture: 36°31′56″ N, 138°06′39″ E; and Shonai-gawa River in Nagoya City, Aichi Prefecture: 35°12′59″ N, 136°57′48″ E) were studied as unisexual populations. In the Sakura-gawa and Chikuma-gawa River populations, no males were found (i.e., fully unisexual populations); a few males were found in the Shonai-gawa River population, but the percentage of males was extremely low (approximately 0.2%) (see Fig. 1, Appendix 1).

Twenty virgin females (uncoupled, but mature adults) and 20 mated females were collected from the bisexual population from the Asahi-gawa River (Sept. 6, 2000). Twenty virgin females were also collected from the respective unisexual populations from the Chikuma-gawa and Sakura-gawa rivers (Sept. 13, 1998 and Sept. 7, 2001, respectively). Although 13 virgin females were collected from the mostly unisexual population of the Shonai-gawa River (Sept. 7, 2000), mated females could not be collected from this population. The females that were collected just after emergence and could not come into contact with the others were treated as virgin individuals. In the bisexual population, the females that were collected some time after emergence were treated as mated individuals. In a previous

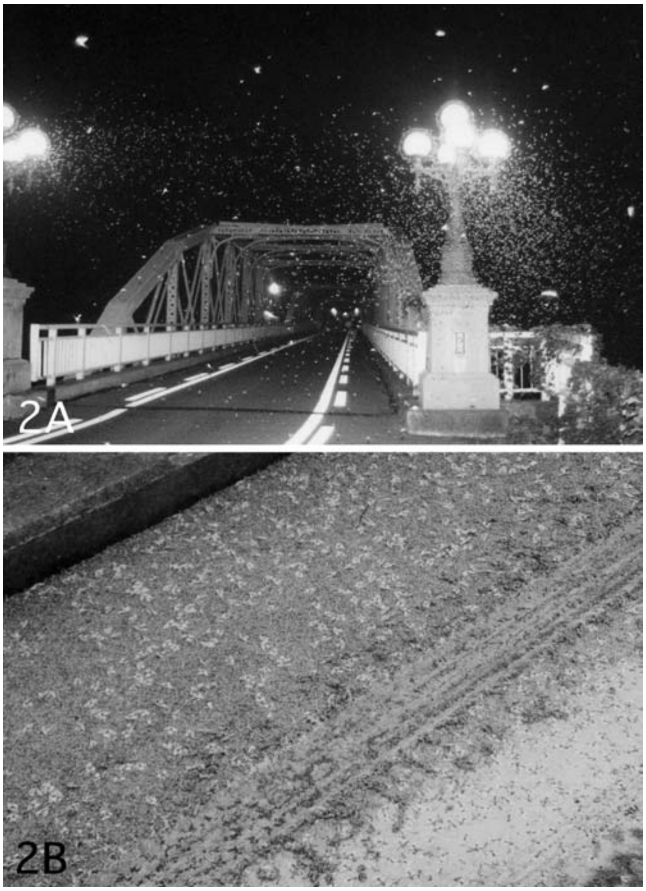

Fig. 2. Mass emergence of *Ephoron shigae*. At evening (just after sunset) for only a few days in early to middle September every year, huge numbers of imagos (or subimagos) emerge synchronously. At the riverside, especially on bridges, huge aggregations are attracted by lights on roads and interfere with traffic (**A**). Thickly accumulated dead mayflies on the road, resembling snow, sometimes cause car accidents. After cars pass over the layer of mayflies, "ruts" are made (**B**). The numerous whitish spots in the lower right corner of the photo are eggs or egg masses

studies of this mayfly (Watanabe et al. 1989; Watanabe 1996), it was revealed that males generally emerged earlier than females, that emerging females were caught at once by males and formed pairs, and that the mating rate of females in the period following the emergence of most of the males, i.e., at the latter half of the mass emergence, reached almost 100%. In our field research on the bisexual population (Asahi-gawa River, Okayama Prefecture; Abukuma-gawa River, Fukushima Prefecture; and Hino-yousui, Tokyo Prefecture), a similar pattern was observed. For reference, a related species, *Ephoron eophilum*, was also used: 6 mated females were collected from the bisexual population of the Sakura-gawa River (July 5, 2003; near the collecting site of *E. shigae*, 36°05′54″ N, 140°10′03″ E).

Eggs were obtained in the respective fields from virgin or mated females collected under each of the conditions described above and incubated separately in batches [immersed in distilled water, at 20°C ± 0.5°C using a cooled incubator (PC1-100; Iuchi, Tokyo, Japan)]. At 8 weeks (2 months) after oviposition, developmental rates of fertilized or unfertilized eggs collected from the four different populations were analyzed for every egg batch. In the embryogenesis of this mayfly, diapause occurs at the last embryonic stage (Watanabe et al. 1993; Nakamura et al. 1999; Nakamura and Endo 2001): equivalent to "stage 13" in embryos of a near relative burrowing mayfly *Ephemera japonica* (cf. Tojo and Machida 1997a,b), and the release of diapause (i.e., the hatching) requires delicate environmental conditions such as water temperature and light conditions. Under the incubation conditions in the laboratory, a long incubation time was required for completion of hatching, and differences occurred in developmental rates and hatchability with short-term experiments such as several months to a half-year. In natural conditions, several years may be required for hatching in rare cases (Sekiné and Tojo, in preparation). Consequently, the developmental rate of eggs was adopted in this study rather than the hatching rate. The judgment as to whether the egg was developing was relatively simple because the developed and undeveloped eggs were clearly different in color (Fig. 3). Significant differences in developmental rate between fertilized and unfertilized eggs from different populations were tested by analysis of variance (ANOVA) and sequential Bonferroni tests.

Results

Distribution of *Ephoron shigae* and the sex ratio of the respective populations, with findings from some new localities

The geographic distribution and sex ratio of *Ephoron shigae* have been extensively surveyed, and 12 bisexual and 7 unisexual populations (containing populations in which "very few males were found") were described by Watanabe and Ishiwata (1997) (see Fig. 1, Appendix 1). In this article, we added new population data (see Appendix 1) based on our field research and surveys carried out after Watanabe and Ishiwata (1997): 4 bisexual and 11 unisexual (no males found) populations, and a practically unisexual population (the percentage of males very low; less than 1%). These different population types overlapped geographically, as shown in Fig. 1.

Developmental rate of fertilized and unfertilized eggs of *Ephoron shigae*

Table 1 summarizes the developmental rate of fertilized and unfertilized eggs of *Ephoron shigae* collected from four different populations. Table 1 also includes data on the developmental rate of fertilized eggs of a related species, *E. eophilum*.

In the bisexual population (Asahi-gawa River) of *E. shigae*, the fertilized eggs from 20 mated females developed almost to the final embryonic stage in 2 months. The per-

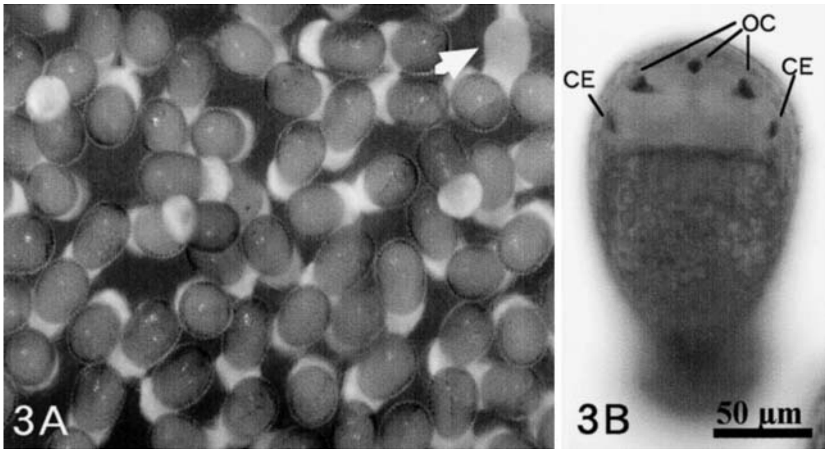

Fig. 3. Unfertilized eggs at 8 weeks after oviposition. Embryos develop equivalent to "stage 13" in embryos of a near relative mayfly *Ephemera japonica*, but an egg (*arrow*) at the stage of the photo frame does not develop (**A**). **B** Enlargement of a developed egg, dorsoanterior view. Compound eyes (*CE*) and three ocelli (*OC*) can be clearly observed

Table 1. Developmental rate of fertilized or unfertilized eggs of *Ephoron shigae* and fertilized eggs of a related species, *Ephoron eophilum*

	Percentage of embryos developing to the final embryonic stage				ANOVA test / sequential Bonferroni test					
	No. of females examined	No. of eggs examined	Mean ± SD	(Minimum–maximum)	Vs. 1	Vs. 2	Vs. 3	Vs. 4	Vs. 5	Vs. 6
***Ephoron shigae* (Takahashi)**										
Asahi-gawa River[a]										
1. Fertilized eggs	20	79,786	98.4 ± 0.73	(97.1–99.4)	–	* / *	* / *	* / *	* / *	ns / ns
2. Unfertilized eggs	20	60,017	Undeveloped		* / *	–	* / *	* / *	* / *	* / *
Chikuma-gawa River[b]										
3. Unfertilized eggs	20	62,856	89.0 ± 4.59	(81.7–96.9)	* / *	* / *	–	ns / ns	ns / ns	* / *
Sakura-gawa River[b]										
4. Unfertilized eggs	20	75,350	84.2 ± 1.96	(81.1–87.4)	* / *	* / *	ns / ns	–	ns / ns	* / *
Shonai-gawa River[c]										
5. Unfertilized eggs	13	27,111	78.5 ± 5.95	(69.3–86.8)	* / *	* / *	ns / ns	ns / ns	–	* / *
***Ephoron eophilum* Isgiwata**										
Sakura-gawa River[a]										
6. Fertilized eggs	6	2,167	98.1 ± 1.38	(96.1–99.7)	ns / ns	* / *	* / *	* / *	* / *	–

ANOVA, analysis of variance
Ephoron shigae were collected from four different populations, the Asahi-gawa, Chikuma-gawa, Sakura-gawa, and Shonai-gawa rivers; *E. eophilum* were collected from Sakura-gawa River
[a] Bisexual population
[b] Unisexual population: no males were found
[c] Practically unisexual population: the percentage of males was approximately 0.2%
* $P < 0.0001$; ns, not significant
Source: After Tojo et al. (2002)

centage of embryos developing to the final stage was 98.4% ± 0.73% (mean of 20 egg batches ± SD). This value was almost equal (no significant difference) to that in fertilized eggs of *E. eophilum* (98.1% ± 1.38%) from the Sakura-gawa River population (all known population of this species consist almost equally of males and females). In contrast, no unfertilized eggs from 20 virgin females developed to this stage (0%). The clear difference in the developmental rate reveals that mating is a necessary condition in the reproduction of mayflies in bisexual populations ($P < 0.0001$ by ANOVA and sequential Bonferroni tests; see Table 1).

In the unisexual populations (Chikuma-gawa and Sakura-gawa rivers), the unfertilized eggs from the respective 20 virgin females mostly developed to the final embryonic stage in 2 months. The percentage of embryos developing to the final stage was 89.0% ± 4.59% and 84.2% ± 1.96% in the Chikuma-gawa and Sakura-gawa river populations, respectively. These developmental rates were lower than that of the fertilized eggs from mated females in the bisexual population (Asahi-gawa River; $P < 0.0001$ by ANOVA and sequential Bonferroni tests). In the mostly unisexual population of Shonai-gawa River, many unfertilized eggs from 13 virgin females also developed to the final embryonic stage, but the percentage was somewhat lower than that of unfertilized eggs from virgin females in the fully unisexual population in the Chikuma-gawa or Sakura-gawa River (see Table 1).

Discussion

Experimental evidence of geographic parthenogenesis

The geographic parthenogenesis of the mayfly *Ephoron shigae* was revealed by the previous study (Watanabe and Ishiwata 1997), in which 12 bisexual populations and 7 unisexual populations were reported, and these different population types overlapped geographically. In the present study, information from new localities was added by our field research and surveys for the population types of the mayfly, comprising 4 new bisexual populations and 11 new unisexual populations, including a practically unisexual population (Natori-gawa River; the percentage of males was very low, <1%).

In addition, this study examined the parthenogenetic ability of several populations. In the unisexual populations examined, including the mostly unisexual population, parthenogenetic reproduction was confirmed. It is expected that thelytokous parthenogenesis is an indispensable mode of reproduction in the fully unisexual populations and the main mode in mostly unisexual population (the Shonai-gawa River population contained very few males, and we could not determine their reproductive contribution in this study): obligatory parthenogenesis, where parthenogenesis is the normal mode of reproduction, appears in unisexual populations of *E. shigae*. Thus, it can be concluded that the burrowing mayfly *E. shigae* exhibits geographic thelytokous parthenogenetic reproduction with a difference in parthenogenetic ability between populations. This study becomes supporting evidence of the previous study (Watanabe and Ishiwata 1997).

Origin of obligatory parthenogenesis

Parthenogenesis is found in most animal groups (Suomalainen 1950, 1962; Glesener and Tilman 1978; Suomalainen et al. 1987) and has been reported in some mayflies (Degrange 1960; Huff and McCafferty 1974; Gibbs 1977; Mingo 1978; Humpesch 1980; Sweeney and Vannote 1987; Glazaczow 2001, Ball 2001, 2002). However, in most mayflies parthenogenesis is only facultative, and is occasional or accidental (i.e., tychoparthenogenesis), such as in the North American burrowing mayfly *Ephoron album* (Say) (Britt 1962; cf. Degrange 1960; McCafferty and Huff 1974; Pescador and Peters 1974). In general, the percentage of hatching success for parthenogenetic eggs was usually much lower than for fertilized eggs in these cases (Huff and McCafferty 1974). Glazaczow (2001) mentioned that only a small percentage of unfertilized eggs hatch and that their development is slower and not well synchronized, and Brittain (1982) argued that parthenogenesis has no importance in population dynamics. Recently, however, Landolt et al. (1997) observed that half the unfertilized eggs developed in a Swiss burrowing mayfly, *Palingenia longicauda* (Olivier), and so parthenogenesis may be very important. Obligatory parthenogenesis is known to exist in several mayfly species (Clemens 1922; Froehlich 1969; Gibbs 1977; Bergman and Hilsenhoff 1978; Sweeney and Vannote 1987; Gillies and Knowles 1990), and Humpesch (1980) reported that the rate of hatching among unfertilized eggs was usually greater than 80% in the obligatory parthenogenesis populations. Evidence of geographic parthenogenesis, i.e., unisexual and bisexual populations of the same species existing in the same geographic area, such as *Ephoron shigae* in this study, has been verified for two ephemerellid mayflies: the eastern North American *Eurylophella funeralis* (McDunnough) (Sweeney and Vannote 1987) and the east European (e.g., Polish, Czech) *Ephemerella notata* Eaton (Jazdzewska 1976; Glazaczow 1994, 2001). The hatching success of unfertilized eggs of *Eurylophella funeralis* was inversely related to the proportion of males in the bisexual populations (Sweeney and Vannote 1987).

The results of the present examination and those of Sweeney and Vannote (1987) agree in terms of the tendency for a relation between hatching success (or developmental rate) and the sex ratio of the population: for *Ephoron shigae*, the developmental rate of unfertilized eggs from virgin females in the mostly unisexual population was also somewhat lower than that of unfertilized eggs from virgin females in the two fully unisexual populations. Thus, it is suggested that the developmental rate of unfertilized eggs of *E. shigae* is also inversely related to the proportion of males in the population rather than to the geographic location or any other factor. The feature of hatching success examined in these two mayflies, *E. shigae* and *Eurylophella funeralis*, may have originated as follows. One or more individuals in a normally bisexual population acquired parthenogenetic ability (e.g., accidentally), and facultative parthenogenetic success has gradually increased over generations. As a consequence, the sex ratio of the population also has gradually become biased toward females. Cuellar (1977) and Templeton (1982) suggest that many unisexual populations of invertebrate and vertebrate animals arise largely from tychoparthenogenesis in bisexual populations. Thus, tychoparthenogenesis is probably the best working hypothesis for the origin of unisexual populations of *Ephoron shigae* and *Eurylophella funeralis* (Sweeney and Vannote 1987). For *E. shigae*, the evolution of parthenogenesis from bisexual reproduction is also indicated by the presence of some (two or three) well-developed micropyles in each egg from females in unisexual populations: Sakura-gawa, Chikuma-gawa, Hirose-gawa, and Natori-gawa rivers (Fig. 4).

Geographic distribution and sex ratio of *Ephoron shigae*: mysterious establishment of broadly overlapped geographic parthenogenesis

Unisexual populations in *Eurylophella funeralis* seemed to occur largely at the periphery of their geographic range in North America (Sweeney and Vannote 1987). Glazaczow (2001) also reported that males of *Ephemerella notata* decrease and disappear toward the periphery of their distribution. Geographic studies on other invertebrates in

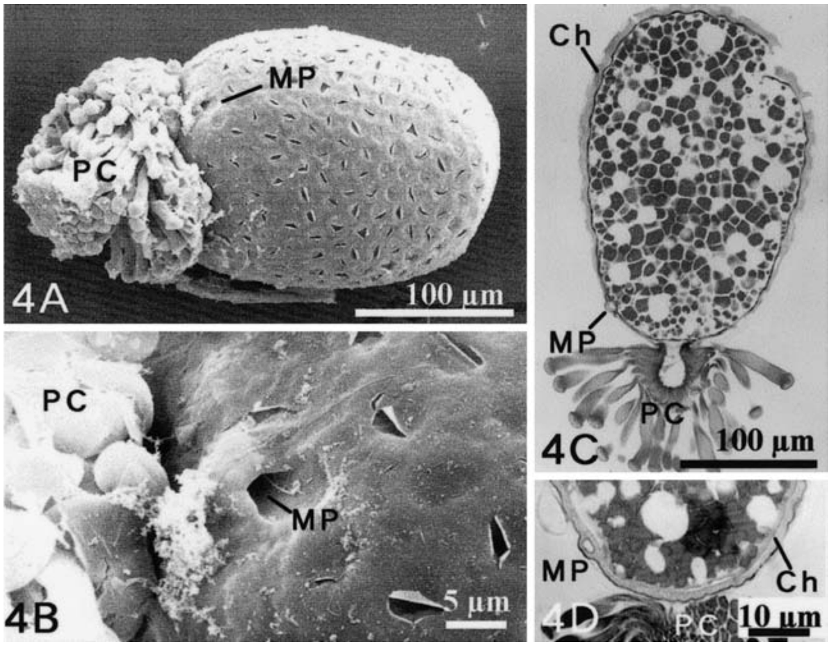

Fig. 4. Eggs of *Ephoron shigae* in an unisexual population, Sakura-gawa River (**A**). Females in the unisexual populations also oviposit eggs with some well-developed micropyles (*MP*). Two or three micropyles are on the posterior region (near the polar cap, *PC*). The micropylar opening is about 1.5 µm in diameter (**B**), and the micropylar canal obliquely penetrates the chorion (*Ch*; **C, D**). Refer to Tojo and Machida (1998, 2003) for scanning electron microscopy (SEM) observation and histological methods (such as sectioning)

temperate regions have shown that unisexual populations generally occur at the periphery of the species range (Glesener and Tilman 1978). Suomalainen (1950, 1962) and Suomalainen et al. (1987) also stated that there are not a few examples of geographic parthenogenesis in the animal kingdom, and parthenogenetic populations tend to occur under severe conditions such as at higher latitudes and altitudes and xeric as opposed to mesic conditions. Cuellar (1977) claimed that certain habitats where the ability to disperse is advantageous to colonization, such as islands and island-like habitats or the periphery of a species, encourage parthenogenesis. Although Wright (1978) and many other workers believe that hybridization provides a better explanation for the origin of unisexual populations, especially because many parthenogenetic vertebrate species have the morphology, karyotype, and allozymes of hybrids, the possibility of a hybrid origin in *Ephoron shigae* seems unlikely in consideration with its distributional properties.

In contrast to *Eurylophella funeralis*, for *Ephoron shigae* the distributions of the bisexual and unisexual populations overlapped broadly in their geographic range of distribution. For example, in Nagoya City (Aichi Prefecture) in central Japan, the two populations were found to inhabit neighboring streams (Hatta, personal communication). Watanabe and Ishiwata (1997) mentioned that no difference in topographical or hydrological characteristics between rivers with unisexual and those with bisexual populations could be found in any environmental data (e.g., data from the yearbook of water quality of the Japanese River Association).

Ephoron shigae may provide good material for studying the differentiation and the establishment of parthenogenesis, the dispersal of parthenogenetic individuals, and the relationship between parthenogenetic ability in each population.

Acknowledgments We acknowledge the valuable suggestions and support of Dr. R. Machida (University of Tsukuba; UT) and Dr. T. Makioka (UT) throughout this study. We express our thanks to Dr. N.C. Watanabe (Kagawa University), Mr. S. Ishiwata (Kanagawa Environmental Research Center), Dr. F. Hayashi (Tokyo Metropolitan University), Dr. M. Myohara (National Institute of Agrobiological Sciences, NIAS), Dr. M. Hatakeyama (NIAS), and Dr. T. Tsutsumi (Fukushima University; FU) for their valuable advice. We are also indebted to Dr. H. Nakamura (Shinshu University, SU), Dr. K. Nakamura (Utsunomiya University), Dr. K. Hatta (Nagoya Women's University), Mr. K. Hoshi (NACS-J), Ms. I. Mori (Okayama Prefectural Nature Conservation Center), Miss A. Endo (FU), and Messrs. K. Miyairi, K. Oka, K. Suzuki, and T. Tanizawa (SU), for their cooperation in collecting materials. This study was supported by grants from the River Environment Fund (12-1-3-7) of the Foundation of River & Watershed Environment Management.

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Appendix 1. Detailed locality information of *Ephoron shigae* shown in Fig. 1. The geographic distribution is updated with some new localities (in boldface type) after Watanabe and Ishiwata (1997)

Number in Fig. 1	River	Locality	Prefecture	Latitude	Longitude	Reference
1	Kitakami-gawa	Esashi	Iwate	39°11′ N	140°10′ E	Watanabe and Ishiwata (1997)
2	Eae-gawa	Wakuya	Miyagi	38°32′ N	141°08′ E	Ishiwata (1996)
3[a]	**Hirose-gawa**	**Sendai**	**Miyagi**	**38°12′ N**	**140°53′ E**	**Present study**
4[b]	**Natori-gawa**	**Sendai**	**Miyagi**	**38°12′ N**	**140°53′ E**	**Present study**
5[c]	**Surikami-gawa**	**Date**	**Fukushima**	**37°49′ N**	**140°30′ E**	**Present study; cf. Tsutsumi et al. (1998)**
6[c]	Abukuma-gawa	Fukushima	Fukushima	37°45′ N	140°28′ E	Watanabe and Ishiwata (1997); cf. Tojo et al. (2002)
7[a]	**Yamanoiri-gawa**	**Nihonmatsu**	**Fukushima**	**37°38′ N**	**140°25′ E**	**Present study**
8[c]	**Abukuma-gawa**	**Koriyama**	**Fukushima**	**37°21′ N**	**140°22′ E**	**Present study**
9[a]	**Nitta-gawa**	**Minami-Souma**	**Fukushima**	**37°40′ N**	**140°55′ E**	**Present study**
10[a]	**Ukedo-gawa**	**Namie**	**Fukushima**	**37°30′ N**	**140°56′ E**	**Present study**
11[a]	**Same-gawa**	**Iwaki**	**Fukushima**	**37°56′ N**	**140°44′ E**	**Present study**
12[c]	Naka-gawa	Gozenyama	Ibaraki	36°33′ N	140°20′ E	Nakamura et al. (1986)
13[a]	**Sakura-gawa**	**Tsuchiura**	**Ibaraki**	**36°06′ N**	**140°09′ E**	**Present study**
14[a]	Kinu-gawa	Utsunomiya	Tochigi	36°34′ N	139°53′ E	Shioyama (1978)
15[c]	Ara-kawa	Ageo	Saitama	35°56′ N	139°32′ E	Ishiwata (1996)
16	Yoro-gawa	Ichihara	Chiba	35°29′ N	140°07′ E	Ishiwata (1996)
17[c]	Obitsu-gawa	Kimitsu	Chiba	35°19′ N	139°55′ E	Ishiwata (1996)
18[c]	Isumi-gawa	Ootaki	Chiba	35°17′ N	140°15′ E	Ishiwata (1996)
19[c]	Tama-gawa	Hino	Tokyo	35°40′ N	139°24′ E	Ishiwata (1996)
20[c]	**Hino-yosui**	**Hino**	**Tokyo**	**36°24′ N**	**138°15′ E**	**Present study; Tsuruta, personal commincation**
21[b]	Sagami-gawa	Atsugi	Kanagawa	35°25′ N	139°23′ E	Nozaki (1983)
22	Agano-gawa	Agano (Yasuda)	Niigata	37°45′ N	139°14′ E	Ishiwata (1996)
23[a]	**Chikuma-gawa**	**Ueda**	**Nagano**	**36°24′ N**	**138°15′ E**	**Present study**
24[a]	**Chikuma-gawa**	**Chikuma**	**Nagano**	**36°32′ N**	**138°07′ E**	**Tojo et al. (2002); present study**
25	**Tenryu-gawa**	**Komagane**	**Nagano**	**35°43′ N**	**137°58′ E**	**Present study; Kubota, personal communication**
26	Nagara-gawa	Mizuho (Hozumi)	Gifu	35°23′ N	136°43′ E	Watanabe and Ishiwata (1997)
27	Kiso-gawa	Inazawa (Sobue)	Aichi	35°15′ N	136°42′ E	Watanabe and Ishiwata (1997)
28	**Nagara-gawa**	**Kaizu**	**Gifu**	**35°13′ N**	**136°40′ E**	**Present study; cf. Chiba (2000)**
29	Toyo-gawa	Shinshiro	Aichi	34°53′ N	137°29′ E	Watanabe and Ishiwata (1997)
30[a]	Oto-gawa	Okazaki	Aichi	34°56′ N	137°12′ E	Ban et al. (1994)
31[a]	Yahagi-gawa	Toyota	Aichi	35°04′ N	137°11′ E	Shiraki et al. (1993); Shiragane, personal communication
32[b]	Shonai-gawa	Nagoya	Aichi	35°15′ N	137°02′ E	Watanabe and Ishiwata (1997); Tojo et al. (2002)
33	Miya-gawa	Watarai	Mie	34°26′ N	136°37′ E	Yamashita (1981)
34	Kumozu-gawa	Tsu (Onoki)	Mie	34°39′ N	136°24′ E	Ishiwata (1996)
35[c]	**Katsura-gawa**	**Kyoto**	**Kyoto**	**35°00′ N**	**135°41′ E**	**Present study**
36	Uji-gawa	Uji	Kyoto	34°53′ N	135°49′ E	Ishiwata (1996)
37	Yura-gawa	Fukuchiyama	Kyoto	35°18′ N	135°08′ E	Kusakabe et al. (1978)
38	Takeda-gawa	Fukuchiyama	Kyoto	35°14′ N	135°09′ E	Ishiwata (1996)
39[c]	Yodo-gawa	Takatsuki	Osaka	34°49′ N	135°38′ E	Ishiwata (1996)
40[c]	Muko-gawa	Takarazuka	Hyogo	34°47′ N	135°22′ E	Tanaka (1993)
41	Maruyama-gawa	Toyooka (Hidaka)	Hyogo	35°29′ N	134°43′ E	Nishimura et al. (1975)
42	Kako-gawa	Kakogawa	Hyogo	34°47′ N	134°54′ E	Morishita (1979)
43[c]	Yumesaki-gawa	Himeji	Hyogo	34°52′ N	134°40′ E	Watanabe and Ishiwata (1997)
44	Ibo-gawa	Tatsuno (Ibo)	Hyogo	34°55′ N	134°33′ E	Morishita (1979)
45	Hino-gawa	Hoki (Mizoguchi)	Tottori	35°20′ N	133°27′ E	Ishiwata (1996)
46	Hii-gawa	Unnan (Kisuki)	Shimane	35°18′ N	132°54′ E	Nishimura et al. (1979)
47	Kando-gawa	Izumo	Shimane	35°17′ N	132°44′ E	Nishimura et al. (1979)
48	Gono-kawa	Gouzu (Sakurae)	Shimane	35°58′ N	132°20′ E	Watanabe and Ishiwata (1997)
49	Gono-kawa	Miyoshi	Hiroshima	34°48′ N	132°51′ E	Watanabe and Ishiwata (1997)
50[c]	Yoshii-gawa	Akaiwa (Yoshii)	Okayama	34°55′ N	134°06′ E	Watanabe and Ishiwata (1997); Mori, personal communication
51[c]	Asahi-gawa	Okayama	Okayama	34°41′ N	133°56′ E	Watanabe et al. (1989)
52[c]	Takahashi-gawa	Soja	Okayama	34°43′ N	133°39′ E	Watanabe and Ishiwata (1997); Mori, personal communication
53	Ota-gawa	Hiroshima (Asaminami)	Hiroshima	34°30′ N	132°31′ E	Ishiwata (1996)
54	Furu-kawa	Hiroshima	Hiroshima	34°28′ N	132°29′ E	Watanabe and Ishiwata (1997)
55	Saba-gawa	Houfu	Yamaguchi	34°04′ N	131°34′ E	Watanabe and Ishiwata (1997)
56	Fushino-gawa	Yamaguchi	Yamaguchi	34°09′ N	131°28′ E	cf. Watanabe and Ishiwata (1997)
57	Yoshino-gawa	Ishii	Tokushima	34°06′ N	134°27′ E	Watanabe and Ishiwata (1997)
58	Katsuura-gawa	Tokushima	Tokushima	33°58′ N	134°48′ E	Watanabe and Ishiwata (1997)
59	Koto-gawa	Takamatsu	Kagawa	34°18′ N	134°01′ E	Watanabe and Ishiwata (1997)
60[a]	Hiji-kawa	Ohzu	Ehime	33°31′ N	132°33′ E	Ishiwata (1996)
61	Monobe-gawa	Noichi	Kochi	33°35′ N	133°42′ E	Ishiwata (1996)

Appendix 1. *Continued*

62[a]	**Shimanto-gawa**	**Shimanto (Nishitosa)**	**Kochi**	**33°26′ N**	**133°05′ E**	**Torii, personal communication; cf. Tojo et al. (2002)**
63	Shimanto-gawa	Shimanto (Nakamura)	Kochi	33°01′ N	132°51′ E	Furuya (1990)
64	Kamimutsuro-gawa	Saga (Kamimutsuro)	Saga	33°27′ N	130°13′ E	Ishiwata (1996)
65	Tabuse-gawa	Saga	Saga	33°18′ N	130°17′ E	cf. Watanabe and Ishiwata (1997)
66	Yamakuni-gawa	Nakatsu	Oita	33°35′ N	131°11′ E	Watanabe and Ishiwata (1997)
67	Chikugo-gawa	Hita	Oita	33°19′ N	130°57′ E	cf. Watanabe and Ishiwata (1997)
68[a]	Ono-gawa	Oita (Shirataki)	Oita	33°09′ N	131°39′ E	Sato (1987)
69	Oita-gawa	Oita (Akegawara)	Oita	33°12′ N	131°35′ E	Watanabe and Ishiwata (1997)
70	Banjo-gawa	Saiki	Oita	32°57′ N	131°51′ E	Sato (1987)
71[a]	**Midori-gawa**	**Kashima**	**Kumamoto**	**32°43′ N**	**130°44′ E**	**Oda et al. (2002); Ueki, personal communication; present study**

[a] Unisexual population: no males were found
[b] Practically unisexual population: the percentage of males was very low (< 1%)
[c] Bisexual population