Two cues for sex determination in *Gammarus duebeni:* Adaptive variation in environmental sex determination?

Alison M. Dunn,[1] *John C. Hogg, and Andrew Kelly*[2]
Centre for Biodiversity and Conservation, School of Biology, University of Leeds, LS2 9JT, United Kingdom

Melanie J. Hatcher
School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, United Kingdom

Abstract

In the crustacean *Gammarus duebeni,* sex is determined by the interaction of a number of environmental, genetic and parasitic factors, which may, in turn, influence sex ratios and population dynamics. We produce novel evidence that environmental sex determination (ESD) in *G. duebeni* depends primarily on the interaction of two environmental cues: day length and temperature. Whereas previous work found that male-biased sex ratios were produced under long day conditions and female biases under short days, we show that, at the lower temperatures normally experienced by this species at northern latitudes, the reverse pattern can occur. We measured ESD in four U.K. populations and found among-population variation in the level of ESD and in the cues that determined sex. In the light of these findings, we conclude that patterns of ESD across the four populations may reflect selection based on differences in breeding season and discrete/overlapping generations.

In animals with environmental sex determination (ESD), offspring become male or female in response to environmental cues experienced during development. ESD is found in organisms from diverse phyla, including fish, reptiles, nematodes, and crustaceans (Adams et al. 1987; Korpelainen 1990). Charnov and Bull (1977) proposed an adaptive explanation for ESD: in a patchy environment where patch quality has a differential effect on male and female fitness, ESD provides a mechanism that enables an individual to match its sex to its greatest expected future fitness. Commonly, we think of patchiness as a spatial dimension, but environments can also differ temporally (Adams et al 1987). The most well-studied example of temporal ESD is that of the Atlantic silverside *Menidia menidia* (Conover and Kynard 1981; Lagamarsino and Conover 1993). Cool temperatures at the start of the breeding season lead to a female-biased sex ratio, whereas warmer temperatures later in the year cue the production of males (Conover and Kynard 1981). Conover (1984) proposed an adaptive explanation for ESD in the silverside: the reproductive success of females is strongly dependent on adult body size, as shown by big females laying more eggs. Body size is not as important for the fitness of males. Males are promiscuous group spawners

that show no male/male competition for mates, and the correlation between body size and sperm number and the number of offspring produced is presumably not as strong. Body size reflects growth period, and temperature acts as a cue that indicates whether offspring will experience a long or short growing season, which thereby enables individuals to match their sex to their greatest expected future fitness (Conover 1984).

Temporal ESD has also been reported in the amphipod crustacean *Gammarus duebeni.* This species has a wide distribution and is of interest to ecologists and evolutionary biologists, because sex is determined by the interaction of a number of environmental, genetic, and parasitic factors, which may, in turn, influence sex ratios and population dynamics (Naylor et al. 1988*a,b;* Hatcher and Dunn 1995; Hatcher et al. 2000). In *G. duebeni,* sex determination is influenced by photoperiod (Bulnheim 1978; Naylor et al. 1988*a,b*) resulting in male and female production peaks at different times of the year. Sex determination in *G. duebeni* can be regarded as a classic example of ESD (Valenzuela et al. 2003)—environmental cues experienced during development irreversibly influence the sex of an individual (Charnov and Bull 1977). Adams et al. (1987) proposed an adaptive explanation for ESD in *G. duebeni* that is similar to that for ESD in *M. menidia* (Conover 1984). In *G. duebeni,* sex determination is influenced by the day length experienced during early development, 3–4 weeks after release from the mother's brood pouch (Bulnheim 1978; Naylor et al. 1988*b*). For *G. duebeni,* the time of year (i.e., temporal patch) when young are released into the environment affects future growth opportunities (Adams et al. 1987; Naylor et al. 1988*a*). Large size is more important for the reproductive success of males than females, because males compete for the females they guard in precopula (Naylor and Adams 1987), and small males may fail to mate. Female fecundity increases with size. However, small females can always be guarded, whereas very large females may have reduced mat-
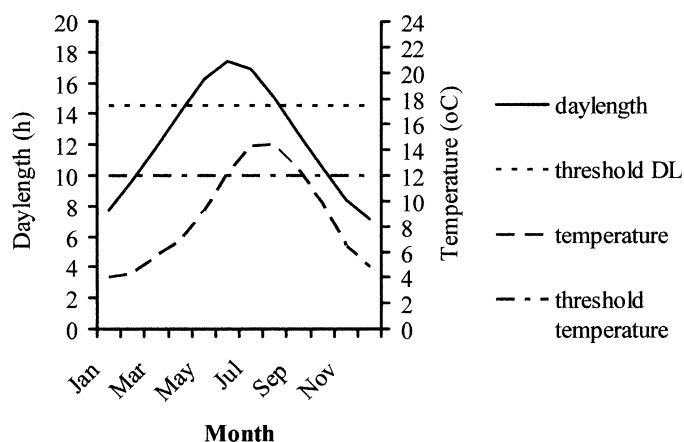
Fig. 1. Seasonal day lengths and temperatures at Budle Bay and the threshold day length and temperature cues. Animals with ESD show a threshold response to environmental cues (Conover and Heins 1987; Naylor et al. 1988*b*) rather than a continuous change in sex ratio as the cue varies. The threshold day length (DL) for ESD (14.5 h; Naylor et al. 1988*b*) is indicated. In the current study, we did not establish the threshold temperature for ESD but exposed broods to high and low temperatures within the range experienced during field breeding. For the purposes of the illustration, we estimate the critical temperature to fall midway between 9°C and 15°C; at 12°C.
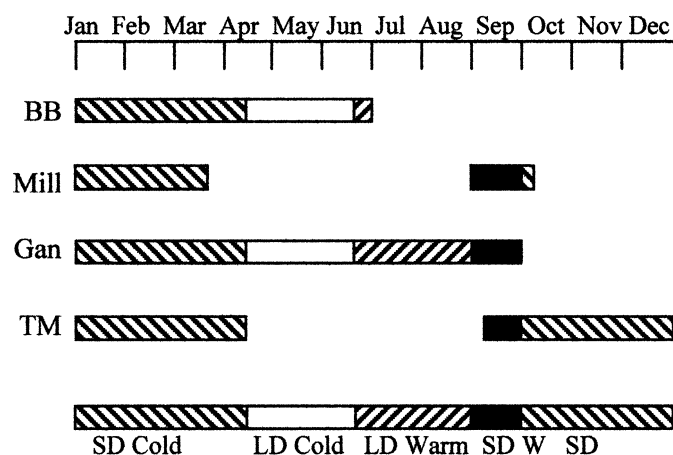

Fig. 2. Breeding season for *G. duebeni* populations from Budle Bay (BB), Millport (Mill), Gansey Beach (Gan), and Totton Marsh (TM) and the temperature and day length cues that animals experience over the year. Breeding season data were taken from the literature and personal observations as follows: Budle Bay (Naylor et al. 1988*a*); Millport (Kelly et al. 2001); Gansey Beach (Hynes 1954; unpubl. data); and Totton Marsh (Watt and Adams 1993). The threshold day length was 14.5 h (Naylor et al 1988*b*). The threshold temperature was estimated to lie at 12°C. SD = short day, LD = long day.

ing success if they are too large to be held in precopula (Hatcher and Dunn 1997). Photoperiod could provide a proximate cue for the time available for growth with selection favoring a system enabling males to be produced early in the year.

The extent to which date of birth predicts future size at first mating depends on life history parameters, including the length of breeding season and the potential for overlapping generations. For both *M. menidia* (Conover and Heins 1987) and *G. duebeni* (Watt and Adams 1994), a higher level of ESD occurs in populations where the month of birth has a strong impact on an individual's future size-related fitness. The potential for future growth and the associated fitness gains of being born early or late matter less in very short growing seasons or where there are overlapping generations. Lower levels of ESD have been recorded in *M. menidia* populations with a very short breeding season (Conover and Heins 1987) and in *G. duebeni* populations where generations overlap (Watt and Adams 1994).

However, in *G. duebeni,* there is a mismatch between the ESD response to photoperiod in the laboratory and sex ratios observed in the field, which led Watt and Adams (1993) to propose that a second environmental cue may affect sex determination in this species. In addition, previous studies of ESD may have been confounded by the presence of parasitic sex ratio distorters in populations of *G. duebeni* (Terry et al. 1998). Feminization by microsporidian parasites overrides the normal mechanism of environmental sex determination such that infected females produce broods that are >90% female (Bulnheim 1978; Terry et al 1998). In addition, abiotic factors—including temperature and salinity—can affect the density of parasitic sex ratio distorters and, consequently, their transmission and feminization efficiency (Dunn and

Hatcher 1997*a;* Hurst et al 2000). Parasite prevalences of 6–30% (Dunn and Hatcher 1997*b*) have been reported in *G. duebeni* populations where ESD has been studied, which may confound the results of previous ESD studies.

Here we examine whether a second environmental cue for sex determination can resolve this conundrum by measuring the effects of both temperature and photoperiod on sex determination in *G. duebeni,* controlling for the influence of feminizing parasites. Both temperature and day length vary seasonally and are thus potential cues for date of birth and, hence, predicted size at first mating. In the field, there is a time lag between day length and temperature; day length increases and peaks before temperature in spring and decreases before temperature in autumn (Fig. 1). Therefore, the use of these two cues may provide more accurate information on the time of year and, hence, on the period for growth. We also look for evidence of adaptive variation in sex determination by measuring ESD in four U.K. study populations that vary in breeding season and generation overlap; Budle Bay, Northumberland (55°40′N, 1°43′W), White Bay, Millport, Isle of Cumbrae (55°47′N, 04°56′W), Gansey Beach, Isle of Man (54°05′N, 4°44′W), and Totton Marsh, Hampshire (50°55′N, 1°29′W). Based on the life history of each population, we predict the sex ratios which should be produced under different environmental cues and use breeding experiments to test these predictions (some day length and temperature combinations do not occur during the breeding season of some populations, so we do not predict sex ratios for these combinations of cues).

The breeding season is shortest in the Budle Bay population, where breeding occurs between January and July, and there are no overlapping generations (Fig. 2; Naylor et al. 1988*a).* This population will experience short days and cold temperatures at the start of the breeding season, long days

Table 1. Numbers of broods reared from each field site and average, maximum, and minimum numbers of offspring per brood.

Site	No. of broods	Average brood size	Maximum brood size	Minimum brood size
Budle Bay	76	14.4	33	3
Millport	93	15.1	33	2
Gansey Beach	98	6.9	24	1
Totton Marsh	66	14.7	43	2

and cool temperatures later in the breeding season, and a short period of long days and warm temperatures at the tail end of the breeding season. We predict strong selection for ESD in this seasonal population. Future fitness will be maximized by male sex determination in young born early in the season and female sex determination in later-born young. Therefore, we predict that low temperatures and short day length at the start of the breeding season should cue male-biased sex ratios. Later in the breeding season, long day length and cool temperatures should cue female sex determination. Females should also be cued by long day length and warm temperatures at the very end of the breeding season.

ESD should also be adaptive for animals from the Millport population. Breeding is also seasonal in this population, and there are two bursts of reproductive activity (Fig. 2; Kelly et al. 2001). This population will experience short days and cool temperatures during the first period of reproductive activity and short days and warm temperatures during the second phase of reproduction. Animals born during the early period of breeding will have a long growing season and should maximize their expected future fitness by becoming male. Therefore, we predict male-biased sex ratios in short days and cool temperatures. Animals born in the later period of breeding (September–October) should become female. Therefore, we predict female-biased sex ratios in short days and warm temperatures. Both periods of reproduction occur during short days, so it is of interest to test for an effect of temperature in this population.

For the Gansey population, the breeding season is not well defined but appears to be relatively long, commencing in January (Hynes 1954; unpubl. data). This population will experience all day length/temperature combinations over the breeding season—short days and cool temperatures early in the breeding season, long days and cool temperatures in spring, long days and warm temperatures in summer, and short days and warm temperatures toward the end of breeding in the autumn. We predict a response to environmental cues for this population that is similar to Budle Bay, although the level of ESD may be lower, because it is probable that generations overlap. Therefore, we predict that low temperatures and short day length at the start of the breeding season should cue male-biased sex ratios. Later in the breeding season, long day length and cool temperatures should cue female sex determination. Females should also be cued by long day length and warm temperatures during the summer. At Gansey Beach, the breeding season continues into autumn, and we predict that females should be cued by short

days and warm temperatures at the end of the breeding season.

In contrast with the other populations, at Totton Marsh, generations overlap strongly (Watt and Adams 1993). We predict a low level of ESD for this population, because there will be little or no selection for sex differential production over the breeding season, and a 50:50 sex ratio would be favored under most circumstances (Hamilton 1967). Therefore, we predict no difference in sex ratios under the different day length and temperature treatments.

Methods

Adult *G. duebeni* were collected at random using a fine mesh net from each of the four field sites. To control for the confounding effects of microsporidian sex-ratio distorters, only uninfected females were used in the experiments. Because these sex-ratio distorters are transovarially transmitted (passed from mother to offspring via the eggs; Terry et al. 1998), the infection status of individual females was determined by screening their embryos for the presence of microsporidia (Terry et al. 1998). Brooding females were anaesthetized in carbonated water, and the embryos were flushed from the brood pouch, permeated with 5 mol L^{-1} HCl and fixed in acetone at $-20°C$. The embryos were stained with 4,6-diamidino-2-phenyl-indole (a fluorescent dye for DNA) diluted $1:500$ in 0.2 mol L^{-1} NaH_2PO_4. The embryos were screened for parasites using a Zeiss Axioplan fluorescence microscope, and any infected mothers were discarded from the experiment.

The effect of temperature and photoperiod on sex determination was examined by rearing *G. duebeni* broods in four different environments that we termed long day cold (16 h light:8 h dark, 9°C), long day warm (16 h light:8 h dark, 15°C), short day cold (8 h light:16 h dark, 9°C), and short day warm (8 h light:16 h dark, 15°C). These temperatures and day length treatments were chosen to reflect environments experienced in the field (Figs. 1, 2) and to maximize successful rearing in the laboratory. A total of 100 individual females from each site were placed in 150-ml pots that contained brackish water (specific gravity = 1,005°) with rotted sycamore leaves and *Enteromorpha* sp. (marine alga) for food and shelter. Females were assigned at random to one of four day length/temperature treatments and allowed to acclimatize to the experimental temperature for 6 weeks (at least one gonotrophic cycle). A male was then added to each pot, pairs were monitored daily, and the male was removed once eggs were visible in the female's brood pouch and mating had occurred. A female broods her young for 3–4 weeks until she moults, when the juveniles are released from the brood pouch. When the juveniles were released, broods were reared in the same day length/temperature treatment as their mother. Juveniles were kept in 150-ml pots, with a maximum of four juveniles (siblings) per pot, and food (*Enteromorpha* sp. and rotted sycamore leaves) was provided in excess. The young were sexed at 3 months on the basis of external sexual characteristics. Broods were successfully reared from a total of 76 females from Budle Bay, 93 females from Millport, 98 females from Gansey Beach, and 66 females from Totton Marsh (Table 1).

Table 2. ANOVA table for the sex ratio of *G. duebeni* broods from four field populations raised under different temperatures and day lengths. NS = not significant.

Source	SS	df	F	p
Day length	7.73	1	6.01	<0.01
Temperature	8.03	1	6.23	<0.01
Population	17.55	3	13.4	<0.001
Day length × temperature	20.28	1	31.63	<0.001
Day length × population	4.16	3	1.16	NS
Temperature × population	0.99	3	0.277	NS
Day length × temperature × population	6.42	3	1.78	NS
Error	374.84	317		
Total	440	332		

The data were analyzed by analysis of variance using the generalized linear modeling package GLIM (Numerical Algorithms). Mean monthly temperature data were obtained from the UK Meteorological Office for the Boulmer weather station (55°25′N, 01°36′W), which is ~2 km from the Budle Bay field site. Seasonal day lengths were calculated using Sunang (SusDesign). Three sets of analyses were carried out. First, we investigate the effect of treatment on sex ratios. Sex ratios are percentage data and so were analyzed specifying a binomial error. Overdispersion was corrected for using Pearson's χ^2 (Crawley 1993). Second, we investigated the effect of treatment on survival specifying a binomial error structure taking the brood size at the start of rearing (i.e., before any animals had died) as the binomial denominator. Finally, because survival was affected by treatment for some of our populations, we went on to control for possible sex differential survival with respect to treatment. We constructed an adjusted data set in which we assigned the dead individuals in each brood to the rarer sex and tested for ESD on the adjusted data, taking brood size at the start of rearing as the binomial denominator.

Results

Our initial analysis of the entire data set showed that brood sex ratios differed significantly with day length, temperature, and between the different populations. Furthermore, the interaction between photoperiod and temperature significantly affected sex ratios (Table 2). For example, in the Budle Bay and Millport populations, under warm conditions, long days cued male-biased production and short days cued female biases, but, under cold conditions, this pattern was reversed (Fig. 3). An examination of Fig. 3 shows that the pattern of sex ratios under different environmental cues was not consistent between populations (see Table 2 for the significant effect of population on sex ratio). Therefore, we analyzed the data for each population separately.

Budle Bay—In animals from the Budle Bay population, there was no significant main effect of either temperature or day length on sex ratio (Table 3). However, brood sex ratios were significantly affected by the interaction between day
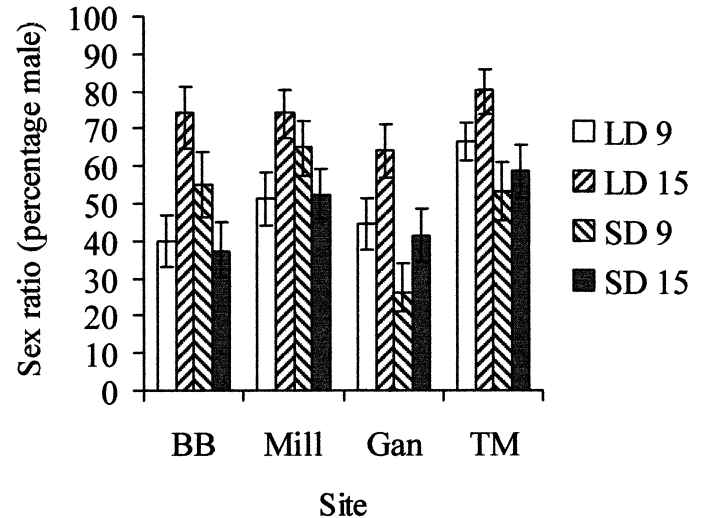

Fig. 3. Sex ratio ± 95% confidence interval (data backtransformed from GLIM maximum likelihood estimates) of *G. duebeni* broods from the four field populations under different day lengths and temperatures. BB = Budle Bay, Mill = Millport, Gan = Gansey Beach, TM = Totton Marsh, LD 9 = long day at 9°C, LD 15 = long day at 15°C, SD 9 = short day at 9°C, and SD 15 = short day at 15°C.

length and temperature. At 15°C, broods were male biased under long days (74.3% male) and female biased under short days (37.3% males). At 9°C the opposite pattern was found; long day broods were female biased (39.7% male) and short day broods were male biased (55.2% male).

Millport—Animals from Millport, Isle of Cumbrae showed a similar ESD response to photoperiod and temperature to that at Budle Bay, although all sex ratios were >50% (Fig. 3). Brood sex ratios were significantly affected by the interaction between day length and temperature, but there was no significant main effect of either temperature or day length on sex ratio (Table 4). At 15°C, broods were more male biased under long days (74.3% male) and less male biased under short days (52.5% males). At 9°C, the opposite pattern was found; long day broods were less male biased (51.5% male) and short day broods were more male biased (65% male).

Totton Marsh and Gansey Beach—The response to environmental cues of animals from Totton Marsh and Gansey Beach differed markedly from that of animals from the Bu-

Table 3. ANOVA table for the sex ratio of *G. duebeni* broods from Budle Bay raised under different temperatures and day lengths. NS = not significant.

Source	SS	df	F	p
Day length	1.74	1	1.33	NS
Temperature	1.11	1	0.85	NS
Day length × temperature	11.30	1	8.66	<0.01
Error	84	72		
Total	98	75		

Table 4. ANOVA table for the sex ratio of *G. duebeni* broods from Millport raised under different temperatures and day lengths. NS = not significant.

Source	SS	df	F	p
Day length	0.15	1	0.015	NS
Temperature	1.88	1	1.884	NS
Day length × temperature	8.52	1	6.794	<0.05
Error	104	89		
Total	115	92		

Table 5. ANOVA table for the sex ratio of *G. duebeni* broods from Gansey Beach raised under different temperatures and day lengths. NS = not significant.

Source	SS	df	F	p
Day length	8.26	1	6.108	<0.05
Temperature	5.07	1	3.649	NS
Day length × temperature	0.07	1	0.057	NS
Error	121.49	94		
Total	134.9	97		

dle Bay and Millport populations. The sex determination of Totton Marsh and Gansey Beach animals was significantly affected by day length (Tables 5, 6) with more males produced under long days than under short days. Neither temperature (main effect) nor the day length × temperature interaction affected sex ratio.

Mortality—Brood survival was high at all sites (average percentage of brood surviving, data back transformed from GLIM maximum likelihood estimates: Budle Bay = 77%, Millport = 88%, Gansey Beach = 75%, and Totton Marsh = 62%). For Budle Bay, survival was significantly affected by the day length × temperature interaction ($F_{1,73}$ = 14.8, p < 0.01), but there was no significant main effect of day length ($F_{1,75}$ = 0.27, p > 0.05) or temperature ($F_{1,74}$ = 0.01, p > 0.05). Survival was lowest (67%) in long day warm conditions, which, in the field, are only experienced for ~2 weeks at the end of the breeding season. For both Millport and Gansey Beach, survival was significantly affected by day length and was lower in short day than in long day treatments (Millport $F_{1,92}$ = 4.13, p < 0.05; Gansey $F_{1,97}$ = 10.97, p < 0.01). Survival for these populations was unaffected by temperature (Millport $F_{1,91}$ = 0.52, p > 0.05; Gansey $F_{1,96}$ = 0.1, p > 0.05) or by the interaction between day length and temperature (Millport $F_{1,90}$ = 0.44, p > 0.05; Gansey $F_{1,95}$ = 0.49, p > 0.05). Survival for Totton Marsh animals was unaffected by environmental treatment (day length $F_{1,64}$ = 0.2, p > 0.05; temperature $F_{1,65}$ = 3.47, p > 0.05; day length temperature interaction $F_{1,63}$ = 0.34, p > 0.05).

Sex differential mortality—Our data, as well as those of previous studies (Bulnheim 1978; Naylor et al 1988*b*), provide strong evidence for ESD in *G. duebeni.* However, we should consider the alternative explanation, that the observed sex ratios resulted from sex differential mortality in the different treatment regimes. We went on to control for this by assigning the dead individuals in each brood to the rarer sex and testing for environmental effects on sex ratio for the adjusted dataset. This provides a conservative test for ESD, because we are effectively checking that any sex ratio variation with respect to environmental treatments is robust to the strongest possible reversal of sex differential mortality that we can impose on the data set. If the sex ratios were the result of ESD, we would expect the adjusted data to show the same statistical results as the real, unadjusted data. If, however, the sex ratios were the result of sex differential mortality, we would predict the significant effects of envi-

ronmental treatments found for the real data to disappear when examining the adjusted data. The analysis of the adjusted data set for Budle Bay found a marginally significant effect on sex ratio of the day length × temperature interaction ($F_{1,75}$ = 3.42, p = 0.07), which indicates that ESD is possibly in operation here, although much of the sex ratio variation could be explained by sex differential mortality alone. For Millport, the adjusted data gave the same results—the nonadjusted data : sex ratio was significantly affected by the interaction between day length and photoperiod ($F_{1,90}$ = 4.74, p < 0.05). This indicates that sex ratio variation in the Millport population cannot be accounted for by sex differential mortality and therefore is the result of ESD. For Gansey Beach, analysis of the adjusted data showed a significant day length × temperature interaction ($F_{1,95}$ = 4.21, p < 0.05), whereas, for the nonadjusted data, only day length affected sex ratio (Table 5). This indicates that ESD operates in the Gansey population, because, even if we assume that all dead individuals were of the rare sex, we find that day length significantly affects sex ratio via the interaction term. Analysis of the adjusted data for Totton Marsh found no significant effect of day length on sex ratio ($F_{1,61}$ = 0.1, p > 0.05), which suggests that sex differential mortality alone could account for the variation in sex ratio.

Level of ESD—We calculated the level of ESD for each population as the difference between the highest and the lowest sex ratio (percentage males) produced under the different environmental conditions (Conover and Heins 1987; Watt and Adams 1993). We provide two estimates. The first estimate was based on the complete range of conditions tested in the laboratory (maximal level of ESD). However, in seasonally breeding populations, not all of these temperature/photoperiod combinations will be experienced during the breeding season (Fig. 2). Therefore, we also calculated a second estimate for each population (operational level of

Table 6. ANOVA table for the sex ratio of *G. duebeni* broods from Totton Marsh raised under different temperatures and day lengths. NS = not significant.

Source	SS	df	F	p
Day length	5.12	1	4.17	<0.05
Temperature	2.44	1	2.09	NS
Day length × temperature	0.64	1	0.558	NS
Error	71.6	62		
Total	79.8	65		

Table 7. The level of ESD (the difference between the highest and lowest sex ratio [% male] produced under the different environmental conditions) for each population.

Population	No. of mothers	No. of offspring	Maximum level of ESD	Operational level of ESD
Budle Bay	76	846	37	35
Millport	93	1,227	23	14
Gansey Beach	98	677	38	38
Totton Marsh	66	577	27	06

ESD) as the difference between the highest and the lowest sex ratio produced under the conditions that would be experienced during a breeding season in the field. Both estimates indicate that the level of ESD varies between populations with ≤38% of animals having sex determined environmentally (Table 7). The operational estimate indicates a greater range in levels of ESD, from 6% at Totton Marsh to 38% at Budle Bay.

Discussion

G. duebeni shows between population variation in the mechanism of ESD—both the level of ESD and the cues which affect sex ratios differ in animals from the different populations. Our results for animals from Totton Marsh and Gansey Beach are in accord with previous studies of ESD with long day conditions cueing more male-biased sex ratios and short days cueing female production (Bulnheim 1978; Naylor et al. 1988b; Watt and Adams 1994). However, a markedly different pattern was observed for animals from Budle Bay and Millport, with temperature and photoperiod interacting to determine sex: sex ratios are male biased in long day warm conditions, but the pattern is reversed in cold conditions.

Developmental switching is controlled by two cues in aphids and nematodes. In *Aphis fabae*, photoperiodic counters for female morph and sex determination are modulated by temperature and populations differ in their response to photoperiod cues (Vaz Nunes and Hardy 1999). Morph development in the parasitic nematode *Strongyloides ratti* is controlled by two developmental switches: host immune status and environmental temperature (Harvey et al. 2000). In *Romanomermis culicivorax*, sex is cued by temperature and parasite density within the host (Tingley and Anderson 1986). To our knowledge, the only other record of a dual cue for ESD comes from the fish *Poecilia sphenops*, in which sex determination is cued by the interaction between temperature and salinity (Baron et al. 2002).

The use of two environmental cues for sex determination may be adaptive if it allows more precise predictions of growth opportunity before reproduction and so of expected future fitness. Our data led us to reevaluate the level of ESD in *G. duebeni* and to reconsider its adaptive significance. Analysis of the adjusted data provides a conservative test for ESD and for two of our populations this analysis clearly indicates that the environmental cues significantly affect sex

determination. For Budle Bay and Totton Marsh, we cannot discount the alternative explanation that the observed sex ratios result from sex differential mortality under the different environments. However, although some sex specific lethal mutations are activated by temperature (Baker and Ridge 1980), to our knowledge there are no reports of day length–activated sex specific mortality. Given that our conservative test provides clear evidence for ESD in two of four populations, ESD appears to be the most parsimonious explanation for the sex-ratio patterns observed across all four populations.

In our study populations, the breeding season is shortest in the Budle Bay population, where breeding occurs between January and July and there are no overlapping generations (Fig. 2; Naylor et al. 1988a). Here, our data support our predictions in that low temperatures and short day length at the start of the breeding season will lead to male biased sex ratios. This will be adaptive, because male sex determination in early born individuals will maximize the advantages of long growth opportunities. Long day length and cool temperatures cue female sex determination, and this will lead to production of females when growth period is shorter. This strategy of seasonal production of males and females was first postulated by Naylor et al. (1988b); however, in the absence of the temperature cue, it was a puzzle how photoperiod could cue male production early in the breeding season. Our data resolve this issue. In contrast with our predictions, the greatest percentage of males was produced under long day lengths and warm conditions (in accord with Naylor et al 1988b), which will lead to the production of small males at the end of the breeding season. However, these conditions are experienced for a very short period at the tail end of the breeding season, so very few individuals will suffer the cost of maladaptive male sex determination.

In a previous study of the Millport population, Terry et al. (1998) concluded that *G. duebeni* from Millport did not show ESD. However, that study only investigated the effects of day length. When we considered the interaction of day length with temperature, we found clear evidence for ESD in the Millport population, although at a lower level than that at Budle Bay. In accord with our predictions, in the field, ESD will lead to the production of male-biased sex ratios early in the breeding season in response to short day length and cool temperatures. During the second period of reproductive activity, short day length and warm temperatures will cue the production of more females, which is in accord with our predictions. At the end of the second period of reproduction, short day length and cool temperatures should cue males, which would be maladaptive. However, the precise pattern of sex differential productivity in the field will depend on the threshold for the two cues. The threshold day length for ESD in *G. duebeni* has been determined (14–15 h; Naylor et al. 1988b), but the threshold temperature for ESD is not known; for illustrative purposes, we have estimated it to lie midway (12°C) between our experimental temperatures. If our estimate is correct, then animals in the Millport population experience short day lengths and cool temperatures at the end of the second period of breeding. However, if the critical temperature lies below our estimate, for example at 10°C, then ESD would lead to female sex

determination during the later period of reproduction in September–October, in accord with adaptive predictions. It is also interesting to note that populations vary in the threshold day length for ESD (Naylor et al 1988*b*). A detailed investigation of the threshold temperature for different populations is required to further our understanding of the evolution of ESD in *G. duebeni*.

In contrast with the two northern populations above, ESD is cued only by photoperiod in animals from the Gansey Beach and Totton Marsh populations. The Gansey population remains somewhat puzzling. The breeding season is relatively long, commencing in January (Hynes 1954; unpubl. data). Because the effect of photoperiod is not modulated by temperature, females should be produced early in the season, contrary to our predictions from adaptive arguments. It is probable that generations overlap here that would reduce the selective pressure for ESD, and yet it has the highest ESD level of all the populations studied (Table 7). The observed patterns may be explained if females produced early in the year can reach sexual maturity during the same year and can then mate with adult males that were born the previous year. In this case, individuals produced early in the breeding season would maximize their reproductive potential by female sex determination response to short day length. The Gansey population is located on the west coast of England and is subject to the warming influences of the gulf stream in winter, so animals born early in the year may grow more rapidly here than in the other field sites. A detailed study of the breeding and life history of this population is required to test this hypothesis.

Previous studies have reported conflicting findings for ESD at Totton Marsh. Although Naylor et al. (1988*b*) found no evidence for a sex-ratio response to day length in Totton Marsh, Watt and Adams (1994) reported that 16% of animals were responsive to day length. Our maximal level of ESD is even higher at 27% but does not apply to the situation in the field (the operational level of ESD is only 6%), where most reproduction takes place from late September to early April, when day lengths are short (Watt and Adams 1993, Fig. 2). Whereas long days lead to a strong male biases (80%) in the laboratory, only short days will be experienced in the field and will lead to sex ratios close to 50% (under short days, the sex ratio produced at Totton Marsh did not differ significantly from $50:50$, $\chi_1^2 = 3.64$, $p > 0.05$). As the breeding season is long at Totton Marsh and generations overlap strongly (Watt and Adams 1993), there will be little or no selection for sex differential production over the breeding season so a $50:50$ sex ratio would be favored under most circumstances (Hamilton 1967). The observation that animals from the Totton Marsh population have a relatively high level of plasticity for sex determination when tested in the laboratory is surprising, given that, in nature, only one of the four environments is experienced throughout most of the breeding season, and this suggests that the observed level of ESD may be ancestral. In the absence of a fitness advantage, it has been suggested that there should be selection against plasticity of sex determination in short-lived species, because skewed sex ratios can strongly affect an individual's future mating prospects (Bull and Bulmer 1989). However, although there is no obvious fitness advantage to ESD in the

Totton Marsh population, there may be little selection against plastic sex determination, because the environmental conditions experienced in the field lead to sex ratios that do not differ from $1:1$.

Our results suggest plasticity of sex determination. The occurrence of different cue responses and ESD levels between geographically close populations suggest the ability of *G. duebeni* to adapt to local conditions. Similarly, studies of *M. menidia* and *M. peninsulae* have shown adaptive variation in the level of ESD. Levels of ESD from 0% to ~70% have been reported for different populations of *M. menidia* (Conover and Heins 1987), and breeding experiments have confirmed that the level of ESD changes in response to selection.

Our observations also illustrate the need for caution in extrapolating from laboratory observations to patterns in the field. Although the maximal level of ESD for Totton Marsh animals is high (27%), breeding in the field occurs only during short days, so there will, in fact, be little sex ratio variation (the operational level of ESD is only 6%). Early studies of the response of Budle Bay animals to photoperiod were undertaken at temperatures that animals do not experience during the breeding season in the wild and led to sex-ratio predictions that are in fact opposite to patterns observed under field-equivalent temperatures.

This work contributes to the increasing evidence that environmental factors can interact to influence developmental processes including sex determination. ESD occurs in broad range of species from aquatic and terrestrial environments (Korpelainen 1990), and current studies suggest that its underlying mechanisms are complex and vary between species or even between populations (Lagomarsino and Conover 1993; Prahlad et al 2003; this study). It is increasingly important to identify the environmental factors that interact to influence sex, determination especially with regard to current concerns over large-scale environmental change. Predicting the ecological consequences of environmental change will prove to be difficult for species that use multiple environmental cues and exhibit between-population variation in their response to these cues.

References

ADAMS, J., P. J. GREENWOOD, AND C. NAYLOR. 1987. Evolutionary aspects of environmental sex determination. Int. J. Invertebr. Reprod. Dev. **11:** 123–136.

BAKER, B. S. AND K. A. RIDGE. 1980. Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. Genetics **94:** 384–423.

BARON, B., F. R. BUCKLE, AND S. ESPINA. 2002. Environmental factors and sexual differentiation in *Poecilia sphenops* Vlenciennes (pisces: Poeceliidae). Aquacult. Res. **33:** 615–619.

BULL, J. J., AND M. G. BULMER. 1989. Longevity enhances selection of environmental sex determination. Heredity **63:** 315–320.

BULNHEIM, H. P. 1978. Interaction between genetic, external and parasitic factors in sex determination of the crustacean amphipod *Gammarus duebeni*. Helgol. Wiss. Meeres. **31:** 1–33.

CHARNOV E. L., AND J. J. BULL. 1977. When is sex environmentally determined? Nature **266:** 828–830.

CONOVER, D. O. 1984. Adaptive significance of temperature-dependent sex determination: In a fish. Am. Nat. **123:** 297–313.

————, AND S.W. HEINS. 1987. Adaptive variation in environmental and genetic sex determination in a fish. Nature **326:** 496–498.

————, AND B.E. KYNARD. 1981. Environmental sex determination: Interaction of temperature and genotype in a fish. Science **213:** 577–579.

CRAWLEY, M. J. 1993. GLIM for ecologists. Blackwell Scientific.

DUNN, A. M., AND M. J. HATCHER. 1997*a.* The effect of salinity on transmission of a microsporidian infecting *Gammarus duebeni.* Parasitology **115:** 381–385.

————, AND M.J. Hatcher. 1997*b.* Prevalence, transmission and intensity of infection by a microsporidian sex ratio distorter in natural *Gammarus duebeni* populations. Parasitology **114:** 231–236.

HAMILTON, W. D. 1967. Extraordinary sex ratios. Science **156:** 477–488.

HARVEY, S. C., A. W. GEMMILL, A. F. READ, AND M. E. VINEY. 2000. The control of morph development in the parasitic nematode *Strongyloides ratti.* Proc. R. Soc. Lond. B **267:** 2057–2063.

HATCHER, M. J., AND A. M. DUNN. 1995. Evolutionary consequences of cytoplasmically inherited feminizing factors. Phil. Trans. R. Soc. Lond. B **348:** 445–456

————, AND ————. 1997. Size and pairing success in *Gammarus duebeni:* Can females be too big? Anim. Behav. **54:** 1301–1308.

————, ————, AND C. TOFTS. 2000. The co-existence of hosts and sex ratio distorters in structured populations. Evol. Ecol. Res. **2:** 185–205.

HURST, G. D. D., A. P. JOHNSON, J. H. G. SCHULENBURG, AND Y. FUYAMA. 2000. Male killing *Wolbachia* in *Drosophila:* A temperature-sensitive trait with a threshold bacterial density. Genetics **156:** 699–709.

HYNES, H. B. N. 1954. The ecology of *Gammarus duebeni* Liljeborg and its occurrence in fresh water in Western Britain. J. Anim. Ecol. **23:** 38–84.

KELLY, A., A. M. DUNN, AND M. J. HATCHER. 2001. Population dynamics of a vertically transmitted, parasitic sex ratio distorter and its amphipod host. Oikos **94:** 392–402.

KORPELAINEN, H. 1990. Sex ratios and conditions required for environmental sex determination in animals. Biol. Rev. **65:** 147–184.

LAGOMARSINO, I. V., AND D. O. CONOVER. 1993. Variation in environmental and genotypic sex determining mechanisms across a latitudinal gradient in the fish *Menidia menidia.* Evolution **47:** 487–494.

NAYLOR, C., J. ADAMS, AND P. J. GREENWOOD. 1998*a.* Population dynamics and adaptive sexual strategies in a brackish water crustacean *Gammarus duebeni.* J. Anim. Ecol. **57:** 493–507.

————, ————, AND P. J. GREENWOOD. 1998*b.* Variation in sex determination in natural populations of a shrimp. J. Evol. Biol. **1:** 355–368.

PRAHLAD, V., D. PILGRIM, AND E. B. GOODWIN. 2003. Roles for mating and environment in *C. elegans* sex determination. Science **302:** 1046–1049.

TERRY, R. S., J. E. SMITH, AND A. M. DUNN. 1998. Impact of a novel, feminizing microsporidium on its crustacean host. J. Eukaryot. Microbiol. **45:** 497–501.

TINGLEY, G. A., AND R. M. ANDERSON. 1986. Environmental sex determination and density dependent population regulation in the entomogenous nematode *Romanomermis culicivorax.* Parasitology **92:** 431–449.

VALENZUELA, N., D. C. ADAMS, AND F. J. JANZEN. 2003. Pattern does not equal process: Exactly when is sex environmentally determined. Am. Nat. **161:** 676–683

VAZ NUNES, M., AND J. HARDIE. 1999. The effect of temperature on the photoperiodic ''counters'' for female morph and sex determination in two clones of the black bean aphid, *Aphis fabae.* Physiol. Entomol. **24:** 339–345.

WATT, P. J., AND J. ADAMS. 1993. Adaptive sex determination and population dynamics in a brackish water amphipod. Estuar. Coast. Shelf Sci. **37:** 237–250.

————, AND J. ADAMS. 1994 Adaptive variation in sex determination in a crustacean, *Gammarus duebeni.* J. Zool. Lond. **232:** 109–116.