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Experimental hybridization of alpine and lowland forms of *Boeckella dilatata*, a calanoid copepod

ANDREA E. BYROM*, CAROLYN W. BURNS & GRAHAM P. WALLIS
Department of Zoology, University of Otago, P.O. Box 56, Dunedin, New Zealand

The freshwater calanoid copepod *Boeckella dilatata* Sars inhabits lakes and ponds of glacial origin in central and southern South Island, New Zealand. In lowland lakes and ponds, this copepod is small and transparent while in alpine bodies of water, it is larger and bright red. The life history traits of alpine and lowland populations of this copepod also differ. In this paper we present the results of experimental hybridization between individuals from an alpine and a lowland population. Crosses between a transparent, lowland morph of *B. dilatata* and a red, alpine morph produced viable F_1. Intermorph matings showed no sign of reduced clutch size in either alpine or lowland females. Back- and inter-crossing of the F_1 showed that this generation was fertile and that subsequent F_2 were viable. Clutch sizes of F_1 hybrid females backcrossed to lowland males, however, were significantly smaller than those of females in other F_1 crosses; in addition, a high variance in clutch size was observed in all F_1 crosses, which is also interpreted as reduced fitness. F_1 hybrid breakdown was also apparent from the observation that clutch sizes of females in backcrosses with a parental constitution of 25 per cent alpine were significantly smaller than clutch sizes of females in pure crosses. These observations are attributed to heritable differences in life history characteristics between alpine and lowland populations of *B. dilatata*. We conclude that the red alpine and transparent lowland morphs of this copepod are best regarded as conspecifics in the early stages of speciation and we discuss the results as they relate to current concepts of speciation. F_1 and F_2 offspring all showed red pigmentation of similar intensity to the original alpine parental generation and we conclude that either pigmentation in *B. dilatata* is a polygenic character with dominance or is induced by a factor present in the alpine water.

Keywords: *Boeckella*, copepod, genetic divergence, hybrid breakdown, hybridization, speciation.

Introduction

Investigations into life-history traits of geographically separated freshwater and marine zooplankton enable insights into genetic variation within and among natural populations, which may provide a basis for subsequent speciation. Freshwater zooplankton in particular have improved our understanding of the interaction beween genetic and ecological processes because of their short generation times and ease of manipulation (Mort, 1991a, b). The genetic basis of differences in life-history traits between geographically separated populations of marine and freshwater copepods has been the focus of recent research. Allan (1984) performed interbreeding experiments between populations of the freshwater cyclopoid *Mesocyclops edax* from Florida and Michigan to examine heritable life-history variation within the species and Wyngaard (1986) revealed considerable additive genetic variance in body size in the Florida population and in maturation time in the Michigan population. Differences between Florida and Michigan populations were interpreted by both of these authors as reflecting different responses to selection in two widely separated geographic localities. More recently, Wyngaard (1988) produced evidence of geographical variation in dormancy in *M. edax*, using interbreeding experiments between the Florida and Michigan populations. In populations of the marine harpacticoid copepod

*Correspondence: Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, B. C. V6T 1Z4, Canada.

Tigriopus californicus, Burton (1987, 1990) demonstrated hybrid breakdown, using developmental time as a fitness parameter.

The freshwater calanoid copepod *Boeckella dilatata* Sars inhabits lakes and ponds of glacial origin in central and southern South Island, New Zealand (Jamieson, 1988). In lowland lakes and ponds, this copepod is less than 1.0 mm in prosomal length, is transparent ('white'), frequently carries approximately five eggs per clutch and breeds continuously throughout the year (Burns, 1979). In alpine bodies of water, *B. dilatata* is larger (prosomal length up to 1.5 mm), bright red, generally produces ten or more eggs per clutch and produces overwintering resting eggs (Burns, 1988). The two 'morphs' are not sympatric anywhere throughout their natural range but are regarded currently as conspecifics, based on Bayly's (1964) revision of the taxonomic status of the genus *Boeckella*.

The present study forms part of a wider investigation of the genetic basis of differences between alpine and lowland morphs of *B. dilatata*. In this paper we determine the interbreeding capabilities of an alpine and a lowland population of this species, examining the fecundity of intermorph matings, and viability and fecundity of F_1 produced from these matings.

Examination of variability within the species at two polymorphic isozyme loci (*Pep* and *Pgi*), using the techniques of Hebert and Beaton (1989), reveals allele frequency differences among red and white populations which are on average no greater than population differences within morphs (A. E. Byrom, unpublished data). *B. dilatata* is associated only with glacial lakes and ponds which historically would have been connected (Jamieson, 1988). Consequently, the genetic basis of adaptive divergence that may have occurred since lowland and alpine populations of *B. dilatata* became isolated, is of interest if speciation is regarded as a result of population differentiation.

Failure of *B. dilatata* individuals of different morphs to copulate would preclude recognition of the two morphs as conspecifics under the biological species concept (BSC; Mayr, 1982), the recognition concept of species (RC; Paterson, 1985) and the cohesion species concept (CSC; Templeton, 1989). The production of inviable or sterile F_1, or extensive hybrid breakdown (characterized by a reduced mean and increased variance in clutch size of F_1 hybrids), would question the status of the two morphs of *B. dilatata* as conspecifics under the BSC and CSC. The phylogenetic species concept (Cracraft, 1983) would regard such hybridization data as irrelevant and would view the two morphs as distinct species because they are fully diagnosable on the basis of colour and clutch size.

Materials and methods

Collection, rearing and design

Red alpine *B. dilatata* (R) were collected from Gem Lake (169°06′E 45°34′S, altitude 1400 m) on 17 January 1990 and were kept at 8°C (to slow their development) until a few days before the start of the experiment. Mortality during this period was less than 1 per cent. White lowland *B. dilatata* (W) were collected from Lake Hayes (168°49′E 44°59′S, altitude 329 m) on 10 March 1990 and were kept at 15°C until the start of the experiment. Copepods from both populations were fed to excess on concentrated suspensions of *Cryptomonas* sp. and *Rhodomonas* sp.; these algae are regarded as good foods for *Boeckella* (Burns & Xu, 1990). The algae were cultured in a modified MBL medium (Stemberger, 1981) at 20°C in a 16 h light:8 h dark regimen.

In this paper we follow Allan (1984) in using clutch size as a measure of fitness. Egg diameter does not vary substantially with body size in calanoid copepods (Maly, 1984), including *B. dilatata* (C. W. Burns, unpublished data), so clutch size was regarded as a suitable fitness parameter for the parental and subsequent F_1 generations. Twenty replicates of each of four parental generation (P_1) crosses were set up; two intramorph crosses and two intermorph crosses as follows: W × W, W × R, R × W and R × R (the first letter designating the female parent). The W × R cross is referred to as 'cross A', and the R × W cross as 'cross B'. For each cross, one female and one male were placed in each of 20 60-ml jars, each jar containing 15 ml of water. Intermorph crosses were carried out in a 50:50 mix of water from the two lakes. As soon as nauplii hatched, they were removed and placed in one of four communal containers (one communal container per cross).

After the F_1 reached maturity, 10 replicates of each of 14 possible classes of F_1 cross were set up (Table 1). F_1 generation pure and hybrid offspring produced from the four parental generation crosses were used in this part of the study; animals were chosen at random from the four communal containers in the previous experiment. One male and one female were placed in each of 10 jars for each of the 14 F_1 crosses. Water in the jars was a mix of alpine and lowland water, in proportions determined by the origin of F_1 individuals present. The random selection of male and female pairs of F_1 crosses would include some sib-sib matings for the R × R, W × W, A × A and B × B crosses (crosses 1, 2, 13 and 14). Based on the distribution of numbers of F_1 produced among the 20 single-pair matings within each of the four parental crosses, we calculate the expected proportions of sib-sib matings will be 10 per

Table 1 Pure, back and hybrid crosses of F_1 generation *Boeckella dilatata*. A = F_1 hybrids from the W × R parental cross; B = F_1 hybrids from the R × W parental cross

Pure cross	Backcross A	Backcross B	Interhybrid	Intrahybrid
1 R×R	3 A×R	7 B×R	11 A×B	13 A×A
2 W×W	4 A×W	8 B×W	12 B×A	14 B×B
	5 R×A	9 R×B		
	6 W×A	10 W×B		

cent, 8 per cent, 6 per cent and 10 per cent for the four crosses, respectively. These are low enough to discount the effects of inbreeding depression.

B. dilatata has six naupliar (N1–6) and six copepodite instars (CI–CVI), reaching maturity in the CVI instar. Eggs are carried in a clutch in an egg sac at the base of the abdomen for a few days before they hatch into nauplii (subitaneous eggs) or are shed (resting eggs). Clutch size of *B. dilatata* from Lake Hayes does not vary with temperature (range 8.5–18°C) in the presence of adequate food (Jamieson & Burns, 1988). Females can produce several clutches of eggs from one mating, and they generally mate very soon after moulting into the adult (CVI) instar, so it was necessary to ensure that they had not already been fertilized before being paired with a male. For F_1 crosses, immature CV females were always paired with adult males and were allowed to mature in the presence of their mate. All hybridization experiments were carried out in a constant temperature room at 15°C, which is ± 2°C of the temperature experienced by both morphs at the time of their collection (Burns, 1979; R. Nichol, personal communication). Copepods were given an equal feeding regimen of 1.0 ml concentrated *Cryptomonas* sp. suspension (5.0 mg dry weight *Cryptomonas* sp. per litre) per jar every 2 days. Replicate jars from all the crosses were spatially randomized.

At 2-day intervals for a 45-day period, P_1 and subsequent F_1 jars were checked for the following factors: (i) clutch production by the female, including the presence of infertile eggs (dead females were recorded but not replaced); (ii) condition of the male (males that died within the first 6 days were replaced); and (iii) number of nauplii present.

In P_1 crosses, each female was allowed to produce two clutches of offspring, and the number of nauplii produced from two broods was recorded for the 20 females in each cross. If a P_1 female did not produce any offspring in the 45-day period, she was omitted from subsequent analyses because she may have been post-reproductive (Jamieson & Burns, 1988) and a failure to develop egg masses could not be regarded as a true indicator of the fitness of the morph. In F_1 crosses, offspring from a single clutch only were counted, as clutch size of *Boeckella* spp. does not vary significantly between early and late clutches (Jamieson & Burns, 1988). As soon as one clutch of offspring was produced by a female, the jar was put aside and placed on a lowered feeding regimen. Qualitative observations of F_2 survival were made for the first 3–5 days. As all CV females matured to stage CVI within a few days of one another (unlike the P_1 experiments), it is unlikely that slight differences in maturation time would contribute significantly to a failure of any females to produce clutches. Thus if a female failed to produce offspring in the 45-day period, this was recorded as 'zero' and the two pure F_1 crosses (R × R and W × W; Table 1) provided a direct comparison with other F_1 back- and hybrid crosses. Females that died during P_1 and F_1 experiments were also excluded from subsequent analyses of clutch size.

To stabilize variation in clutch size within crosses, clutch size data were transformed by taking the square root of the clutch size per two broods for each P_1 female in the four crosses and the square root of the clutch size per single brood for each F_1 female in the 14 crosses. All data were analysed initially by analysis of variance (ANOVA).

Pigmentation of F_1 and F_2 offspring. The colour of offspring from both intermorph and pure P_1 crosses was observed during F_1 development and can be expressed in terms of the pigmentation of the parental generation. The colour of the F_2 generation was noted for the first 3–5 days after the nauplii hatched. All observations of F_1 and F_2 pigmentation were based on visual assessment only.

Production of infertile egg masses. Production of infertile egg masses by P_1 and subsequent F_1 females was recorded for each cross.

P_1 clutch size analysis

Influence of morph on clutch size. A one-factor ANOVA was used to determine the influence of female morphotype on clutch size. This analysis was followed by a Tukey's test, which groups together mean clutch sizes which are not significantly different from one another (Zar, 1984).

Relative influence of each sex on clutch size. A two-factor ANOVA was performed to determine which sex (male or female) had a significant effect on clutch size, with the two sexes as the two factors.

Influence of intermorph mating on clutch size. A two-factor ANOVA was performed to compare the influence of the female on clutch size, with the influence of intermorph mating on clutch size. The two factors were: (i) the morph of the female (red or white); and (ii) the type of cross (intermorph or pure cross).

F_1 clutch size analysis

A one-factor ANOVA was performed to determine whether there were any significant differences in mean clutch size among the 14 crosses. Further analysis of clutch sizes of F_1 females was performed using contrasts, to allow comparisons among various combinations of crosses and to compare groups of pooled means with other groups (Zar, 1984) (see Table 3).

Results

Parental generation crosses

Influence of morph on clutch size. Red (alpine) females produced significantly larger broods than white (lowland) females, whether mated with an alpine male or a lowland male (Table 2).

Relative influence of each sex on clutch size. Female morphotype had an influence on clutch size (d.f. 1, 1; $F = 44.6$; $P = 0.0001$), whereas male morphotype had no effect (d.f. 1, 1; $F = 1.4$; $P = 0.24$).

Influence of intermorph mating on clutch size. Intermorph crosses (R × W and W × R) did not differ from intramorph crosses (d.f. 1, 1; $F = 0.3$; $P = 0.58$).

Pigmentation of the F_1 generation. Nauplii from the pure white (lowland) cross (W × W) all lacked pigmentation and offspring from the pure red (alpine) matings (R × R) were all as red as their parents. F_1 hybrids from the intermorph R × W cross appeared as red as the alpine parental generation. Females involved in this cross always carried red-pigmented eggs and hatched nauplii were always red. F_1 hybrid nauplii from the intermorph W × R cross, like their lowland mother, always lacked pigmentation and females involved in this cross always carried non-pigmented eggs. At approximately the time that the nauplii metamorphosed to CI, however, all the F_1 hybrids from the W × R cross developed red pigmentation and, as adults, appeared as red as the alpine parents.

F_1 generation crosses

There was heterogeneity among mean clutch sizes of females in F_1 crosses (d.f. 13, 110; $F = 5.039$; $P = 0.0001$; Fig. 1). Contrasting various pure crosses, backcrosses and hybrid crosses highlighted significant factors contributing to the variability in F_1 clutch size (Table 3).

Pigmentation of the F_2 generation. Visually, F_2 nauplii from the W × W F_1 cross were as white as the P_1 and F_1 *B. dilatata*; nauplii from the R × R F_1 cross were as red as their parents and grandparents. Nauplii from backcrosses involving a lowland female and a hybrid male (crosses 6 and 10; Table 1) lacked pigmentation. We do not know if these nauplii developed red pigmentation on reaching the CI developmental stage. All other nauplii (from pure red and red hybrid mothers) appeared to be as red as the original alpine parental generation.

Infertile clutch production. When mated with a white (lowland) male (crosses 4 and 8; Table 1), some hybrid females produced infertile egg masses consisting of a sac of yolk with no recognizable eggs. These masses were not shed but appeared to be reabsorbed by the female and, a short time later, a new infertile mass was produced. Of the 10 A females mated with a white male (cross 4), four produced three or four infertile egg masses. Of the 10 B females mated with a white male (cross 8), seven produced at least one infertile mass; no females in other F_1 crosses produced them.

Discussion

Parental generation intra- and intermorph crosses of *B. dilatata* showed that the two populations can hybridize and produce viable offspring and that intermorph matings do not produce significantly smaller clutch sizes than intramorph matings. Thus under the BSC,

Table 2 Results of comparisons of square-root transformed mean clutch sizes per two broods among alpine (R) and lowland (W) parental generation *Boeckella dilatata* females mated with alpine and lowland males

Cross	R×R	R×W	W×R	W×W
Back-transformed mean CS/2 broods	25.20	20.70	11.18	10.65

Tukey's multiple contrast test groupings; braces join means that are not significantly different at the 5% level.
CS = clutch size.

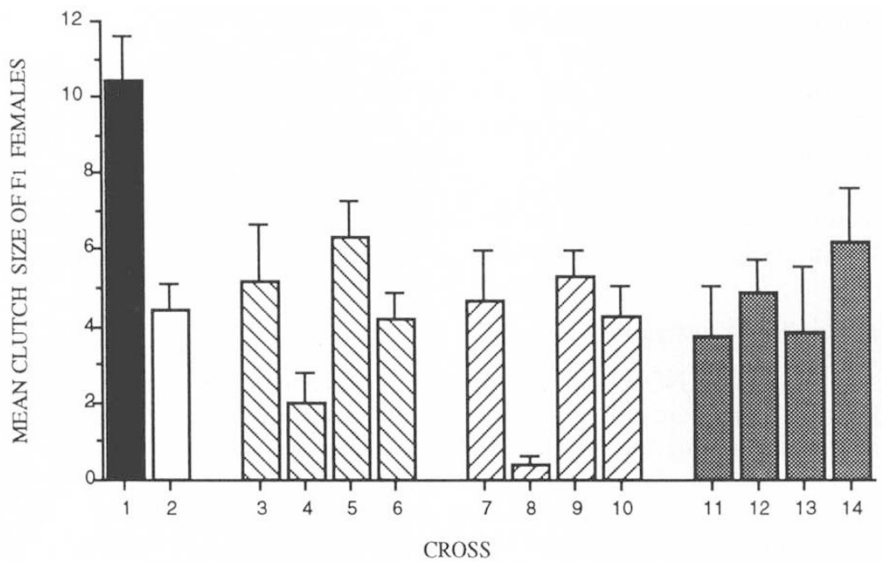

Fig. 1 Mean clutch sizes of F_1 *Boeckella dilatata* females: pure, back- and hybrid crosses (see Table 1). (■) Pure red (R × R) cross; (□) pure white (W × W) cross; (▨) backcrosses involving A hybrids; (▨) backcrosses involving B hybrids; (▨) inter- and intrahybrid crosses. Error bars indicate S.E.M.

and certainly under Paterson's (1985) RC they should be regarded as conspecifics.

The observation that *B. dilatata* female morphotype in the P_1 generation crosses has a significant effect on clutch size whereas male morphotype has no effect, is in accordance with findings made using *M. edax* (Allan, 1984). Interestingly, clutch sizes of F_1 generation *B. dilatata* are not consistent with this general observation. In both types of backcross, clutch sizes of red females are not significantly different from those of white females and the origin of the female has little influence on the number of offspring produced. That is, fitness as measured by clutch size is reduced to the level of pure white crosses rather than being intermediate. However, non-hybrid male morphotype in both types of backcross has a significant effect on the clutch sizes of hybrid females.

The finding that clutch sizes of intermorph hybrid females are more like the low values of the W × W matings rather than intermediate to the W × W and R × R matings has two broad explanations: hybrid breakdown and/or dominance of genes controlling the trait of low clutch size in the lowland population. Contrasts (k)–(p) show that the latter explanation is unlikely to be a major one (Table 3). Specifically, crosses with 25 per cent parental alpine genetic background produce even smaller clutches than the W × W F_1 cross and crosses with 50 per cent alpine genes and 75 per cent alpine genes have clutch sizes scarcely larger than the W × W F_1 cross. This strongly suggests that hybrid breakdown is the main cause of these observations.

In terms of the non-hybrid parent, clutch sizes of females in both types of backcross (A and B) show the same relative order: crosses involving red females have the largest clutch size, followed by those involving red males, then white females, with white males being smallest. This pattern is unlikely to occur by chance ($^1/_{4!}$; $P < 0.05$) and suggests consistency in the nature of F_1 hybrid breakdown.

As the two morphs of *B. dilatata* in nature differ in some aspects of their life history characteristics (e.g. the large clutch size and resting egg production of the red morph), it would be expected that there would be a

Table 3 Results of contrasts among various combinations of crosses involving F_1 *Boeckella dilatata* showing significant factors contributing to variability in clutch sizes of F_1 females

Comparison	Cross (see Table 1)	Back-transformed mean CS ± S.E.	*P* (from contrast)
(a) Red males in a pure cross (R × R) vs. red males mated with hybrid females	1 vs. 3,7	10.40 ± 1.20 vs. 4.95 ± 0.95	0.0003
(b) White males in a pure cross (W × W) vs. white males mated with hybrid females	2 vs. 4,8	4.44 ± 0.65 vs. 1.19 ± 0.45	0.0002
(c) Red females in a pure cross (R × R) vs. red females mated with hybrid males	1 vs. 5,9	10.40 ± 1.20 vs. 5.71 ± 0.56	0.02
(d) White females in a pure cross (W × W) vs. white females mated with hybrid males	2 vs. 6,10	4.44 ± 0.65 vs. 4.24 ± 0.50	0.87
(e) White males mated with hybrid females vs. red males mated with hybrid females	4,8 vs. 3,7	1.19 ± 0.45 vs. 4.95 ± 0.95	0.0001
(f) Red females mated with hybrid males vs. white females mated with hybrid males	5,9 vs. 6,10	5.71 ± 0.56 vs. 4.24 ± 0.50	0.23
(g) Interhybrid vs. intrahybrid matings	11,12 vs. 13,14	4.33 ± 0.76 vs. 5.06 ± 0.94	0.59
(h) A females in inter- and intrahybrid matings vs. B females in inter- and intrahybrid matings	11,13 vs. 12,14	3.83 ± 0.85 vs. 5.56 ± 0.82	0.05
(i) All A females vs. all B females	3,4,11,13 vs. 7,8,12,14	3.81 ± 0.62 vs. 4.14 ± 0.64	0.67
(j) All A males vs. all B males	5,6,12,13 vs. 9,10,11,14	4.74 ± 0.47 vs. 4.92 ± 0.55	0.76
(k) Pure white cross (W × W) vs. backcrosses with parental constitution of 25% alpine	2 vs. 4,6,8,10	4.44 ± 0.65 vs. 2.76 ± 0.43	0.03
(l) Pure white cross (W × W) vs. crosses with parental constitution of 50% alpine	2 vs. 11,12,13,14	4.44 ± 0.65 vs. 4.69 ± 0.60	0.68
(m) Pure white cross (W × W) vs. backcrosses with parental constitution of 75% alpine	2 vs. 3,5,7,9	4.44 ± 0.65 vs. 5.31 ± 0.56	0.82
(n) Pure red cross (R × R) vs. backcrosses with parental constitution of 25% alpine	1 vs. 4,6,8,10	10.40 ± 1.20 vs. 2.76 ± 0.43	0.0001
(o) Pure red cross (R × R) vs. crosses with parental constitution of 50% alpine	1 vs. 11,12,13,14	10.40 ± 1.20 vs. 4.69 ± 0.60	0.0001
(p) Pure red cross (R × R) vs. backcrosses with parental constitution of 75% alpine	1 vs. 3,5,7,9	10.40 ± 1.20 vs. 5.31 ± 0.56	0.001

great deal of variation in clutch size among F_1 hybrids, as was the case (Figure 1). Lacey *et al.* (1983) and Gillespie (1974) have proposed models that estimate the fitness of individuals from the combined effects of mean and variance. As such a high variance is unexpected within a population (Gillespie, 1974; Slatkin, 1974; Lacey *et al.*, 1983), and is unstable (Burton, 1987, 1990), this may again indicate some breakdown in the F_1 generation hybrids.

The substantially smaller clutch sizes of hybrid females backcrossed to lowland (as opposed to alpine) males, and the high incidence of infertility in these

matings, indicates that the trait is strongly influenced by the morph of the male. F_1 hybrid breakdown is particularly evident from these observations.

Because there was no difference in clutch size between females of the two different F_1 hybrid lines (originating from intermorph parental crosses R × W and W × R), or among the clutch sizes of females mated with hybrid males, hybrids produced from both intermorph crosses probably had similar fitness. This, and the fact that F_1 hybrids produced reasonable numbers of offspring, suggests that it is best to continue regarding the red and white morphs of *B. dilatata* as conspecifics under the BSC. Further support for this conclusion comes from two polymorphic isozyme loci data from six *B. dilatata* populations (four red and two white populations, inclusive of the Gem Lake and Lake Hayes populations) (A. E. Byrom, unpublished data). This analysis revealed differences in allele frequencies among the six populations but morphological differences made little or no contribution to genetic differentiation within the species as a whole.

Two hypotheses can be proposed to explain the observed red pigmentation of F_1 and F_2 *B. dilatata*. Firstly, red pigmentation of adult F_1 copepods produced from intermorph crosses may suggest that dominant nuclear genes are responsible for red pigmentation and this is supported by continuation of red pigmentation into the F_2. The absence of F_2 *B. dilatata* with white or intermediate pigmentation (aside from those with white mothers) indicates that several loci are involved. Bocquet (1951) claims that in the harpacticoid copepod *Tisbe*, body pigmentation patterns in females are controlled by at least seven loci. Secondly, red pigmentation in *B. dilatata* may be environmentally induced by a factor present in the Gem Lake water (even at 25:75 dilution).

The non-pigmented nauplii produced by white P_1 and F_1 females crossed with red males suggests an additional maternal effect on the pigmentation of early developmental stages that disappears after approximately the final naupliar stage. Further research is required to investigate the physiological and genetic basis of pigmentation in *B. dilatata* and to determine selective forces operating to produce the bright red pigmentation observed in the alpine morph of this species.

The experimental procedure outlined in this paper could be regarded as a conservative test of conspecificity because females were not provided with a choice of males with which to mate. The two morphs of *B. dilatata* interbred freely, however, and produced a viable and fertile F_1 which, in turn, produced a viable F_2. Therefore, despite the observed indications of hybrid breakdown, under the BSC, and certainly under the RC and CSC, the two morphs of *B. dilatata* should still be regarded as a single species. The observed hybrid breakdown suggests, however, that red and white populations of *B. dilatata* to some extent represent distinct coadapted gene pools.

Investigations such as these allow insights into genetic variation within and among natural populations, and into the genetic basis of adaptive divergence occurring among allopatric populations, the precursor of speciation.

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