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LIMITS TO THE SOUTHERN BORDER OF *DROSOPHILA SERRATA*: COLD RESISTANCE, HERITABLE VARIATION, AND TRADE-OFFS

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Abstract.—There are a number of evolutionary hypotheses about why species distributions are limited, but very little empirical information to test them. We present data examining whether the southern distribution of *Drosophila serrata* is limited by cold responses. Species comparisons were undertaken for cold resistance, development time, and viability at 15°C and 25°C for *D. serrata* and other species with a more southerly distribution (*D. melanogaster, D. simulans*, and *D. immigrans*). Relative to the other species, *D. serrata* had a long development time at both temperatures and a low level of cold resistance. Using isofemale lines collected in different seasons, central and marginal populations were compared for cold resistance, as well as development time and viability at 14°C. The border population had a relatively higher resistance to cold shock in postwinter collections, but there was no population differentiation for prewinter collections or for the other traits. The presence of variation among isofemale lines within the border populations suggests that genetic variation as measured in the laboratory is unlikely to limit range expansion. Population cages were used in the field to determine if *D. serrata* persisted over winter at borders. Although all cages yielded adult offspring at northern sites, only a few produced offspring at or just south of the border. In contrast, all cages with *D. simulans* produced adult offspring, suggesting that climatic factors limited *D. serrata* numbers. Offspring from surviving adults showed a phenotypic trade-off between fecundity and cold resistance. Comparisons of the cold resistance of field males and females with their laboratory-reared offspring provided evidence for heritable variation in field-reared flies. Overall, the results suggest that cold stress is important in limiting the southern distribution of *D. serrata*, but it seems unlikely that a lack of genetic variation restricts range expansion.

Key words.—*Drosophila serrata*, evolutionary hypotheses, field heritability, genetic variation, species borders, stress resistance.

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Many species have restricted distributions with clear-cut borders, leading to the question of why borders exist. When borders are determined by physical barriers that prevent a species from extending its range, answers to this question are obvious. However, borders commonly occur where abrupt changes in physical features are not evident. In this case, it is unclear why evolutionary changes in border populations do not allow continual expansion of a species range. Unfortunately, although a number of evolutionary hypotheses exist to explain the limited distribution of species (Hoffmann and Blows 1994; Hoffmann and Parsons 1997), there are almost no empirical data to evaluate them.

To remedy this situation, we have initiated a long-term study of species borders in *Drosophila serrata*. This species and its sibling *D. birchii* are endemic to Australia and Papua New Guinea (Ayala 1965). *Drosophila birchii* is largely confined to tropical rainforests in northern Australia and New Guinea, whereas *D. serrata* is more widespread and occurs as far south as Sydney. This species is more resistant to desiccation and cold than *D. birchii* (Hoffmann 1991; Hercus and Hoffmann 1999), which is consistent with the increased likelihood of *D. serrata* encountering these stresses across its broader distribution. The range of *D. serrata* is unlikely to be limited by a lack of resources because *D. serrata* has been collected in a range of habitats (e.g., Bock and Parsons 1978; Bock 1977) and is able to utilize a variety of host species, including fruit (Atkinson 1985; R. D. van Klinken, unpubl. data), fungus, cactus (Barker et al. 1984), and flowers (J.S.F. Barker, unpubl. data). Many of the hosts that *D. serrata* uses occur well below its southern distribution.

In this paper we are concerned with three main issues. First, we consider which traits are likely to be associated with the southern border of *D. serrata*. One approach is to compare related species with different distributions. Such species comparisons have been widely used to suggest traits involved in limiting species distributions in *Drosophila* (Parsons 1982; Kimura and Beppu 1993). Previously, *D. serrata* has been compared to species such as *D. melanogaster* and *D. simulans*, which are found continuously distributed along the eastern coast of Australia. For desiccation resistance, the ranking from most to least resistant was *D. melanogaster* > *D. simulans* = *D. serrata* > *D. birchii* (Hoffmann 1991). However, the relative performance of *D. serrata* under cold has not been examined in detail. Therefore, we compared the cold responses of *D. serrata* and other species in the laboratory. In addition, we examined the persistence of *D. serrata* and *D. simulans* in winter at the border using field population cages. Food was provided to ensure that persistence in the cages was only likely to be limited by climatic factors.

Another way of identifying potential traits is to test for adaptive differences between central and marginal populations. Such differences may indicate traits that are under selection in the marginal environment and thus likely to constrain distributions (Bateman 1967). Therefore, we compared the cold responses of isofemale lines from different *D. serrata* populations. As selection pressures may differ between seasons, collections were made both before and after winter. Laboratory adaptation was minimized by executing all comparisons within a few generations of laboratory culture.

The second issue we consider is the presence of genetic variation for cold resistance because of its likely importance

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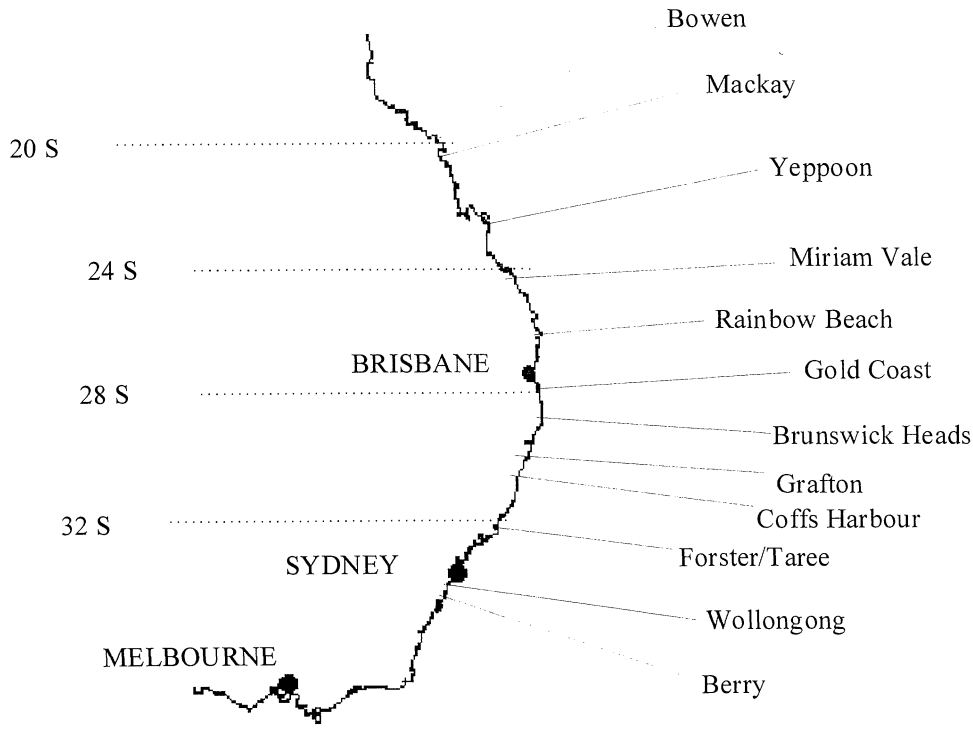

FIG. 1. Map of eastern Australia showing collection sites for *Drosophila serrata*. This species was only collected in late summer/autumn below Forster. Population cages were placed at Berry, Wollongong, and Forster.

in the *D. serrata* border. There is very little information on this issue even though genetic variation is commonly thought to limit range expansion. In previous work on *D. serrata* (Blows and Hoffmann 1993), laboratory selection indicated that genetic variation for desiccation resistance was limited in a marginal population. However, the overall level of resistance was similar in all populations, thus suggesting that directional selection for this trait had not occurred at the border (Hoffmann 1991; Blows and Hoffmann 1993). Because the southern border of *D. serrata* coincides with changes in minimum temperature rather than rainfall or humidity patterns (N. L. Jenkins, unpubl. data), it is also unlikely that desiccation resistance is of principal importance.

Here we test if heritable variation in cold responses was limiting in populations using flies reared in the laboratory and field environments. To test heritable variation under laboratory conditions, comparisons were undertaken using isofemale lines. Comparisons of variation within and among lines provide an indication of genetic variation (Hoffmann and Parsons 1988; David et al. 1994; Imasheva et al. 1997; Krebs and Loeschcke 1997), although this approach does not allow distinction to be made between additive and nonadditive genetic effects. To examine field heritable variation, flies were collected from the border population and three other populations both prior to and after winter. Field parent–laboratory offspring comparisons were used to test for genetic variation in cold resistance (cf. Prout and Barker 1989; Riska et al. 1989).

The final issue we consider is the possibility of a trade-off between cold resistance and early fecundity. Trade-offs may limit range expansion when genes under selection at borders are constrained because of pleiotropic effects (Blows and Hoffmann 1993). For cold resistance in *Drosophila*, there is evidence from selection experiments that increasing levels of resistance are associated with a decrease in fecundity (Watson and Hoffmann 1996). Here we test for such an association at the phenotypic level in *D. serrata*. We show that flies with a reduced reproductive output have relatively higher cold resistance.

MATERIALS AND METHODS

Species Comparisons

For these comparisons, isofemale lines were initiated from flies collected by banana baiting in March 1996, from Wollongong, Forster, and Coffs Harbour (Fig. 1). Sites are named according to the closest large town, although the *D. serrata* were collected in scrub and open woodland rather than in residential areas, where the species is rarely found. Four isofemale lines each of *D. serrata, D. melanogaster, D. simulans*, and *D. immigrans* were maintained for each site, with the exception of *D. simulans* from Coffs Harbour, for which there were only three lines.

Pilot studies were conducted to determine the lowest temperature at which *D. serrata* developed in the laboratory. At 12 ± 1°C, eggs failed to hatch, while larval death was high even at 13 ± 1°C. Full development from egg to eclosion only occurred at 14 ± 1°C. We initially intended to measure development time at this temperature. However, due to problems with the temperature cabinet, egg-to-adult development time and viability were measured at 15 ± 1°C, rather than the 14 ± 1°C used for the population comparisons (see below). These traits were also measured at 25 ± 1°C. The low temperatures we used are encountered by *D. serrata* at mar-

ginal sites in winter. Data loggers placed in the field in 1997 at Forster between mid-May and mid-June indicated an average temperature of 14.6°C, with an average daily maximum of 16.3°C and daily minimum of 13.1°C (A. Magiafoglou, unpubl. data). Equivalent temperatures at Coff's Harbour were 2–3°C higher than these values. Moreover, *D. serrata* do breed at Forster in winter and larvae can be collected at this site in midwinter (A. Magiafoglou, unpubl. data).

Approximately 80 adults from each isofemale line were placed in empty bottles covered with watchglasses containing 8 ml medium colored by green food dye. These bottles were left for approximately 1 h at 25°C to obtain eggs. Eggs were placed in 40-ml vials containing 8-ml culture medium at a constant density of five eggs per vial. Eight to 12 replicates were set up per line. Development time was scored from the number of flies emerging at intervals of 12 h or less, and the number of adults emerging per vial provided an estimate of viability.

Species were also compared for cold resistance. Adults were sexed using CO_2 anesthesia when three to four days old and females were cold stressed when seven days old. This testing age corresponds to the onset of peak fecundity of *D. serrata* at 25°C (Birch et al. 1963), as well as maximum fecundity in the other species. Flies were cold shocked in empty plastic vials at a density of 10–20 flies per vial, in a Braun (Melsungen, Germany) refrigerated water bath. The temperature within the water bath was maintained to within ±0.1°C using a Braun thermoregulator in addition to the refrigeration unit. Initial comparisons showed that, within the ranges used, the age of flies when anesthetized and the density of flies within the plastic vials did not influence survival. Species were exposed to −2°C for varying intervals (30–540 min). All species were tested at 30, 60, 90, 120, 150, 180, and 240 mins. *D. melanogaster* and *D. simulans* were additionally stressed for 300, 420, 480, and 540 mins. There were seven to 20 replicates per isofemale line.

Population Comparisons

Females were collected by banana baiting at 11 sites along the eastern coast of Australia, ranging from Bowen to Wollongong (Fig. 1). Collections were divided into four groups: during winter and after winter in 1993 (June 7–30, November 8–10, and December 10–15), referred to as postwinter 1993; prewinter 1994 (April 22–28 and May 22–26); postwinter 1994 (October 19–30); and prewinter 1995 (April 3-10). Low densities of *D. serrata* at field sites often made collections difficult, and for this reason collections had to be combined in some cases. This also prevented all sites from being sampled on all occasions. Three to five isofemale lines per site were used to represent each geographic area.

All laboratory populations were maintained on agar-sucrose-dead yeast-potato (culture) medium at 25 ± 1°C in constant light. Isofemale lines were generated from single field females placed in 40-ml vials containing 8 ml culture medium. Progeny of these females were then set up in bottles. The isofemale lines were subsequently reared in 250-ml bottles containing 50 ml of medium at a density of 100–120 adults per bottle. Two bottles were maintained for each isofemale line. To minimize the potential effects of laboratory adaptation, all experiments were performed within six laboratory generations.

For the population comparisons, development time and viability were measured at 14 ± 1°C for three collections: postwinter 1993, postwinter 1994, and prewinter 1994. There were 10 replicates for each isofemale line. Replicates were arranged in a block design throughout the temperature cabinets and rotated once a day to minimize the effects of minor fluctuations within the cabinet. Other details were the same as for the species comparisons.

Because there is a lot of variability between experiments testing the resistance of *Drosophila* to cold stress (cf. Watson and Hoffmann 1996), preliminary experiments were undertaken prior to each comparison of populations to determine which stress levels resulted in approximately 50% mortality. The stress levels applied to the flies were −2°C for 2 h or −1°C for 30 min. Mortality was scored 1 h and 24 h after the cold shock. The few individuals that were dead after one hour were assumed to have been injured during transfer and therefore discounted. Between four and six replicates were cold stressed for each line.

Population Cages

Drosophila serrata isofemale lines derived from field females collected from Forster and Coffs Harbour in March 1996 were combined to form mass bred stocks for each field site. Twenty-two lines were combined for the Forster population and 26 for the Coffs Harbour population. Five to 10 F_1 females per line were used to initiate each population. For cage experiments with *D. simulans*, laboratory F_1 progeny of four isofemale lines derived from field females collected at three sites (Forster, Coffs Harbour, and Taree) were combined to produce a mass-bred population. Fifteen to 20 females from each of the 12 lines were used to initiate the population.

Plastic containers (210 × 130 × 170 mm) were used to construct population cages. To facilitate air flow, 65-mm holes were cut into the sides and covered with netting. To allow addition of food and transfer of flies, a section of stocking was attached to one side of the cage. Wire gauze attached to the outside of the population cages prevented other organisms from piercing the netting. Plastic crates (420 × 420 × 270 mm) lined with wire mesh were placed upside down over the population cages and pegged to the ground to prevent disturbance. Each crate held three population cages. Data loggers have shown that the temperature within these population cages varies from ambient conditions by less than 0.5°C when cages are placed in the shade (A. Magiafoglou, unpubl. data).

Population cages were placed at three field sites (Fig. 1), one further south than *D. serrata* had ever been collected (Berry), one where *D. serrata* had been found only in autumn (Wollongong), and one where this species occurs all year round (Forster). The Berry site was at the Graham Park Campus of Wollongong University, the Wollongong site was in a suburban backyard, and the Forster site was within the Seal Rocks Camping Reserve. Three crates were placed at each site. Each crate contained three population cages, one containing the *D. serrata* Forster population, one with the *D. serrata* Coffs Harbour population, and one with *D. simulans*.

Fifty flies (approximately 1:1 sex ratio) were placed in each population cage. These were the first generation of the mass bred population (laboratory F_2). Where possible, crates were placed under cover of natural foliage to minimize direct sunlight. At the Wollongong site, crates were placed under the back veranda of the house, which had a clearance of about 1.5 m and was also shaded by a large tree. Food in each cage consisted of two plastic dishes (diameter 70 mm) each containing 25 ml of culture medium, one-third of an apple, and approximately 120 mm of banana. The food was supplemented with an equivalent amount of fruit after 72 days.

Population cages were placed in the field on 5 June 1996, food was added on 16 August, and cages were retrieved on 19 September. Upon collection, cages were scored for integrity (particularly holes in the netting and stocking), and the life cycle stage reached by the next generation was ascertained. A field generation was assumed to have been completed if the total number of adults (dead and alive) exceeded 50. Intact cages only ever contained the species released into them at the start of the experiment, despite the presence of several *Drosophila* species at the sites.

Flies obtained from *D. serrata* cages were used to test for changes in fecundity and cold stress. Flies were combined across replicates within sites and the F_2 generation from these flies was compared to the laboratory mass-bred stocks from which they had originated. Five populations were compared: Forster maintained in the laboratory (FL), Coffs maintained in the laboratory (CL), Forster from the population cages at the Forster field site (FF), Coffs from the population cages at Forster (CF), and Coffs from the population cages at the Berry field site (CB). Unfortunately, none of the Forster flies survived at Berry and there were no live adults at Wollongong even though adults from one of the Coffs Harbour cages had produced offspring at this site (see Results). For the FL and CL populations, flies used were of the fifth laboratory generation (fourth generation since combining the isofemale lines). Approximately 50 females and 50 males were used to set up each population, although the number was lower when there were insufficient flies in the field cages (see Results).

After the two generations of laboratory culture, flies were tested for fecundity and cold resistance. Upon eclosion, females were collected over a 12-h period and placed individually in vials with a single male. When females were 24–36 h old, pairs were placed in vials with spoons containing treacle medium, which were replaced every 24 h for seven days. Eggs laid on the spoons were counted and total fecundity over seven days calculated. This process was repeated with a second set of F_2 offspring from different sets of vials. In each case, 33–40 females were tested per population.

After measuring fecundity for seven days, all females were aged in vials for a further 24 h. The females, now 9.5–10.5 days old, were then tested for cold resistance as described above, except that they were stressed singly in 1.5-ml microcentrifuge tubes rather than in groups. The cold stress applied was -2°C for 60 min. Because cold resistance and fecundity were measured for each individual, the phenotypic association between these traits was examined.

Heritable Variation for Cold Resistance

Flies were collected from Wollongong, Forster, Taree, Coffs Harbour and Grafton using banana-baited traps. Three collections were made: two postwinter 1995 (October 12–23 and December 6–20) and one prewinter 1996 (March 5–25). Field *D. serrata* were identified following ether anesthesia. Due to low numbers, flies from the October and December 1995 collections were combined into a postwinter 1995 collection. At Wollongong, only a few *D. serrata* were found in March 1996 (eight females and nine males) and these flies were not considered further.

All males and females from the postwinter 1995 collections and up to 70 per sex per population from the March 1996 collection were set up in vials to obtain F_1 progeny. Laboratory stocks were maintained at 25 ± 1°C in constant light. Isofemale lines were generated from single field females placed in 40-ml vials containing 8 ml culture medium, with 0.02 ml antibiotics (streptomycin/penicillin). These antibiotics were needed to reduce bacterial infection, which can make it difficult to establish strains of field *D. serrata*.

Field males were crossed to virgin females from isofemale lines originating from an earlier collection (April 1995). The isofemale lines were derived from a similar geographic location. A single isofemale line was used for each collection site, in the case of Grafton, Coffs Harbour, and Forster. The isofemale line from the Forster site was also used in crosses with the Taree males. The isofemale lines were reared in 250-ml bottles with 50 ml of medium, at a density of 100–120 adults per bottle.

Field females and field males (crossed with laboratory females) were set up individually in vials and flies were tipped into fresh vials every two days. Upon eclosion, F_1 adults were sexed without anesthesia and set up in single pairs to obtain the F_2 generation. Where possible replicate pairs of F_1s were obtained from different vials to minimize common environment effects. Field females were cold stressed after seven days and field males after eight days at 25°C. All laboratory-reared flies were cold stressed when seven days old. We stressed two to four F_1 progeny (one to two of each sex) and two to eight F_2 progeny (one to four of each sex).

Flies were stressed singly in 1.5 ml-microcentrifuge tubes at -2°C for 2 h. Mortality was scored after 1, 12, 24, and 48 h after cold shock. The few individuals ($< 1\%$) that were dead after one hour were discounted. By scoring flies on three occasions rather than only one, we could score the resistance of an individual more accurately. The time taken for flies to die reflects the opportunity flies have for countering a cold shock because we have found that flies can lay fertile eggs and mate directly after cold stress even when they die within two days. In addition, we have found that any flies that survive beyond 48 h live as long as unstressed flies.

Statistical Analyses

To compare species, ANOVAs were undertaken with isofemale lines nested within the species-by-location term. For development time and viability, vial means provided the raw datapoints for analysis. Although mortality and viability data were initially angular transformed prior to analysis, transformation did not affect the conclusions; only the ANOVAs on untransformed data are presented. To compare populations, similar ANOVAs were undertaken, except that in this case isofemale lines were nested within populations. ANO-

VAs were also used to compare the mortality of females following a cold shock. The proportion of flies that died was used in the analysis rather than angular-transformed proportions because these led to the same conclusions. In all cases, Tukey post hoc tests were used to compare the different treatments.

Variation among isofemale lines was used to obtain estimates of the heritability of traits (Hoffmann and Parsons 1988; Krebs and Loeschcke 1997). When measurements are made on groups, as in the present case, the heritability is estimated as $S^2_A/(S^2_A + xs^2)$, where S^2_A is the variance component among lines, x is the group size, and s^2 is the variance component within lines. We reduced the dataset to a balanced design to estimate these components. Unfortunately, heritability estimates obtained with this technique need to be treated cautiously because the estimate of S^2_A is likely to be inflated by common environment effects, which can only be controlled with additional information (cf. Krebs and Loeschcke 1997). Pearsons correlation coefficients were computed using the means of the isofemale lines to investigate correlations among traits. These correlations are related to genetic correlations because different isofemale lines represent different genotypes. Because only a few isofemale lines were tested for each population, we pooled lines across populations when computing correlations. However, this approach confounds variation within and between populations and was only used when traits showed no differences among populations.

ANOVAs were used to compare fecundity scores among populations derived from the field cage experiment. The cold resistance of these populations was compared with contingency analyses by computing G-statistics. Logistic regression was used to investigate the effect of oviposition on the cold resistance of females. The significance of the regression coefficients was determined from Wald statistics, which follow the chi square distribution.

Spearman rank correlations were used to examine heritable variation in cold resistance. Because the mortality of the females was scored at different stages, the heritability of this trait could not be computed. Instead, the presence of heritable variation was tested by examining correlations across generations on the length of time flies took to die. This provides a measure of fitness following cold shock, given that flies that live 24 h or longer had a greater opportunity for reproducing. Flies were given the lowest rank if they did not recover. The next lowest rank was assigned to individuals that lived for only 24 h, and so on. Because there is a sex difference in time to mortality, correlations were limited to within-sex comparisons.

Results

Species Comparisons

ANOVAs (Table 1) indicate highly significant differences among species at both temperatures. At both 25°C and 15°C, *D. serrata* had the slowest development time of the four species tested (Fig. 2). *Drosophila immigrans* developed more slowly than the other cosmopolitan species at 25°C, but not at 15°C. The term for line within species by location indicates significant variation within species at both temperatures.

TABLE 1. ANOVAs comparing species for development time and viability at two temperatures. Development time was scored in days (15°C) or hours (25°C). Viability was scored as the number of flies emerging out of five eggs.

Source of variation	df	Mean squares 25°C	Mean squares 15°C
Development time			
Block	9	58.95	79.87***
Species	3	70,511.43***	1009.22***
Location	2	171.60	1.30
Species by location	6	363.62	13.03
Line within species by location	35	282.00***	8.51***
Error	384/390	70.49	1.19
Viability			
Block	9	0.71	1.23
Species	3	39.63***	9.23
Location	2	1.78	1.52
Species by location	6	9.58*	5.84
Line within species by location	35	3.70***	3.39***
Error	409/404	1.30	1.36

* $P < 0.05$; *** $P < 0.001$.

However, there were no overall effects of location on development time, reflecting weak geographic differentiation relative to the large species differences.

For viability, ANOVAs (Table 1) indicate a significant difference between species only at 25°C. This was due to the relatively lower viability of *D. immigrans* and *D. simulans* at this temperature compared to the other species (Fig. 3). Both *D. melanogaster* and *D. serrata* had a similar viability at both temperatures. As in the case of development time, there were significant line effects at both temperatures and no overall effects of location. Population differences were inconsistent across species. For *D. serrata*, the Coffs Harbour population exhibited a higher viability than Wollongong at 15°C, whereas for *D. immigrans*, the Coffs Harbour population had a higher viability than the other populations at 25°C (Tukey post hoc, $P < 0.05$). For *D. melanogaster*, Coffs Harbour had a lower viability at both temperatures. No population differences were observed for *D. simulans*.

For the cold shock experiment, data were combined across populations because populations differed significantly in only one case (in *D. melanogaster*, Wollongong showed a higher resistance than Coffs Harbour after being stressed for 480 mins) and because population differences were extremely small relative to the species differences. The mean percentage mortality for each species for cold shocks ranging from 30 min to 300 min is shown in Figure 4. The data indicate clear species differences for resistance to cold shock. At time intervals up to 180 min, *D. serrata* had a significantly higher percentage mortality than the other species, ranging from about 50% to 95%. After 240 min at −2°C, both *D. serrata* and *D. immigrans* had 100% mortality. After 180 min, *D. melanogaster* was significantly more resistant to cold than all of the other species. Even after 540 min at −2°C, this species only showed 31% mortality.

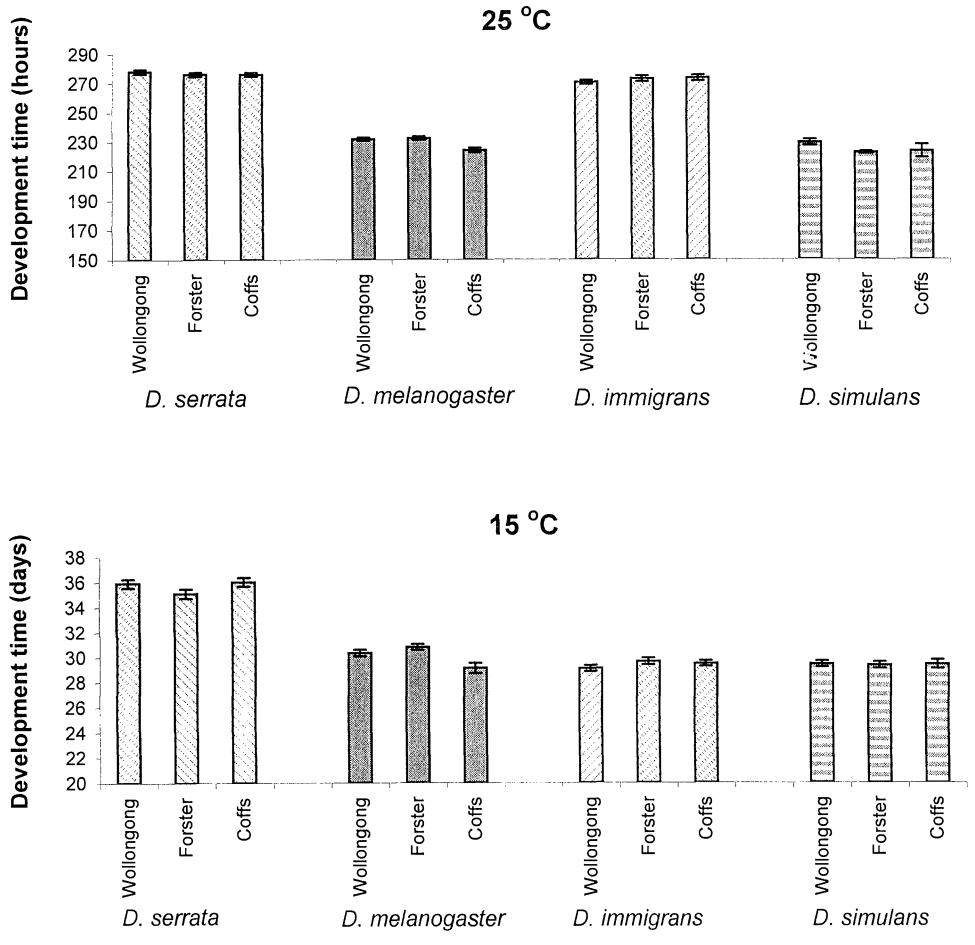

FIG. 2. Species comparisons for mean development time of flies at 25°C and 15°C. Means and standard errors (bars) are based on four isofemale lines.

Population Comparisons

ANOVAs for development time at 14°C (Table 2) indicate a significant difference between populations only in the postwinter 1993 collection. This difference is due to the most northern population (Bowen) having a longer development time than all other populations (Fig. 5), which is significant in a Tukey post hoc test (Tukey, $P < 0.05$). There were significant line effects in all three collections, suggesting genetic variation within populations for this trait. Block effects were also evident for all collections, presumably because of minor variation in rearing temperature.

For viability at 14°C, ANOVAs indicate significant differences among populations in two collections (Table 2). For the postwinter 1993 collection, both the southernmost (Forster) and northernmost (Bowen) populations had a higher viability than the Grafton, Gold Coast, Rainbow Beach, and Yeppoon populations (Tukey post hoc, $P < 0.05$). The Miriam Vale and Brunswick Heads populations were not significantly different from either group in the post hoc tests. For the prewinter 1994 collections, the Brunswick Heads and Miriam Vale populations had the highest viability, but differences among populations were not significant in post hoc tests. There were significant line effects in two collections (Table 2), thus suggesting heritable variation within the populations. These data provide no evidence for clinal changes under cool conditions.

ANOVAs on survival of a cold stress leading to approximately 50% mortality indicate significant differences among the populations for the two postwinter collections, but not the prewinter collections (Table 2). Note that different stresses were required to produce 50% mortality (see Methods). In both postwinter collections, the Forster population had the highest level of resistance (Fig. 6). In the 1993 collection, this population was significantly more resistant than the Grafton, Brunswick Heads, Yeppoon, and Bowen populations. For the post-winter 1994 collection, Forster was more resistant than Coffs Harbour and Gold Coast. This suggests that the isofemale lines derived from the border population may consistently have a higher level of resistance to cold than those from other sites. There were significant-line-within population differences for all collections, thus suggesting heritable variation for this trait under laboratory conditions.

Heritability estimates based on variation among the isofemale lines ranged from 71% to 90%. Because there were no population differences for any of the traits for the postwinter 1994 collection, we examined correlations among traits using the means of all 20 isofemale lines. Viability and development time at 14°C were positively correlated ($r =$

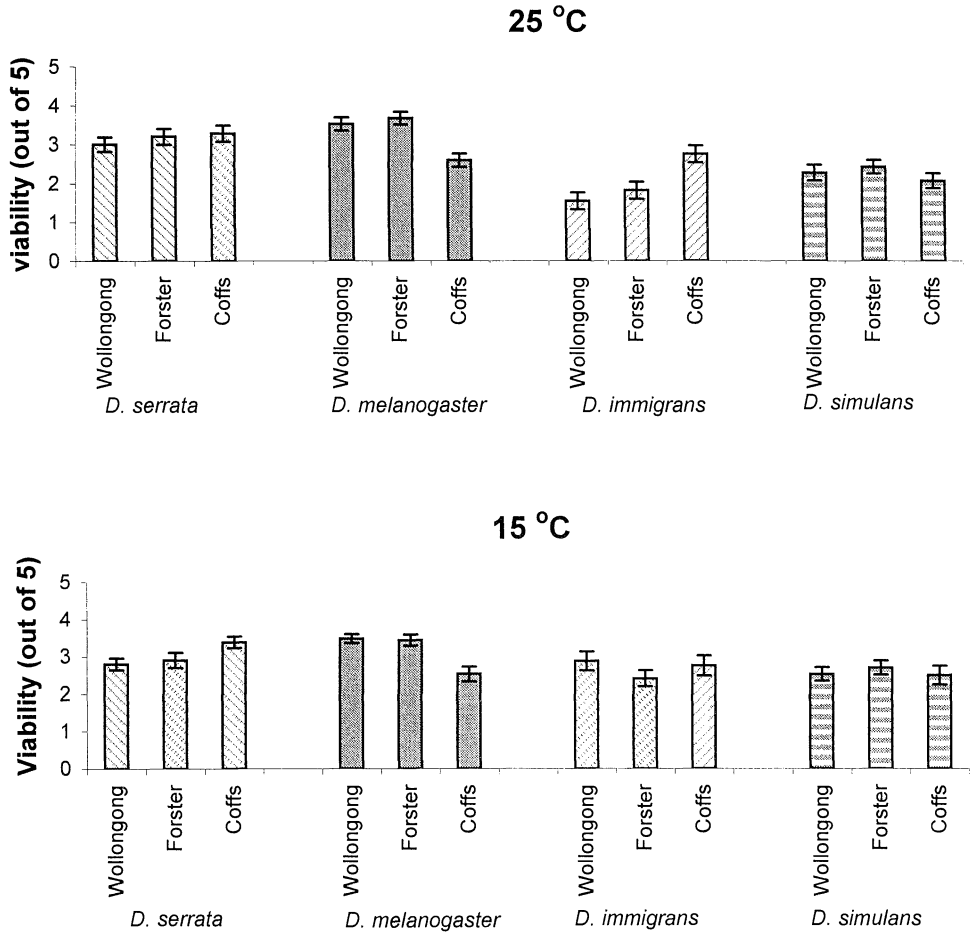

FIG. 3. Species comparisons for egg-to-adult viability (of five eggs) at 25°C and 15°C. Means and standard errors (bars) are based on four isofemale lines.

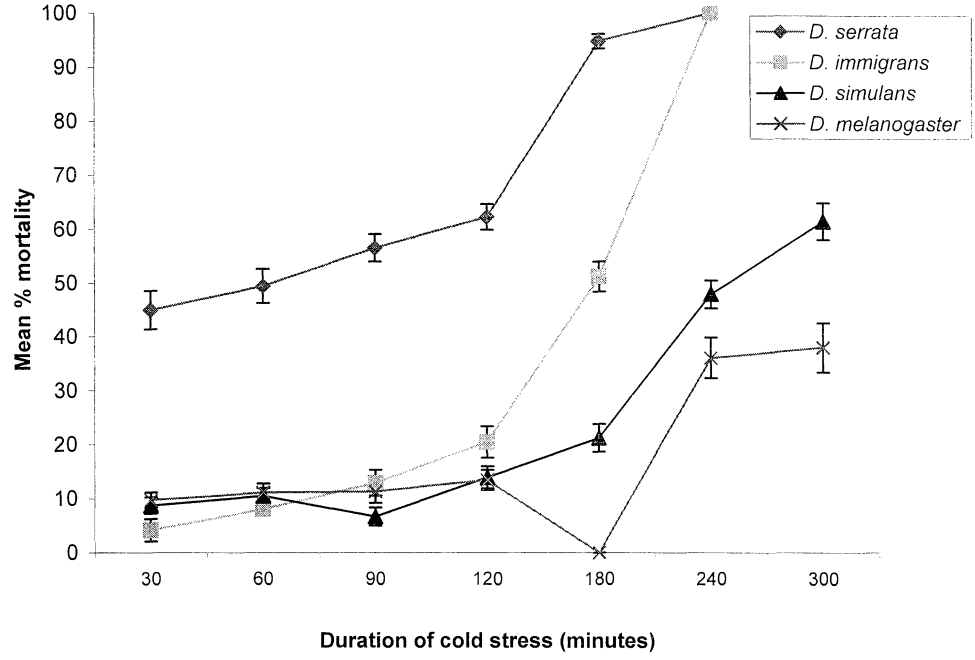

FIG. 4. Species comparisons for survival of a cold stress (−2.0°C) for different lengths of time. Error bars are standard errors from the means of three populations.

TABLE 2. ANOVAs comparing *Drosophila serrata* populations for development time (in days) and viability (adults emerging out of five eggs) at 14°C and cold resistance (proportion of females that died).

	Population		Line within population		Block		Error	
	df	MS	df	MS	df	MS	df	MS
Development time								
Postwinter 1993	7	10.41**	28	2.16**	9	26.98***	278	0.92
Prewinter 1994	7	11.10	27	8.21***	9	102.07***	234	2.36
Postwinter 1994	4	4.26	18	7.39***	9	10.63***	188	1.16
Viability								
Postwinter 1993	7	19.86*	28	6.16***	9	0.87	295	1.15
Prewinter 1994	7	7.90*	27	2.44**	9	1.46	306	1.16
Postwinter 1994	4	0.44	18	1.86	9	1.89	197	1.30
Cold resistance (× 100)								
Postwinter 1993	7	52.29**	30	10.47***	5	63.78***	147	2.60
Prewinter 1994	7	2.66	29	8.24**	3	95.74**	91	3.27
Postwinter 1994	4	86.56**	15	14.60***	3	1.28	137	2.39
Prewinter 1995	5	2.65	18	8.62**	3	18.27**	66	3.26

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

0.38, $P < 0.05$), suggesting that lines with a long development time had a high viability. There was no correlation between cold resistance and viability ($r = 0.05$), but mortality was negatively correlated with development time ($r = 0.42$, $P < 0.05$). This suggests that high resistance is associated with a long development time.

Population Cages

In general, the population cages were successful in providing a secure environment for *Drosophila*. Only two cages had to be discarded because of holes in the gauze, which were most likely produced by ants. The other cages contained only the species that had been originally placed within them. All *D. simulans* cages produced at least one field generation of adults regardless of where they had been located, and there were numerous adults in all cages. In contrast, as expected from their relative abundance and distribution over winter, *D. serrata* were less successful. All *D. serrata* cages produced adult offspring at Forster. However, only the mass-bred population from Coffs Harbour produced adult offspring at the more southerly sites. At Wollongong, dead pupae were found in cages set up with Forster flies, whereas at Berry all cages contained dead pupae but only one of the cages with Coffs Harbour flies produced adults. There were also dead larvae in all *D. serrata* cages at Wollongong and Berry.

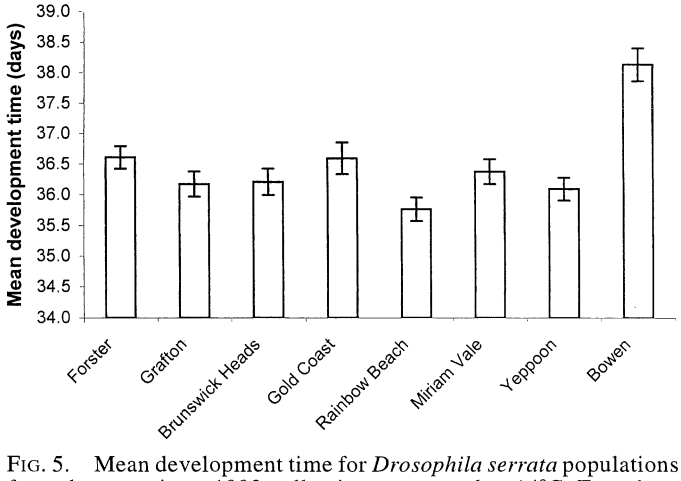

FIG. 5. Mean development time for *Drosophila serrata* populations from the postwinter 1993 collections measured at 14°C. Error bars are ± 1 standard error computed from 4–5 line means.

The number of live flies found in the *D. serrata* population cages after winter, and subsequently used as founders of the experimental generations, varied between sites and replicates. At Berry, one Coffs mass-bred population cage yielded 33 females and 14 males. At Forster, two Coffs mass-bred population cages yielded 31 females and 23 males and 28 females and 27 males, respectively and three Forster mass-bred population cages yielded three females and one male, eight females and six males, and seven females and four males.

Females from the F_2 laboratory generation were scored for fecundity and cold resistance. The mortality results (Table 3) are presented separately for the replicates that were carried out at different times with offspring from different sets of vials. Mean fecundity was significantly lower for the second replicate (ANOVA, $F_{1,361} = 243.38$, $P < 0.001$). Variation among populations was also significant ($F_{4,361} = 3.59$, $P < 0.01$). In post hoc tests, the FF differed significantly from the two laboratory populations, but not from the other field-derived populations (CF and CB).

For cold resistance, data could not be pooled across replicates because there was a difference in mortality levels between replicates ($G = 4.35$, df = 1, $P = 0.033$). Populations did not differ in their survival of a cold stress in the first replicate ($G = 5.43$, df = 4, $P = 0.25$), but did in the second ($G = 11.73$, df = 4, $P = 0.019$) when survival was relatively higher for both the CF and CB populations relative to Forster populations (Table 3).

These data were also used to examine the association between fecundity and cold resistance. When each replicate and population was treated as an independent datapoint, there was a significant, positive association between the percentage mortality and mean total fecundity for the population ($r = -0.59$, $t = 2.087$, df = 8, $P < 0.05$). This suggests a trade-off between fecundity and cold resistance. The association between these traits was further assessed at the phenotypic

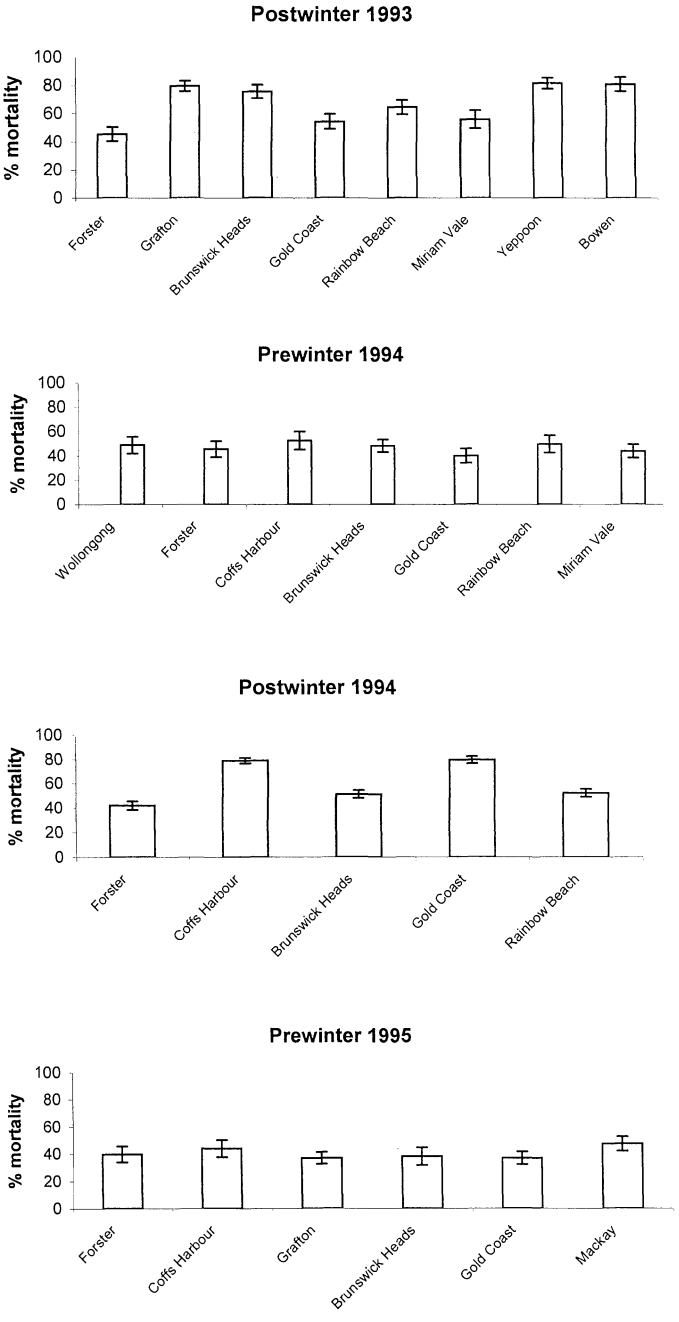

FIG. 6. Mortality of *Drosophila serrata* populations after a cold shock. Error bars are ± 1 standard error based on means of 3–5 lines. The postwinter 1993 and prewinter 1994 collections were stressed for 30 min at −1.0°C, whereas the postwinter 1994 and prewinter 1995 collections were stressed for 120 min at −2.0°C.

level using the data from individual flies. Mortality after cold shock was treated as a dichotomous variable and populations and replicates as categorical variables in a logistic regression. The results (Table 4) indicate a significant effect for fecundity. The slope of the regression was negative and, because survival was coded as 1 and death as 0, this negative regression indicates a negative phenotypic association between fecundity and cold resistance.

TABLE 3. Fecundity and percentage mortality after a cold shock (−2°C for 60 min) for F_2 offspring from *Drosophila serrata* population cages.

Population	Replicate	*N*	% mortality	Mean fecundity
Coffs Harbour (laboratory)	1	35	40.0	241.2
	2	36	33.3	132.9
Coffs Harbour (field)	1	39	46.2	213.4
	2	38	18.4	120.5
Coffs (field at Berry)	1	34	52.9	242.6
	2	40	25.0	123.0
Forster (laboratory)	1	35	54.3	233.0
	2	39	43.6	149.8
Forster (field)	1	33	30.3	195.6
	2	38	50.0	126.4

Heritable Variation for Cold Resistance

Spearman rank correlations were used to assess heritable variation for cold resistance (Table 5). Comparisons were restricted to within the same sex, making a total of eight estimates per population per collection. For the postwinter collection, there were five negative and 27 positive correlations, ranging from −0.179 to 0.586 (mean 0.189, median 0.195). Nine of these were significant and all are positive. Because of the failure of many field females to reproduce, the small number of families available for the Forster and Coffs Harbour postwinter field females makes this a weak test of heritable variation. Nevertheless, one of the Coffs Harbour comparisons for field flies was significant, and for the Forster population, the comparison of field males to F_2 males was significant. There were three significant comparisons involving field flies for the Taree population.

For the March 1996 collection, there were six negative and 26 positive correlations, of which six were significant. Coefficients range from −0.152 to 0.577 (mean, 0.112; median, 0.107). There were three significant comparisons for the Forster population, including the field female to F_1 female comparison. The other three significant comparisons were from the Grafton population, including two involving field flies. In general these data suggest that there is some heritable variation for cold resistance in field flies as well as laboratory flies because most of the correlations are positive (including all the significant correlations). Overall, there is no evidence that heritable variation is greater under laboratory conditions compared to field conditions; on average, comparisons from Table 5 involving field flies had a rank coefficient of 0.16

TABLE 4. Results of logistic regression testing the effects of fecundity and population on the survival of adult *Drosophila serrata* after a cold shock of −2°C for 60 min.

Variable	df	Regression coefficient	Wald statistic
Population	4	—	3.98
Pop (1)	1	−0.397	1.36
Pop (2)	1	0.106	0.09
Pop (3)	1	−0.249	0.51
Pop (4)	1	0.177	0.26
Replicate	1	0.083	0.08
Fecundity	1	−0.059	8.94**

** $P < 0.01$.

TABLE 5. Spearman rank correlations (r_s) computed between the resistance of field *Drosophila serrata* and their laboratory-reared progeny to a cold shock (−2°C for 120 min). The numbers of families for the comparisons (N) are given.

	Forster		Taree		Coffs Harbour		Grafton	
	N	r_s	N	r_s	N	r_s	N	r_s
Postwinter 1995								
Field female–F_1 female	11	0.423	31	0.041	12	0.586*	28	−0.179
Field female–F_2 female	11	0.424	31	0.253	12	0.294	28	0.092
F_1 female–F_2 female	11	−0.073	31	0.348*	12	0.302	28	−0.160
F_1 male–F_2 male	11	0.013	32	0.098	13	0.307	29	0.553**
Field male–F_1 male	28	0.072	58	0.251*	45	0.182	47	0.030
Field male–F_2 male	28	0.320*	59	0.135	44	−0.082	46	0.178
F_1 male–F_2 male	28	0.050	59	0.355**	47	0.460**	47	0.207
F_1 female–F_2 female	28	0.087	60	−0.132	48	0.356**	47	0.270**
March 1996								
Field female–F_1 female	29	0.339*	35	0.204	31	0.257	34	0.294*
Field female–F_2 female	28	−0.107	34	0.116	31	0.144	33	−0.067
F_1 female–F_2 female	28	−0.152	34	−0.151	31	0.012	33	−0.097
F_1 male–F_2 male	28	0.577**	34	−0.042	31	0.150	33	0.322*
Field male–F_1 male	55	0.106	54	0.054	56	−0.101	67	0.073
Field male–F_2 male	55	0.018	56	0.114	57	0.123	66	0.335**
F_1 male–F_2 male	55	0.040	54	0.149	56	0.113	66	0.045
F_1 female–F_2 female	54	0.352**	56	0.080	57	0.107	66	0.072

* $P < 0.05$; ** $P < 0.01$.

compared to 0.14 for the comparisons involving only laboratory flies.

Data from these collections were also used to further investigate the association between cold resistance and reproduction. In both collections, many field females did not produce viable progeny. For the postwinter 1995 collections, more than half (86 of 148) of the females failed to reproduce. This was particularly so for females from Coffs Harbour and Forster populations, where the percentage of females that produced progeny were only 36.1% and 40%, respectively. Females that survived cold shock appeared to be overrepresented among those that failed to produce progeny, although this was not quantified.

For the March 1996 collection, a record was kept of the cold resistance and reproduction of individual females. Of the 217 field females that were cold stressed, 88 (40.6%) failed to produce an F_1 generation. More females that did not produce any viable progeny survived the cold shock than expected by chance ($\chi^2 = 37.08$, df = 8, $P < 0.001$). This suggests a trade-off between cold resistance and fecundity in field females.

DISCUSSION

Cold Responses and the Southern Border

The results of both the population and species comparisons suggest that resistance to cold shock may be an important trait limiting the southern border of *D. serrata*. The difference in cold resistance between the border and most central populations in the postwinter collections suggests seasonal selection for altered resistance. The finding that population differences are not necessarily stable over time highlights the need to study them over several seasons when making adaptive comparisons. Unlike in the case of cold resistance, there were no consistent patterns for the life history traits that could be related to selection at borders. At 14°C, there were no significant differences in development time between border and central populations, whereas for egg-to-adult viability the border population mean was relatively high only in the postwinter 1993 collection. Population differences in cold resistance do not follow body size because Forster females were always larger than those from Coffs Harbour regardless of when they were collected (N. L. Jenkins, unpubl. data).

Changes across collections for cold resistance may be due to gene flow. This process may eliminate the differences between the Forster and Coffs Harbour populations established following winter selection. Marginal populations will often act as sinks (Holt and Gomulkiewicz 1997), so that gene flow is likely to be unidirectional toward Forster and Wollongong, particularly as the Wollongong population may be seasonal because *D. serrata* has only been collected there in late summer/autumn. However, it is also possible that selection decreased resistance in populations away from the margins rather than only increasing it at the margins. Unfortunately levels of cold resistance in pre- and postwinter collections cannot be compared directly, so the direction of selection is not known.

Trade-offs between cold resistance and other traits selected under warmer conditions may also influence levels of resistance in populations. There is now evidence for a trade-off between fecundity and cold resistance in *D. serrata* as well as in *D. melanogaster* and *D. simulans* (Watson and Hoffmann 1996). Changes in fecundity are likely to influence the outcome of competition among *Drosophila* species known to occur in the field (Atkinson 1985) and thereby indirectly influence the distribution of *D. serrata*.

The laboratory data suggest that *D. serrata* is susceptible to cold conditions. The culture boundary of 14°C is above that reported for species that inhabit temperate areas (see Parsons 1978; David et al. 1983). The results indicate that *D. serrata* adults are sensitive to cold stress in the laboratory relative to species with a more southerly distribution. These

differences are not associated with body size because the largest species (*D. immigrans*) had a resistance level intermediate between *D. serrata* and *D. melanogaster/D. simulans*, which are all of a similar size.

Field cages set up at different locations provide some support for the conjecture that cold conditions limit the *D. serrata* border. *Drosophila simulans* population cages successfully produced offspring at the southern site, which is consistent with its broad distribution. In contrast, *D. serrata* produced far fewer offspring over winter at any of the sites, including Coffs Habour. Although *D. serrata* has never been found as far south as Berry, one of the Coffs Harbour population cages produced a few adults. Nevertheless, reproductive output was extremely low at this site and adults failed to emerge and persist in most cages.

Overwintering strategies of *D. serrata* are unknown. Many species of drosophilids appear to survive winter periods as adults because this is the most resistant life stage (e.g., Jefferson et al. 1974; Tucic 1979; Kimura 1988; Kimura and Beppu 1993). *Drosophila serrata* adults are also more cold resistant than larvae (D. Berrigan, unpubl. data). Many species that overwinter as adults undergo reproductive diapause, a strategy that generally increases cold resistance (Kimura 1988; Kimura et al. 1992; Ohtsu et al. 1992). However, we have found no evidence that *D. serrata* follows this strategy, which may reflect an origin in the tropics, where close relatives of *D. serrata* are found. Because *D. serrata* is not associated with human activities, this species is also unlikely to overwinter in buildings, compost heaps, or other habitats thought to be important for overwintering domestic species (David et al. 1983). Although microclimate selection may alter the temperatures experienced by field flies, it has a greater impact in avoidance of heat rather than cold; in winter, microclimate selection only means a difference between 0.1°C and 0.5°C (Leather et al. 1993).

Despite the apparent importance of cold responses in the southern border of *D. serrata*, our first attempt to directly demonstrate selection with field cages over winter failed. A comparison of the F_2s from the field with populations maintained in the laboratory did not provide evidence for an increase in cold resistance. However, this experiment had several limitations. We did not control for the effects of laboratory adaptation. Moreover, it is possible that some inbreeding took place in the field flies, particularly as offspring may have mated with parents. The fact that fecundity declined in the field populations may reflect inbreeding, although it could also reflect direct or indirect selection on fecundity. Ideally, further experiments will need to involve replicate cages held as discrete generations so that offspring can be identified and crossed to overcome inbreeding effects.

Heritable Variation in Cold Responses

Significant variation among *D. serrata* isofemale lines was observed across all collections and in nearly all traits. This indicates that a lack of overall genetic variation is unlikely to be limiting border expansion. However, additive genetic variation, which determines the ability of populations to respond to directional selection, is not directly measured by the isofemale line approach. The parent-offspring comparisons we undertook did test for an additive genetic component and indicated heritable variation for cold resistance in natural populations. In general, offspring from resistant field parents tended to have resistant offspring, although we could not obtain a heritability estimate for this trait.

There were generally too few datapoints to compare levels of heritable variation among the populations, particularly because of the low fecundity of field flies. Nevertheless, the data suggest heritable variation for cold resistance in populations at the border because several field male/female comparisons with laboratory-reared offspring were significant. This suggests that a lack of heritable field variation for cold resistance does not limit range expansion in *D. serrata*. The F_1-F_2 comparisons indicate heritable variation in laboratory-reared flies. However, there was no evidence that variation in the field was lower than in the laboratory. This is consistent with a growing body of data indicating that laboratory and field heritabilities are similar for many traits both in *Drosophila* (Jenkins and Hoffmann 1994; Hoffmann 1999) and in other organisms (Weigensberg and Roff 1996).

Concluding Remarks

This paper represents one step in understanding the southern border of *D. serrata* from an evolutionary perspective. Although cold resistance appears to be under selection in marginal populations, there is no evidence that a lack of genetic variation in this trait limits the southern border. Perhaps gene flow from central to marginal sink populations prevents further range expansion. In addition, trade-offs between increased stress resistance and early reproductive output may select against genotypes with an increased level of cold resistance.

Obviously, a lot of additional information is required to further test the role of cold responses in limiting evolutionary change. In particular, molecular markers could be used to directly assess gene flow, and field experiments with cold resistant lines of *D. serrata* could be used to determine if changes in this trait are sufficient to allow range expansion. Other indirect effects of cold exposure may also play a role in limiting the southern *D. serrata* border. For instance, it appears that in *D. serrata* exposure to cold conditions may contribute to a marked decline in reproductive output in offspring as well as in the parental generation (N. L. Jenkins, unpubl. data).

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