A genetic analysis of path length and pupation height in a natural population of *Drosophila melanogaster*

SHARON J. BAUER AND MARLA B. SOKOLOWSKI
Department of Biology, York University, Downsview, Ont., Canada M3J 1P3

Corresponding Editor: P. B. Moens
Received November 7, 1984
Accepted March 6, 1985

BAUER, S. J., and M. B. SOKOLOWSKI. 1985. A genetic analysis of path length and pupation height in a natural population of *Drosophila melanogaster*. Can. J. Genet. Cytol. **27**: 334–340.

Behaviour–genetic studies using laboratory strains of *Drosophila* are often criticized because the results cannot be generalized to natural populations. The genetic component of variation in two prepupal behaviours was studied for strains derived from a natural population of *Drosophila melanogaster*. These strains showed a second-chromosome based contribution to differences in path length (the distance a larva crawls in a yeasted culture dish) with the long path length phenotype dominant over the short. Differences in pupation height (the distance a larva pupates above the surface of the medium) were affected not only by the second chromosomes but also by the third pair of chromosomes. The second pair influenced the differences in pupation height threefold more than the third. Intermediate pupation heights were found in the reciprocal crosses. While path lengths could be replicated in their absolute scores over different days, pupation heights could only be replicated in their relative scores.

Key words: larval behaviour, *Drosophila*, natural population, genetics.

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Les études de comportement génétique avec souches de *Drosophila* de laboratoire sont souvent critiquées vu que, généralement, les résultats obtenus ne peuvent être appliqués aux populations naturelles. La composante génétique de variation a été étudiée en rapport avec deux comportements pré-pupes chez des souches dérivées d'une population naturelle de *Drosophila melanogaster*. Ces souches ont révélé la contribution d'une seconde paire de chromosomes face aux différentes longueur de trajets accomplis par les larves (c.-à-d. la distance couverte par une larve sur culture en pot contenant de la levure); de fait, les phénotypes à trajets longs ont dominé les phénotypes à trajets courts. Les différences en hauteur de pupation (c.-à-d. la distance au-dessus de la surface du milieu où les larves vivent leur stade nymphal) ont été affectées non seulement par la seconde paire de chromosomes, mais aussi par la troisième paire de chromosomes. Toutefois, les différences en hauteur de pupation ont été trois fois plus influencées par la seconde paire de chromosomes que par la troisième paire. Des hauteurs intermédiaires de pupation ont été observées chez des croisements réciproques. Tandis que les longueurs de trajet pouvaient être répliquées en valeurs absolues à des jours différents, les hauteurs de pupation n'ont pu être répliquées qu'en valeurs relatives.

Mots clés: comportement larvaire, *Drosophila*, populations naturelles, génétique.

[Traduit par le journal]

Introduction

The study of the genetics of behaviour is a relatively new field of investigation (Parsons 1983). Behaviour–genetic studies have been primarily confined to populations of animals which have been reared in the laboratory for many generations. To help relate the behaviour of laboratory populations to that of natural populations, it is important to determine if behavioural variation is found in natural populations and whether this variation has a genetic component similar to that in laboratory populations. It is also important to understand the environmental influences on the behavioural phenotype in both laboratory and natural populations.

Sokolowski (1980) identified a behavioural polymorphism in laboratory populations of *Drosophila melanogaster* larvae. "Rovers" were defined as having significantly longer foraging trails in a yeast-covered petri dish than "sitters." Differences in the length of the foraging trail (called "path length") were attributable to differences in the second pair of chromosomes. The rover phenotype was completely dominant over the sitter (Sokolowski 1984).

Drosophila larvae do not only travel in a horizontal plane, they may also move downward through the medium. Godoy-Herrera (1977) showed inter- and intra-populational variation in digging behaviour (tunnelling by larvae through the medium) in *D. melanogaster* larvae. Godoy-Herrera (1978) successfully selected for low digging activity and also concluded that this behaviour was probably under polygenic control. Sokolowski (1982) found that both the second and third pairs of chromosomes affected digging and that a positive correlation existed between digging behaviour and larval path length. Sokolowski and Hansell (1983) showed that larval foraging path length was also positively correlated with pupation height (the distance

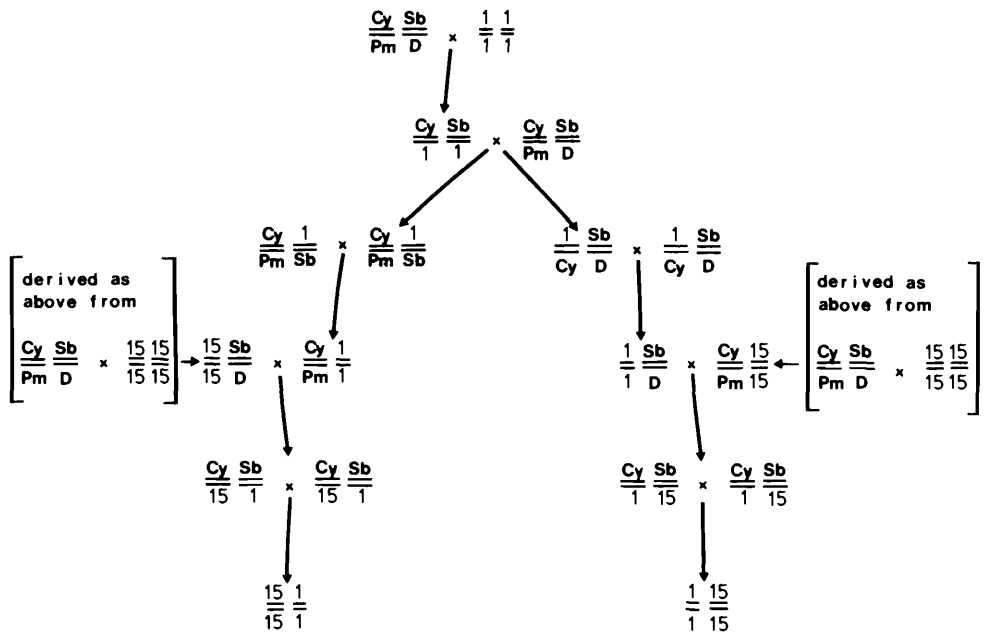

FIG. 1. Breeding scheme used to generate *D. melanogaster* strains that are isogenic for the second and third pairs of chromosomes. The second chromosomes from the balanced lethal strain are represented by *Cy* and *Pm*; the third chromosomes are represented by *D* and *Sb*. Chromosomes originating from isofemale lines B-1 or B-15 are represented by 1 or 15, respectively.

a larva pupates above the surface of the medium). Rover larvae dug significantly deeper and pupated significantly higher than sitter larvae.

Larval foraging behaviour in *Drosophila* is of interest since adult emergence is dependent on the success of the larvae in utilizing available resources and choosing a suitable site for pupation (Ohnishi 1979). Pupation behaviour in *Drosophila* is strongly influenced by the environment. Differences in pupation site preference are influenced by abiotic factors such as moisture (Sameoto and Miller 1968; Sokal et al. 1960), lighting conditions (Manning and Markow 1981; Markow 1979), and temperature (Mensua 1967). Pupation site preferences are dependent on biotic factors such as density (Ringo and Wood 1983; Sokolowski and Hansell 1983), sex (Bauer 1984), developmental time (Sokal et al. 1960), and the species measured (Markow 1979).

When studying the genetic contribution to differences in pupation behaviour, the above factors must be carefully controlled. The methods used to measure pupation site choice behaviour may also influence the results (Sokolowski and Hansell 1983). Using continuous measurements, Markow (1979) found considerable additive genetic variation for pupation height in *D. melanogaster*; Ringo and Wood (1983) were unsuccessful in selecting for high and low pupation height lines of *D. simulans* and concluded that little genetic variation existed in their base population. de Souza et al. (1970) used discrete measurements of pupation site choice (inside versus outside the food cup) and found that a single gene was responsible for larval pupation site choice in *D. willistoni*.

The above studies have primarily been concerned with laboratory strains of *Drosophila*. A common criticism of studies using laboratory strains (especially *Drosophila* mutants) is that the results may not be readily applicable to the field (Parsons 1977*a*). Most *D. melanogaster* mutants are never found in nature; in the laboratory, they are more useful for studying proximate as compared with ultimate hypotheses. Because laboratory populations of flies have undergone a history of inbreeding and selection they may differ from present day natural populations.

The isofemale line technique can be used to study variation in *Drosophila* larval behaviour in natural populations (Ohnishi 1979; Parsons 1977*b*, 1980*a*, 1980*b*; see Parsons (1980*b*), for an extensive discussion of the this technique). Isofemale lines are strains that are initiated by single, inseminated females from nature. Bauer and Sokolowski (1984) used the isofemale line technique to study larval foraging path length in *D. melanogaster*. Significant between-line phenotypic variability was found for larval path length. By using the isofemale line technique, Bauer (1984) also found significant between-line variation for pupation height in a natural population.

The purpose of the present study is (*i*) to determine whether the results of a chromosomal analysis on larval behaviours reported by Sokolowski (1980) and Sokolowski and Hansell (1983), performed on strains maintained in the laboratory for over 30 years, could be extended to natural populations of *D. melanogaster*, (*ii*) to further elucidate the behaviour–genetic analysis of pupation height, and (*iii*) to investigate the stability of pupation behaviour over replicate days.

Materials and methods

A crossing procedure (Fig. 1) manipulated chromosomes from the isofemale lines (described in Bauer and Sokolowski 1984) that had long (B-15) and short (B-1) path lengths. Strains isogenic for the second and third pairs of chromosomes were constructed. The resultant strains were called B1B1, B15B15, B15B1, and B1B15 with the first letter and number giving the origin of the second chromosomes and the second letter and number giving the origin of the third chromosomes.

Figure 1 illustrates the technique (Muller and Oster 1963) used to generate the four strains. A balanced lethal strain (SM6a, Cy/Pm; TM6, D/Sb) that carried inversions on the second and third pairs of chromosomes was used to suppress crossovers. Markers for the second chromosomes were curly (*Cy*) and plum (*Pm*); markers for the third chromosomes were stubble (*Sb*) and dichaete (*D*). Chromosomes from the B-1 isofemale line are indicated by a 1 and from the B-15 isofemale line by a 15 in Fig. 1. A single fly from the original isofemale line (i.e., B-1) was mated to a fly from the balanced lethal strain. The F_1 heterozygote with *Cy* and *Sb* was backcrossed to the balanced lethal strain. The balancers kept desired second and third chromosomes intact through successive generations so that individual second and third chromosomes could be traced. The sex chromosomes were further studied by performing reciprocal crosses between the extreme strains B1B1 and B15B15. The sex chromosomes and the tiny fourth chromosomes consisted of an unknown random mixture of the original isofemale line and the balanced lethal strain.

Larval behaviours

For the purposes of this study, closely aged larvae were required. Only larvae hatching over a 3.5-h period were collected so that the chronological age of the larvae was closely timed (±1.75 h).

The test procedure for larval foraging behaviour (path length) was modified from Sokolowski (1980). For each strain, 100 newly hatched larvae were placed in a plastic culture dish (10 cm in diameter and 1.5 cm in height) containing at least 28 g of a dead yeast – agar medium. The dish was incubated under standard conditions until the larvae had reached 3rd instar (96 h posthatching). Standard conditions consisted of a temperature of 24 ± 1°C, a relative humidity of 60%, uniform overhead illumination (28 cm above the incubator shelf), and a photocycle of 12 h light and 12 h dark with lights on at 0800. All the larvae were then removed from the culture dish by carefully separating the medium with a paint brush. Fifty larvae were randomly chosen for testing. About 1 g of a yeast paste (prepared by combining 8 g Fleischmann's active dried yeast in 25 mL of water) was spread evenly on the surface of a culture dish. A single larva was placed in the center of the culture dish using a paint brush. The larva left a visible trail in the yeast during a 5-min test period. The length of the trail represented the locomotry component of foraging behaviour and was termed path length.

TABLE 1. Analysis of variance (ANOVA) by chromosome and Student–Newman–Keuls (SNK) test of path length in *D. melanogaster*

ANOVA by chromosome	Source			
	DF	SS	*F*	*P*
Second	1	142.80	15.82	<0.0001
Third	1	1.49	0.16	NS
Second × third	1	1.56	0.17	NS
Error	194	1751.34		

Isogenic stock	*N*	Mean± SE (cm)	SNK grouping* ($P < 0.05$)
B1B1	50	8.5±0.4	A
B1B15	50	8.2±0.4	A
B15B1	50	10.0±0.5	B
B15B15	50	10.0±0.4	B

*Under SNK grouping, means with the same letter are not significantly different.

Fifty trails were measured for each strain. The four strains made isogenic for the second and third chromosomes were all tested for path length on the same day. The parentals (B1B1 and B15B15) were tested again on another day along with the progeny of the two reciprocal crosses between the parentals. Tested larvae of the parental strains and reciprocal crosses were placed into individual vials containing medium until the adults emerged and could be sexed (an average of 92% of the larvae reached the adult stage per strain).

Our pilot studies on pupation height have shown the importance of controlling the age of the larvae seeded into the vial and the age and quantity of medium per vial. It is also important not to pour medium on the sides of the vials, to randomize vials over strains, and to randomize vial and strain position in the incubator. Temperature, humidity, illumination, light cycle, and the distance the light is placed over the vials must also be controlled. The following methodology was used to measure and reliably replicate strain differences in pupation height. Ten newly hatched (±1.75 h) larvae were placed into each of 10 randomly chosen vials (2 cm in diameter and 11 cm in height) using a dissecting probe. Each vial contained 5 mL of a 2-day-old dead yeast – agar medium; care was taken that no medium was poured onto the walls of the vials. Larvae were placed on the center of the medium, without puncturing the medium. The vials were stoppered with standard-size cotton balls and randomly positioned in vial racks which were placed in the incubator under standard conditions. After pupation, the distance from the surface of the medium to the point in between the two anterior spiracles of each pupa was measured and was called pupation height. When sexing pupae, males were distinguished from females by the presence of the sex combs, which were easily

TABLE 2. Mean path length for parentals and reciprocal crosses of *D. melanogaster*

Cross	Mean path length ± SE for females (cm)	*N*	Mean path length ± SE for males (cm)	*N*
Parental B1B1	8.2±0.5	24	7.8±0.5	20
Parental B15B15	11.1±0.5	20	11.0±0.4	27
B15B15 ♀ × B1B1 ♂	10.7±0.4	24	10.4±0.4	23
B1B1 ♀ × B15B15 ♂	12.1±0.3	36	10.2±0.4	11

TABLE 3. Analysis of variance (ANOVA) by chromosome and Student–Newman–Keuls (SNK) test for pupation height of *D. melanogaster*

ANOVA by chromosome	Source DF	SS	*F*	*P*
Second	1	34.28	43.07	<0.0001
Third	1	4.96	6.23	<0.01
Second × third	1	0.12	0.16	NS
Error	334	265.80		

Isogenic stock	*N*	Mean± SE (cm)	SNK grouping* ($P < 0.05$)
B1B1	85	2.2±0.2	A
B1B15	86	1.9±0.1	B
B15B1	78	1.5±0.1	C
B15B15	89	1.4±0.1	C

*Under SNK grouping, means with the same letter are not significantly different.

identified on darkened pupae, at the basal joint of the males' foreleg. An average of 82% of the larvae reached the pupal stage per strain.

The four chromosomally manipulated strains were tested simultaneously for pupation height. The two parental strains, B1B1 and B15B15, and their reciprocal crosses were tested and then replicated on another day. One replicate was sexed and no differences in pupation height were found between males and females, so the second replicate was not sexed. In all experiments, except one, 10 vials containing 10 larvae/vial were used. In replicate 2, four vials containing 10 larvae/vial were used for the B15B15 × B1B1 cross. Because random environmental fluctuations have a noticeable effect on pupation height, B1B1 and B15B15 were tested on 5 replicate days during an 8-month period under the same conditions as described above. The results were analyzed to determine whether there was a significant day effect.

Results

Path length

Table 1 shows the results of an analysis of variance (ANOVA) and a Student–Newman–Keuls (SNK) test for path length. Differences in path length in these strains were attributed to the second pair of chromosomes. This result was highly significant ($P < 0.0001$). The third chromosomes did not significantly effect path length. No second × third chromosome interaction was found. A SNK test showed that strains sharing the B1 second chromosomes had significantly shorter path lengths than strains sharing the B15 second chromosomes. The results of the parental and reciprocal crosses are shown in Table 2 with males and females presented separately. There were no significant differences between males and females for path length. The reciprocal crosses were not significantly different from each other or from the long path length parental (B15B15). No sex-linkage, Y chromosomes, or maternal effects were evident in these crosses. The results for each parental strain (Tables 1 and 2) were not significantly different between replicate days, indicating that mean path lengths were repeatable in their absolute scores over 2 days.

TABLE 4. Mean pupation height for parentals and reciprocal crosses of *D. melanogaster*

Cross	Mean pupation height ± SE for females (cm)	*N*	Mean pupation height ± SE for males (cm)	*N*
Parental B1B1	2.2±0.1	49	2.5±0.1	40
Parental B15B15	0.9±0.1	37	1.0±0.1	35
B15B15 ♀ × B1B1 ♂	1.3±0.1	35	1.6±0.1	42
B1B1 ♀ × B15B15 ♀	1.5±0.1	53	1.7±0.1	43

Pupation height

The results of the chromosomal analysis on pupation height are shown in Table 3. The effect of the second pair of chromosomes on differences in pupation height was highly significant ($P < 0.0001$). The effect of third chromosomes on differences in pupation height was also significant ($P < 0.01$). No second × third chromosome interaction was found. From Table 3, it is apparent that the B1 second chromosomes increased pupation height 1.41-fold compared with the B1 third chromosomes which increased it 1.11-fold. The second chromosome had more than three times the effect of the third chromosome on pupation height.

The results of the parentals and reciprocal crosses for pupation height are shown in Table 4 (males and females are presented separately). Males were not significantly different from females for pupation height. Reciprocal crosses did not differ significantly from each other indicating that no sex-linkage, Y chromosome, or maternal effects were evident in these crosses. Pupation heights were intermediate in the reciprocal crosses which were significantly different from both parentals.

TABLE 5. Mean pupation height for parentals and reciprocal crosses in *D. melanogaster**

Cross	Mean pupation height ± SE for replicate 1 (cm)	*N*	Mean pupation height ± SE for replicate 2 (cm)	*N*
Parental B1B1	2.3±0.1	72	2.1±0.1	85
Parental B15B15	0.9±0.1	89	1.3±0.1	70
B15B15 ♀ × B1B1 ♂	1.4±0.1	97	1.6±0.1	32
B1B1 ♀ × B15B15 ♂	1.6±0.1	96	1.6±0.1	98

*Sexes are pooled for both replicates.

Table 5 shows the pooled data for pupation heights as well as the data for the 2nd replicate day tested. The results of the 2 replicate days show similar patterns.

Figure 2 shows the mean ±SE pupation height for the strains B1B1 and B15B15 for each of the 5 replicate days. "Day effects" were analyzed using a mixed-model analysis of variance with strain as a fixed factor and day as a random factor. There was a significant effect of strain ($F = 88.71$; DF = 1, 4; $P < 0.0001$), a significant effect of day ($F = 7.91$; DF = 4, 851; $P < 0.0001$), and a significant interaction between strain and day ($F = 5.12$; DF = 4, 851; $P < 0.0004$). This interaction reflected the fact that the "day effect" differed for each strain. Although significant variation occurred over days, the relative differences between the two strains were maintained.

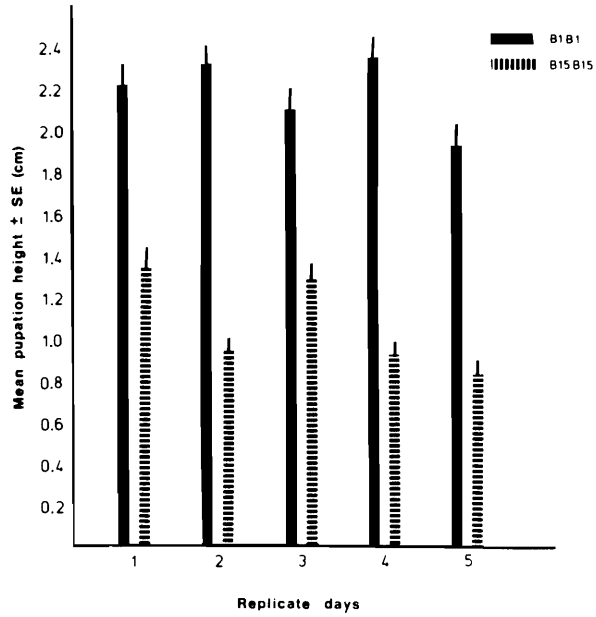

FIG. 2. Mean pupation height (centimetres) ± SE for *D. melanogaster* strains B1B1 and B15B15 for 5 replicate days. $65 < N < 98$ pupae·strain^{-1}·day^{-1}.

Discussion

A genetic component for two prepupal behaviours was found in strains derived from a natural population of *Drosophila melanogaster*. Differences in path length were found to be significantly affected by the second pair of chromosomes. The importance of second chromosomes to differences in this behaviour was also found by Sokolowski (1980). This indicated that the differences found in the laboratory strains were not an artifact of breeding flies under laboratory conditions. Reciprocal crosses between B1B1 and B15B15 showed that the long path length phenotype was dominant over the short. This was in agreement with Sokolowski's (1984) results for laboratory strains and Bauer and Sokolowski's (1984) results for isofemale lines collected from nature. Larval path length was shown to be a stable character whose absolute scores were replicate over days.

The second pair of chromosomes also had a significant effect on differences in pupation height. This result supports that found by Sokolowski and Hansell (1983). The present study shows that differences in pupation height are significantly affected by the third pair of chromosomes with the second chromosomes having three times the effect of the third. This result, and the intermediate pupation heights shown by the reciprocal crosses, indicate that pupation height is under polygenic control. The influence of third chromosomes on prepupation behaviour was also shown by Wong et al. (1985). They found that the third chromosomes influenced "embedding" behaviour (the tendency for a larva to dig into a nonnutritive agar surface and pupate with only its spiracles above the surface of the agar).

Pupation height scores were found to be variable over replicate days under the controlled conditions of the present study. The relative differences between strains were consistent over days. The significant variation in pupation height scores between days shows the sensitivity of pupation behaviour to random fluctuations in the environment. Larval survivorship, larval development, and differences between batches of medium made on different days may have contributed to the

significant variation over days. Sokal et al. (1960) found a significant effect of medium made on different days and that earlier pupating larvae tended to pupate more on the glass wall of the vial than on the medium at the bottom of the vial.

The laboratory stocks studied by Sokolowski and Hansell (1983) showed a positive correlation between path length and pupation height. Sokolowski et al. (1985) also found a positive correlation between the two behaviours in a field population. Sokolowski (1984) found a nonsignificant positive correlation between the two behaviours in a group of 15 isofemale lines from which the isofemale lines B-1 and B-15 were derived. The isofemale line B-15 had longer path lengths and higher pupation heights than B-1. In contrast, in the present study B15B15 had longer path lengths but lower pupation heights than B1B1. Because isofemale lines are not fixed for all genes, the single fly randomly chosen to make the strains isogenic for the second and third pairs of chromosomes may not have represented the isofemale line as a whole. Differences in pupation heights between the isofemale lines B-1 and B-15 and the isogenic strains B1B1 and B15B15 may be a result of random sampling. Path length and pupation height should both be measured on the same larva to further determine whether behavioural correlations reflect linkage and (or) pleiotropy (Sokolowski and Hansell 1983). In the present study, we verify that genes controlling path length and pupation height are in the same linkage group (the second chromosomes). Preliminary data indicates that the second-chromosome genes controlling these behaviours are located on opposite arms. We also show that genes on the third chromosomes influence pupation height. These results, and other unpublished data, indicate that pleiotropy is probably not a factor.

Parsons (1983) stated the importance of integrating behaviour–genetic laboratory studies with ecological ones so that evolutionary questions may be addressed. Why is variation for larval path length and pupation height maintained in natural populations? Recently, we have been able to relate laboratory measures of larval path length and pupation height to the field (Sokolowski et al. 1985). Offspring of pupae collected from different microhabitats in an orchard differed in path length and pupation height in a systematic fashion. Flies derived from pupae on the fruit had significantly shorter path lengths and lower pupation heights than those found off the fruit (on or in the soil). In the laboratory simulation of the field, strains with higher pupation heights in vials pupate farther away from the fruit than those with lower pupation heights in vials (Sokolowski 1985). Pupation height is most likely related to the distance a larva pupates away from the food supply. In the future, we plan to determine what selective pressures act on the intrapopulation variation in larval behaviour and pupal microhabitat choice.

Acknowledgements

We thank Jeff Hall for suggesting and supplying the balancer stocks, S. de Belle, J. Wong, and C. Kent for reading the preliminary manuscript, and V. Wai-Ping for stimulating discussion. R. Bauer and L. Chow provided technical assistance. This research was supported by an Ontario Graduate Scholarship to S. J. Bauer and by a Natural Sciences and Engineering Research Council University Research Fellowship to M. B. Sokolowski.

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