Variations in mitochondrial DNA and gene transcription in freezing-tolerant larvae of *Eurosta solidaginis* (Diptera: Tephritidae) and *Gynaephora groenlandica* (Lepidoptera: Lymantriidae)

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Abstract

Respiration, mitochondrial (mt)DNA content, and mitochondrial-specific RNA expression in fat body cells from active and cold-adapted larvae of the gold-rod gall fly, *Eurosta solidaginis*, and the Arctic woolly bear caterpillar, *Gynaephora groenlandica*, were compared. Reduced amounts of mtDNA were observed in cold-adapted larvae of both *E. solidaginis* and *G. groenlandica* collected in fall or winter, compared with summer-collected larvae. mtDNA increased to levels similar to those of summer-collected larvae after incubation at 10 °C or 15 °C for 5 h. Mitochondrial-specific RNAs (COI and 16S) were observed in fat body cells of both active and cold-adapted *E. solidaginis* larvae. Our results suggest that mitochondrial proteins required for respiration may be restored rapidly from stable RNAs present in overwintering larvae.

Keywords: goldenrod gall fly, Arctic woolly bear, caterpillar, cold-hardiness, freezing, mitochondria, mtDNA, cytochrome oxidase, 16S rRNA, mRNA.

Introduction

Many insects respond to cold winters by acquiring cold-hardiness, suspending development and reducing metabolism. Cold-hardiness prevents freezing through supercooling or enables survival of extreme cold through freezing-tolerance (Salt, 1961). These adaptations vary widely among species, but include the accumulation of polyhydroxy alcohols, antifreeze proteins, and ice nucleating agents, as governed by season and environmental conditions (for reviews see Danks, 1978; Baust & Rojas, 1985; Zachariassen, 1985; Cannon & Block, 1988; Storey & Storey, 1988; Lee, 1989; Block, 1990; Storey, 1990; Lee, 1991; Loomis, 1991; Danks, 1996; Storey & Storey, 1996; Danks, 2000). Diapause suppresses development, typically in response to environmental cues in advance of the adverse conditions, and metabolism is much reduced at the same time (for reviews see Danks, 1987, 2002). In some, but not all species, cold-hardiness is linked to or enhanced by diapause (Danks, 1987; Denlinger, 1991; Danks, 2002). We have investigated mitochondrial status in two species that live in habitats that are especially cold in winter.

The goldenrod gall fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae), inhabits round galls on the stems of goldenrod plants (*Solidago* spp.) during its freezing-tolerant larval stages. *E. solidaginis* third-instar larvae have reduced metabolism in the fall that is indicative of diapause (Lee *et al*., 1995; Layne & Ten Eyck, 1996; Irwin *et al*., 2001) and cryoprotectant synthesis coincides with the onset of diapause (Baust & Lee, 1982; Storey & Storey, 1985; Storey & Storey, 1986; Joanisse & Storey, 1994a,b; Layne & Ten Eyck, 1996). The fate of mitochondria during cold-hardiness in *E. solidaginis* has not been investigated.

The Arctic woolly bear caterpillar, *Gynaephora groenlandica* Wocke (Lepidoptera: Lymantriidae) is found in Canada’s high Arctic, Greenland and in the Northern Yukon (Kukal *et al*., 1988a; Lafontaine & Wood, 1997). *G. groenlandica* caterpillars overwinter within two-layered hibernacula in unsheltered sites on the arctic tundra (Kukal, 1995; Lyon & Cartar, 1996). Larvae are freezing-tolerant in both winter and summer, and synthesize the cryoprotectants glucose and trehalose in haemolymph, while cold-acclimated or previously frozen larvae synthesize glycerol as an additional cryoprotectant (Kukal *et al*., 1988b; Kukal *et al*., 1989). *G. groenlandica* larvae are capable of

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supercooling to −8 °C, and freezing tolerance allows larvae to withstand temperatures that may dip as low as −50 °C (Danks et al., 1994). Loss of mitochondria was detected in fat body and brain tissue of G. groenlandica (Kukal et al., 1989). The number of mitochondria per nucleus dropped from > 100 : 1 in both brain and fat body cells in 15 °C acclimatized larvae, to 0.04 : 1 in brain tissue and < 0.01 : 1 in fat body tissue from larvae acclimatized to −15 °C. This change represented a 10 000-fold decrease in the number of mitochondria per cell in fat body tissue. Kukal et al. (1989) suggested that the ability to degrade and rapidly regenerate mitochondria might be a mechanism used by freezing-tolerant insects during overwintering to conserve energy.

To date, experiments investigating insect mitochondrial degradation have focused on mitochondrial enzyme analyses and morphological data generated by electron microscopy or epifluorescence microscopy (Locke & Collins, 1965, 1967; Kukal et al., 1989), but have not explored the phenomenon of mitochondrial degradation at the DNA or RNA level. We used molecular techniques to analyse mitochondrial copy number and gene transcription in larvae of E. solidaginis and G. groenlandica to assess whether mitochondrial degradation is an adaptive physiological response to cold in both species.

We hypothesized that mitochondrial degradation results in decreased mitochondrial DNA and in altered transcription of mtDNA-specific genes in winter larvae (exposed to cold), compared with active summer-collected larvae. Oxygen consumption was used as a measure of larval metabolic rate under normal (active) and cold-adapted conditions. Relative amounts of mitochondrial DNA and mitochondrial-specific RNAs (cytochrome oxidase I and 16S rRNA) in fat body cells from normal, active larvae, and from cold-adapted larvae held under various conditions were measured, and patterns of respiration, mtDNA content and mitochondrial-specific RNA expression were compared. Variations in mtDNA were analysed by dot blot hybridization while the state of mitochondrial-specific gene transcription was evaluated by Northern blot analyses.

Results

Respiration rates

Average respiration rates for third-instar larvae of E. solidaginis were highest in summer-collected larvae, and decreased significantly in fall-collected and winter-collected larvae (Fig. 1A). Respiration rates were lowest in fall- and winter-collected larvae that had been incubated at 4 °C for 5 h, and increased in both fall- and winter-collected larvae that had been incubated for 5 h at 10 °C or 15 °C (Fig. 1A). Nonparametric analysis of variance showed that variation among column medians was significantly greater than expected by chance (P < 0.0001; Kruskal–Wallis statistic KW = 57.775). Transformed logarithmic data analysed using one-way ANOVA and the Tukey–Kramer test showed significant differences (P < 0.0001) among treatment groups except: fall 4 °C vs. winter 4 °C; fall 10 °C vs. fall 15 °C; fall 10 °C vs. winter 10 °C, and fall 15 °C vs. winter 15 °C.

Average respiration rates for G. groenlandica larvae were highest in active summer larvae and lowest in larvae that had been frozen for five and a half months (Fig. 1B). Comparisons using nonparametric ANOVA showed that...
variation among treatment group medians was not significantly greater than expected by chance \( (P < 0.0632; \text{Kruskal–Wallis statistic } KW = 7.290) \). However, multiple comparisons detected a significant difference between median respiration values from active summer larvae and larvae frozen for five and a half months \( (P < 0.05) \).

E. solidaginis COI DNA sequence and probe

An 800 bp band was selected from several bands produced by PCR using cytochrome oxidase I (COI) primers and E. solidaginis DNA template. The 800 bp band was cloned, sequenced, and identified as a partial COI sequence \( \text{GenBank accession number: AY228754} \). The E. solidaginis COI partial nucleotide sequence shared 87.33% identity with the Mediterranean fruit fly, Ceratitis capitata, COI gene, and 87.0% identity with the Drosophila melanogaster Oregon isolate COI gene, over a length of 600 bp. Protein translation BLAST® showed that the E. solidaginis COI partial amino acid sequence shared identities of 86.0% with C. capitata COI protein and 87.8% with the \text{D. melanogaster COI protein} (data not shown). A single 610 bp band was produced using Primer3 (Whitehead Institute for Biomedical Research) designed 16S primers and \text{D. melanogaster Oregon isolate DNA template}. The 610 bp 16S band was cloned and sequenced, and was found to be > 99% identical to the \text{D. melanogaster 16S mitochondrial gene sequence} in \text{GenBank [Accession NC001709]} (data not shown).

Dot blots

Dot blots for E. solidaginis and G. groenlandica probed with labelled 16S rRNA PCR products resulted in hybridization intensities that varied with collection and treatment groups. E. solidaginis dot blots probed with a mitochondrial DNA-specific probe (16S rRNA PCR product) displayed maximum hybridization intensity in summer larvae and in winter-collected larvae that had been incubated at 10 °C or at 15 °C for 5 h. The lowest hybridization intensities were observed in samples derived from fall-collected larvae that had been incubated at 4 °C for 5 h, and in samples obtained from winter-collected larvae that were maintained under freezing (−20 °C) conditions. Densitometry analysis of the E. solidaginis 16S rRNA dot blots revealed that the fall-collected larvae incubated at 4 °C and the frozen, winter-collected larvae contained approximately half the amount of mtDNA as either summer-collected larvae (53.8%) or winter-collected larvae (49.9%) that were incubated at 10 °C or 15 °C. Moreover, this analysis revealed a trend of increasing mtDNA content as the temperature of incubation increased (Fig. 2A).

Northern blots

Northern blots yielded consistent results with the same pattern of hybridization intensities on replicate membranes. Northern hybridization of the E. solidaginis COI probe with E. solidaginis RNA yielded a prominent band of 1500 nucleotides (nt), and two faint bands of 2370 nt and 2880 nt (Fig. 3A). The 2370 nt and 2880 nt bands were more prominent in the E. solidaginis larvae collected in winter. Hybridization of E. solidaginis COI probe with G. groenlandica...
RNA yielded two bands in all treatment groups corresponding to 1110 nt and 1640 nt (Fig. 3B). Hybridization of the 16S rRNA probe yielded strong bands that varied slightly in size of approximately 1310–1320 nts with RNAs from *E. solidaginis* and bands that varied from 1320 to 1330 nts with RNAs from *G. groenlandica* (Fig. 3C).

**Discussion**

In the present study, oxygen consumption levels were used to provide a measure of larval metabolic rate under normal and cold-adapted conditions. The respiration rates of *E. solidaginis* reported here, and their decrease with temperature, are similar to those described for field-collected larvae from the north-eastern United States (Lee *et al*., 1995; Layne & Ten Eyck, 1996). Respiration rates of larvae of *G. groenlandica* were also comparable with previously reported values (Kukal *et al*., 1988a; Kukal & Dawson, 1989; Kukal *et al*., 1989), and are lower than those of *E. solidaginis* at the same temperature.

Kukal *et al.* (1989) reported a reduction in the number of mitochondria in fat body and brain cells of *G. groenlandica* of greater than 10 000-fold during cold adaptation. Based on analyses of mitochondrial enzyme activities, mitochondrial degradation also has been hypothesized to occur in the goldenrod gall fly (Joanisse & Storey, 1994a,b). The activities of three mitochondrial enzymes (citrate synthase, glutamate dehydrogenase, and NAD-isocitrate dehydrogenase) all decreased by more than 50% in cold-adapted larvae of *E. solidaginis* (Joanisse & Storey, 1994a,b). Further enzyme studies by the same authors, however, demonstrated that levels of β-hydroxybutyrate dehydrogenase, a mitochondrial enzyme involved in fatty acid metabolism, increased in *E. solidaginis* larvae during overwintering, and that the previously noted decreases in mitochondrial enzymes during winter may have been due to enzyme degradation (Joanisse & Storey, 1996). Furthermore, analyses of mitochondrial oxidation of various substrates by warm- and cold-acclimatized *E. solidaginis* larvae suggested that mitochondria from overwintering larvae must retain function and integrity over a wide range of temperatures (Ballantyne & Storey, 1985). Thus, there are conflicting data with respect to the possibility of mitochondrial degradation in *E. solidaginis*.

In the present study, dot blot analyses of *E. solidaginis* mtDNA revealed a pattern of decreased hybridization intensity with cold-acclimatization and increased hybridization intensity when larvae were incubated at temperatures above freezing. The pattern of mtDNA-specific hybridization closely followed the respiration rates displayed by
Table 1. Sizes of processed COI mRNAs and 16S rRNAs from different species of Diptera (first three spp. listed) and Lepidoptera

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>COI mRNA Length (nt)</th>
<th>GenBank No.</th>
<th>Reference</th>
<th>16S RNA Length (nt)</th>
<th>GenBank No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. capitata</td>
<td>Tephritidae</td>
<td>1534</td>
<td>NC000817</td>
<td>Spanos et al. (2000)</td>
<td>1310–1320</td>
<td>AB070264</td>
<td>This work</td>
</tr>
<tr>
<td>E. solidaginis</td>
<td>Tephritidae</td>
<td>1500</td>
<td>AJ226754</td>
<td>This work</td>
<td>1300–1310</td>
<td>AB070264</td>
<td>This work</td>
</tr>
<tr>
<td>B. mori</td>
<td>Bombycidae</td>
<td>1530</td>
<td>AB070264</td>
<td>This work</td>
<td>1310–1320</td>
<td>AB070264</td>
<td>This work</td>
</tr>
<tr>
<td>O. furnacalis</td>
<td>Pyralidae</td>
<td>1534</td>
<td>NC003368</td>
<td>This work</td>
<td>1310–1320</td>
<td>NC003368</td>
<td>This work</td>
</tr>
<tr>
<td>G. groenlandica</td>
<td>Lymnaeidae</td>
<td>1110–1640</td>
<td></td>
<td>This work</td>
<td>1320–1330</td>
<td></td>
<td>This work</td>
</tr>
</tbody>
</table>

E. solidaginis larvae under the various treatment conditions. These results suggest that the amount of mtDNA present in fat body cells of E. solidaginis decreases with cold adaptation and low respiration, and increases rapidly (within 5 h) concomitantly with increased respiration rates. Dot blot analyses of G. groenlandica mtDNA showed decreased hybridization intensity, and therefore decreased mitochondrial copy number, in cold-adapted larvae when compared with summer active larvae. These results suggest that, as in E. solidaginis, the amount of mtDNA present in fat body cells of G. groenlandica larvae is related to the respiration rate, and thus to the physiological condition of the larvae.

We also observed putative, processed mitochondrial-specific RNAs. COI and 16S rRNA are well characterized mitochondrial genes. The COI gene is encoded on the heavy strand of the mitochondrial genome, while the 16S rRNA gene is encoded on the light strand of the mitochondrial genome. Both genes are transcribed together with other heavy or light strand-encoded genes as a large polycistronic RNA from strand-specific promoters located in the ‘control region’ of the mitochondrial genome. The large (approximately 16 kb) polycistronic RNAs are subsequently cleaved and processed into functional mRNAs (Lehninger et al., 1993; Alberts et al., 1994; Lewin, 2000).

Because COI and 16S rRNA genes are highly conserved among vertebrates and invertebrates, expected sizes of E. solidaginis and G. groenlandica COI and 16S rRNA were estimated using published mitochondrial DNA sequences from species of Diptera (Ceratitis capitata [Tephritidae], Drosophila melanogaster [Drosophilidae]), Lepidoptera (Bombyx mori [Bombycidae] and Ostrinia furnacalis [Pyralidae]). The nucleotide sequences of the COI genes of D. melanogaster and C. capitata are both 1534 nt (Table 1). The size of the COI gene of B. mori is 1530 nt, while the O. furnacalis COI gene is 1534 nt. The sizes of the RNAs from E. solidaginis larvae, detected by the 16S rRNA probe, varied in size from approximately 1310 nt to 1320 nt, while the RNAs from G. groenlandica larvae varied in size from approximately 1320 nts to 1330 nts. These values are in agreement with the expected size of 16S rRNA transcripts from both dipteran and lepidopteran species. Because the COI and 16S rRNA nucleotide sequences are highly conserved (87.33% between the E. solidaginis and C. capitata COI genes; 87.0% between the E. solidaginis and D. melanogaster COI genes; and > 99% between the E. solidaginis and D. melanogaster 16S rRNA genes), we are confident that the specific RNAs detected on the Northern blots were indeed the COI and 16S rRNA transcripts.

It is of interest to note that RNAs of the expected size for both COI (approx. 1500 nt) and 16S rRNA (approx. 1320 nt) transcripts were observed in all treatment groups of E. solidaginis. Faint COI-specific RNAs of higher molecular mass (2370 nt and 2880 nt) were observed in the fat body cells of E. solidaginis larvae. These RNAs may represent intermediate stages of COI mRNA processing.

Transcripts of approximately the expected size also were observed in RNAs extracted from all treatment groups of G. groenlandica hybridized with the 16S rRNA. Both the hybridization patterns and the size of COI RNAs derived from G. groenlandica larvae, however, varied from those observed for E. solidaginis RNAs hybridized with the COI probe. We observed two bands of 1640 nt and 1110 nt of more or less equal intensity, as opposed to one major band. The source of these variations is unknown at this time. However, while the size and number of the RNAs detected were different, the overall patterns were similar, in that these bands appear in all treatment groups. Based on the presence of major bands of the expected size (16S rRNA), or at least specificity (COI in G. groenlandica), in all treatment groups (active and cold-adapted), our data suggest that processed mitochondrial-specific RNAs are present in fat body cells of E. solidaginis and G. groenlandica larvae even when they are cold-adapted. One explanation of these data may be that the COI mRNA and the 16S rRNAs are stabilized within the fat body cells of cold-adapted larvae, even though mitochondrial DNA is degraded.

While RNA stability in organelles has not been well investigated or characterized, mRNA stability in oocytes has been
well established. Messenger RNA stored in the cytoplasm of oocytes is activated only upon fertilization (Gilbert, 1994), indicating that mechanisms for long-term storage of mRNA are in place within the nuclei of specialized cells. Proteins encoded in the nuclei interact with 5′- and 3′ mRNA regions forming secondary structures that protect both nuclear-encoded and mitochondrial-encoded mRNA from degradation (Gruissem & Schuster, 1993; Higgs et al., 1999).

In general, RNA degradation within organelles is understood to be a rapid process. The half-lives of mitochondrial RNAs usually range from as short as 25 min for mRNA to several hours for rRNA. In some cases, however, RNA stability has been shown to increase as transcription is inhibited (Attardi et al., 1982; Gruissem & Schuster, 1993). Moreover, changes in organelle mRNA stability may contribute to establishing pools of selected mRNAs (Gruissem & Schuster, 1993). Thus, the presence of COI and 16S RNAs in fat body cells of cold adapted E. solidaginis larvae suggests that these RNAs accumulate and may serve as a mechanism that enables E. solidaginis larvae to respond rapidly to increases in temperature. Polyadenylation (the addition of many adenine residues) of mRNA within mitochondria may also stabilize mRNA (Baker, 1993). Although the mechanism is not known, shortening the poly(A) tail generally reduces the mRNA half-life by targeting it to a degradation pathway (Baker, 1993). This mechanism may explain the slight variations in size of the COI and 16S rRNA transcripts observed in the various treatment conditions of E. solidaginis larvae.

In summary, we observed reduced levels of respiration in cold-adapted larvae of both E. solidaginis and G. groenlandica collected in the fall or winter, compared with active larvae collected in the summer. Mitochondrial (mt)DNA content was reduced, and mitochondrial-specific COI mRNAs and 16S RNAs were present in fat body cells from cold-adapted larvae of E. solidaginis and G. groenlandica. These results confirm the seasonal degradation of mitochondria in G. groenlandica, and suggest that a similar process exists in E. solidaginis. Incubation of fall- or winter-collected E. solidaginis larvae at 10 °C or 15 °C for 5 h resulted in rapid increases in respiration and mtDNA content to levels similar to those of summer-collected larvae. This rapid increase in metabolic activity may be facilitated by the presence of stable mRNAs of mitochondrial-specific genes that can be rapidly translated in response to warmer temperatures to provide proteins essential for increased respiration.

Experimental procedures

Collection of insect larvae

E urost a solidaginis third-instar larvae were collected by H. V. Danks. Insects were collected from two sites located near Ottawa, Ontario: Aylmer, Quebec (about 45°26′35″; 75°47′44″), and Gloucester, Ontario (about 45°20′36″; 75°36′28″). Larvae contained within gall material were shipped in insulated coolers by overnight courier, and were placed in storage upon arrival. Summer-collected larvae were stored at 14 °C, fall-collected larvae were stored at 4 °C, and winter-collected larvae were stored at −20 °C. Larvae were removed from galls as needed for experiments, and weighed using a Sartorius balance (Sartorius Corporation, Edgewood, New York). Summer- and fall-collected larvae were used within five days of collection.

Larvae of E. solidaginis were collected in late summer (August 29/30, 2000), fall (October 29/30, 2000) and winter (January 1, 2001). Respiration rates of summer-collected larvae were measured at 15 °C (designated ‘summer active’ larvae) to establish the baseline metabolism of active larvae. Respiration rates of fall-collected and winter-collected larvae were measured at 4 °C (designated ‘fall 4 °C’ and ‘winter 4 °C’, respectively), 10 °C (designated ‘fall 10 °C’ and ‘winter 10 °C’), and 15 °C (designated ‘fall 15 °C’ and ‘winter 15 °C’).

Larvae of G. groenlandica were collected by Ms. Valerie Bennett and Dr Olga Kukal, on Ellesmere Island, Nunavut (78°53′N; 75°55′W) in June 2000. Larvae were shipped on ice to Victoria by overnight courier and stored at 4 °C upon arrival. G. groenlandica larvae were measured at 15 °C only, due to the small numbers of larvae available. Larvae were designated as follows: summer active larvae (‘summer active’), larvae stored at 4 °C and had spun hibernacula but were not frozen (‘hibernacula’ group), larvae that were frozen for two weeks at −20 °C (‘summer frozen’ group), and larvae frozen for five and a half months at −20 °C (‘winter frozen’).

Respiration measurements

Oxygen consumption of individual larvae was measured using a simple respirometer as described by Lee (1995). Larvae were equilibrated for 1 h at the desired temperature before a 4 h respiration measurement interval began. Statistical analyses of respiration data were completed using raw respiration data and transformed (logarithmic) values, and GraphPad Instat (version 3.01) Graphpad Software, Inc. One-way analysis of variance (ANOVA) tests were performed, as well as the Tukey–Kramer and Dunnett’s multiple comparison tests. A P-value of less than 0.05 was considered significant. At the conclusion of respiration measurements, larvae were dissected and the fat body tissue removed for further use.

Nucleic acid extractions

Larval fat body tissues dissected after respiration measurements were used for RNA or DNA extraction. Larvae were dissected in the presence of phosphate buffered saline (PBS) pH 7.4, and fat body tissues were immediately transferred to sterile 1.5 ml tubes for RNA or DNA isolation. To isolate DNA, larval fat body tissues dissected from whole larvae were homogenized as above, and 470 µl DNA extraction buffer (0.01 M Tris/HCl pH 8.0; 0.1 M NaCl; 0.4 M DTT; 0.05 M EDTA pH 8.0; 2% SDS) and 10 mg/ml proteinase K (Sigma-Aldrich, Oakville, Ontario), to a final concentration of 0.06 mg/ml, were added. Samples were incubated at 55 °C overnight, in a circulating water bath (Haake D8, WVR Canlab, Mississauga, Ontario). DNA was extracted following a standard phenol/chloroform procedure (Sambrook et al., 1989). Precipitated DNA pellets were dried by brief centrifugation under vacuum and resuspended in TE buffer, pH 7.5. DNA concentrations
were determined using GeneQuant pro as above. DNA samples were stored at -80 °C until required. Total DNA was obtained from *D. melanogaster* Oregon isolate by homogenizing whole insects (North-west Scientific Supply, Victoria), and extracting DNA using a standard phenol/chloroform method (Sambrook *et al.*, 1989). To isolate RNA, fat body tissues dissected from whole larvae were homogenized in sterile 1.5 ml tubes using disposable pellet pestles (VWR Canlab). TRIzol reagent (400 µl) (Gibco BRL) was added to each sample and incubated at room temperature for 15 min, before freezing in liquid nitrogen for storage purposes. RNA isolations were carried out using TRIzol reagent inside a biological safety cabinet following manufacturer's instructions. Precipitated RNA was resuspended in autoclaved 0.01% diethyl pyrocarbonate (DEPC)-treated water and incubated at 55 °C for 10 min. RNA concentrations were determined using a GeneQuant pro RNA/DNA calculator (Amersham Pharmacia Biotech, New Jersey, U.S.A). Individual RNA samples from the same collection and treatment group were pooled to increase concentrations of RNA. RNA samples were stored at -80 °C until required for further experimentation.

**Dot blots**

DNAs to be used in dot blot analyses were treated with RNase A (Sigma-Aldrich) at a final concentration of 0.25 µg/ml. Samples were incubated on the benchtop for 1 h, and DNA was again extracted using a standard phenol/chloroform method (Sambrook *et al.*, 1989). DNA pellets were briefly dried by centrifugation under vacuum, the pellets were resuspended in 15 µl sterile TE buffer (pH 7.5) and the concentrations were determined using a GeneQuant pro RNA/DNA calculator (Amersham Pharmacia Biotech, New Jersey, U.S.A). One hundred and fifty nanograms of total RNA/DNA samples from each group was analysed by dot blot analyses. RNA was diluted in 100 µl lysis buffer (0.8 M NaOH, 20 mM EDTA), and 95 µl TE buffer (pH 7.5), and heated at 100 °C for 5 min, followed by 5 min on ice. Replicate samples were blotted on to Hybond-N membranes (Amersham Pharmacia Biotech) using a Bio-Rad Bio-Dot Apparatus (Bio-Rad, U.S.A). Membranes were rinsed briefly in 2× SSC, and dried on the benchtop before being wrapped in plastic wrap and cross-linked by UV transillumination for 5 min. Membranes were stored at 4 °C until used.

**Northern blots**

Replicate RNA samples were analysed by denaturing gel electrophoresis and Northern blot analyses (Sambrook *et al.*, 1989). Five micrograms of each RNA sample were mixed with 10 µl of loading buffer (4.8 M formaldehyde; 39% formamide; 0.88× 3-(N-morpholino)propanesulfonic acid (MOPS) buffer; 0.31 µg/ml ethidium bromide; 1.3 mM EDTA pH 7.5), heated at 70 °C for 10 min, and loaded onto a 1% denaturing agarose gel (1% agarose; 1× MOPS buffer; 0.92 M formaldehyde). A 0.24–9.5 kb RNA ladder (Invitrogen/Life Technologies, Carlsbad, California) was used as a molecular size marker. Gels were run at 5 V/cm in 1× MOPS buffer, using a Fisher Biotech Electrophoresis system (Fisher Scientific, Pittsburgh, PA). Gels were photographed and visualized using the Eagle-eye transilluminator system and software (Stratagene, La Jolla, California) and blotted onto Schleicher & Schuell BAS 85 reinforced nitrocellulose membranes following the method of Sambrook *et al.* (Sambrook *et al.*, 1989). Dried membranes were wrapped in plastic wrap and transilluminated for 5 min to crosslink RNA to the membrane. Membranes were stored at 4 °C until required.

**Probe production**

COI and 16S probes were produced using PCR-generated gene fragments. COI PCR fragments were generated using *E. coli* DNA as template, and the primers COI forward (5′-GGAGGATTTGAATGTATGTTGCC-3′) and COI reverse (5′-CCCGGTAAATATTTAATTTCTC-3′; Dalton Chemical Laboratories, Ontario). A step-up PCR cycle (denaturation at 94 °C for 30 s, annealing temperature for 45 s, and elongation at 72 °C for 45 s; annealing temperatures of 40 °C for 5 cycles, 45 °C for 5 cycles and 50 °C for 30 cycles) generated an 800 bp COI band. 16S PCR fragments were generated using primers designed by the Primer3 program (Whitehead Institute for Biomedical Research). *D. melanogaster* Oregon isolate DNA was used as template for primer design and 16S PCR fragment generation. A touchdown PCR (denaturation at 94 °C for 30 s, annealing temperature for 45 s, and elongation at 72 °C for 45 s; annealing temperatures of 65 °C for 3 cycles, 60 °C for 3 cycles and 55 °C for 25 cycles) with the 16S forward (5′-TGAAGGTTAAATTTAAGATACTCG-GCA-3′) and 16S reverse (5′-GAAACCAACCTTGCTTA-CACC-CG-3′) primers (Life Technologies), generated a 610 bp *Drosophila*16S fragment.

**Pfu polymerase** (Stratagene Corp.) was used in 25 µl PCR amplifications, containing 1–10 ng template DNA, 0.2 mM each dNTP, 1 µM each oligonucleotide primer, and 10× *Pfu* buffer (200 mM Tris/HCl pH 8.8, 20 mM MgSO4, 100 mM KCl, 100 mM (NH4)2SO4, 1% Triton X-100, 1 mg/ml nuclease-free BSA). The integrity of PCR products was confirmed on 1% agarose gels containing 0.25 µg/ml ethidium bromide, and 1× TBE buffer. DNA bands of interest were excised and gel purified using Millipore ultrafree-DA gel nebulizers, and Millipore Microcon-100 microcentrators (Millipore corporation, Bedford, Massachusetts).

The identities of the 16S and COI PCR fragments were confirmed by cloning the gel purified product using the Zero Blunt® TOPO® PCR cloning kit for sequencing (Invitrogen/Life Technologies) following the manufacturer’s instructions. To confirm the cloned insert size, colonies were picked using a sterile toothpick, and added to 200 µl sterile distilled deionized water. One tenth of the reaction volume (2.5 µl) of diluted cells was used as the template in a 25 µl PCR reaction, with *Pfu* enzyme and M13 universal primers. A touchdown PCR cycle was used with annealing temperatures of 68 °C for 3 cycles, 64 °C for 3 cycles, 60 °C for 3 cycles, and 56 °C for 25 cycles, followed by 72 °C for 7 min, and 20 °C for 10 min.

Overnight cultures of positive insert clones were made using 10 ml of LB media inoculated with positive clone using a sterile toothpick. Plasmids were purified from 5 ml of overnight cultures using a Qiaprep Miniprep Kit (Qiagen Corporation, Mississauga, Ontario) following manufacturer’s protocol. Concentrations of resulting plasmid preps was determined by gel electrophoresis and sequenced. Plasmid preparations were adjusted to a concentration of 150 ng/ml, and sequenced using fluorescent-tagged dideoxy chain terminators with an automated DNA sequencer (ABI 377, Applied BioSystems, Foster City, California). Sequences were analysed using Chromas 2.01 software (Technelyum Pty, Ltd, Queensland, Australia). Nucleotide sequence identity was determined by using BLAST® [Basic Local Alignment Search Tool; National Center for Biotechnology Information (NCBI)] and BLAST® sequence alignments (http://www.ncbi.nlm.nih.gov/BLAST).
DNA labelling reactions consisted of 25–100 ng of heat-denatured, gel-purified PCR product, and the Random Primers labelling reaction. Reactions consisted of 25–100 ng of heat-denatured sperm DNA (Promega), and hybridization buffer (5× SSC, 5× Denhardt’s solution, and 0.5% SDS). Hybridizations were carried out at 63 °C overnight. Membranes were washed twice with a 1× SSC/0.1% SDS solution, wrapped in plastic wrap, and placed on phosphor-screens (Fujifilm Imaging Plate; CR ST-VN). Screens were visualized with Molecular Dynamics Laser Scanning Phosphor-imager (Storm), and analysed using ImageQuant software (Molecular Dynamics/Amersham Biosciences, Uppsala, Sweden). Maximum intensity of hybridized bands was calculated using ImageQuant software, version 5.0 (Molecular Dynamics).

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References


