Description of the Californian leech *Helobdella robusta* sp.nov., and comparison with *Helobdella triserialis* on the basis of morphology, embryology, and experimental breeding

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This paper describes the leech ***Helobdella robusta***, which was collected from the Sacramento delta of California. This new species is generally similar to another species found in California, *Helobdella triserialis*, and the two were compared in detail. In the adult, we observed reliable differences in the relative dimensions of the body, the size of the dorsal papillae, the pattern of cutaneous pigmentation, and the structure of the gut. In the embryo, we observed differences in the appearance of the yolk platelets and the size of the adhesive gland. We also observed differences in the rate of embryonic development. All of these differences persist in breeding populations that have been maintained in the laboratory over many generations. Experimental studies indicate that *H. robusta* and *H. triserialis* have little or no proclivity for interbreeding, supporting their distinction as separate species.

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On trouvera ici la description d'une nouvelle espèce de sangsue, ***Helobdella robusta***, trouvée dans le delta du Sacramento, en Californie. La nouvelle espèce ressemble de façon globale à une autre espèce de Californie, *Helobdella triserialis*, et les deux espèces ont été comparées en détails. Les adultes des deux espèces se distinguent par les proportions relatives de leur corps, par la taille de leurs papilles dorsales, par la répartition de leurs pigments cutanés et par la structure de leur tube digestif. Les embryons se distinguent par l'aspect de leurs plaquettes vitellines et par la taille de leur glande adhésive. La vitesse du développement embryonnaire n'est pas la même chez les deux espèces. Toutes ces différences se sont avérées constantes chez des populations reproductrices gardées en laboratoire depuis plusieurs générations. Les travaux expérimentaux indiquent que *H. robusta* et *H. triserialis* n'ont pas tendance à se reproduire entre elles, ce qui corrobore leur statut d'espèces distinctes.
[Traduit par la rédaction]

Introduction

The glossiphoniid leech *Helobdella triserialis* (Ringuelet, 1943) has been the subject of extensive embryological investigations with regard to cell lineage, cell interactions, and segmentation (see reviews by Stent *et al.* 1982; Shankland 1991). We recently discovered and began to study a similar leech species which has proven to be hardier and more prolific when maintained in breeding laboratory populations. The embryonic development of the two species has been extensively compared by cell-lineage analysis, and is identical in most regards (Martindale and Shankland 1990*a*, 1990*b*; Nelson and Weisblat 1991; M. Shankland, S. T. Bissen, and D. A. Weisblat, unpublished observations). We herein name this new species *Helobdella robusta*, and enumerate the features that distinguish it from *H. triserialis*.

Methods

Thirty-nine specimens of *H. robusta* were collected on four different occasions (September 1989 to July 1991) from a man-made waterway that empties into the American River in the Sacramento delta of California. Some of these wild leeches were used to found multiple laboratory breeding colonies which, given a generation time of 2 months (Wedeen *et al.* 1990), have been maintained for over 10 generations. Laboratory colonies are kept in a 1% dilution of artificial seawater (Instant Ocean), and are fed thrice weekly on physid pond snails (Western Scientific; Sacramento, California), which appear to be the major, if not the sole, food source of the wild population.

Helobdella triserialis is similarly maintained in several laboratory breeding colonies that were established in 1976 from individuals collected in the ponds of Golden Gate Park, San Francisco (Stent *et al.* 1982). The original breeding colony has been maintained for nearly 100 generations. In addition, we examined roughly 100 wild specimens of *H. triserialis* that were collected from these same ponds in May 1990.

Embryos of the two species were handled and staged according to Stent *et al.* (1982). Anatomical data were taken from live animals and from specimens that were fixed in 4% formaldehyde in HEPES-buffered saline (pH 7.4).

Results

Helobdella robusta is characteristic of the genus *Helobdella* in all regards (Soós 1969; Klemm 1985). The largest wild specimens exhibited a resting length of 25–30 mm. Animals reared in the laboratory grow to about one-half this length but are otherwise similar. The body is dorsoventrally flattened,

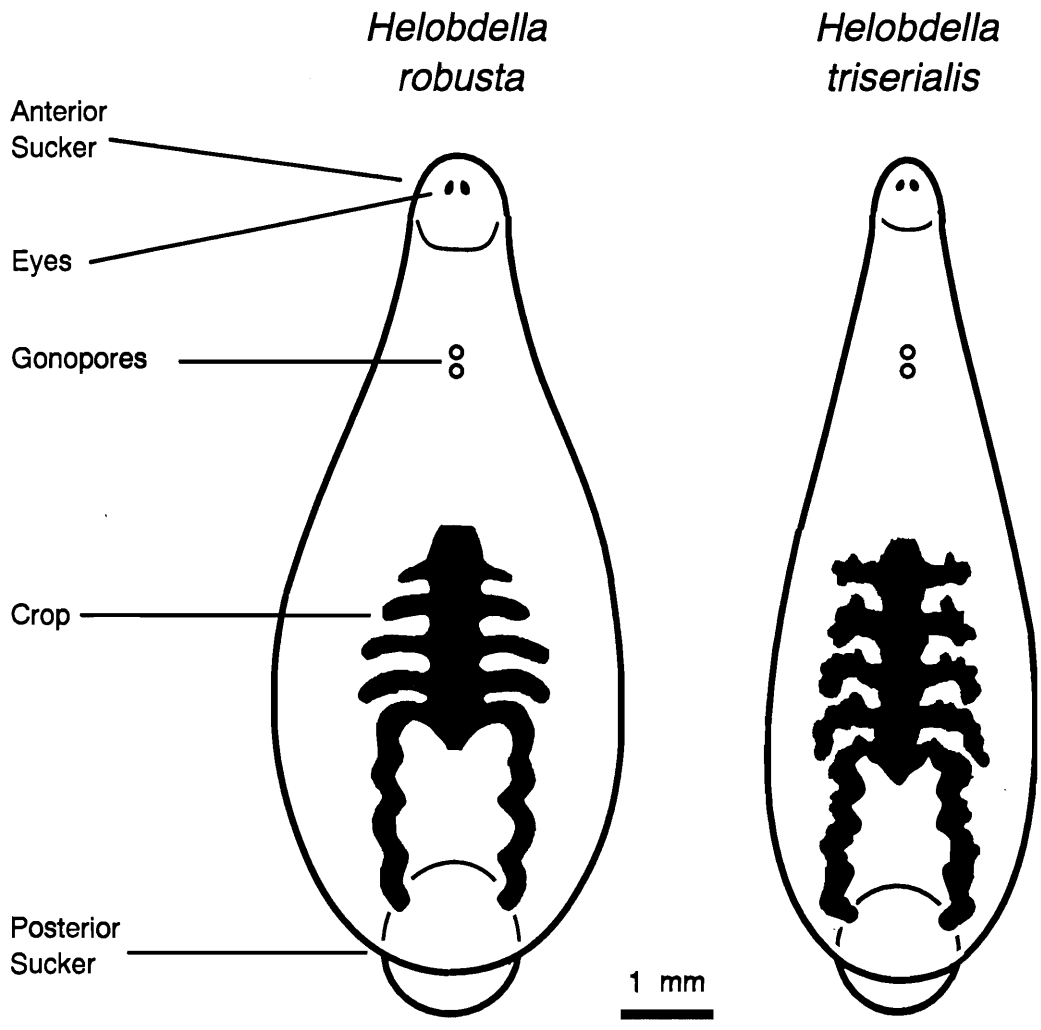

FIG. 1. For specimens of similar body length, *Helobdella robusta* is broader and has larger anterior and posterior suckers than *H. triserialis*. The two species have the same number of crop caeca, but in *H. robusta* the caeca are simple in structure and do not have secondary branches, whereas in *H. triserialis* the engorged caeca have numerous lateral protrusions.

with a sharp lateral margin, and gradually tapers in width with no obvious differentiation of the cephalic region (Fig. 1). The oral pore opens in the center of the anterior sucker on the ventral surface of the cephalic region. Dorsally there is a single pair of dark-brown eyespots which are typically separated by the width of one eye. The salivary glands ramify diffusely, and the digestive tract bears 5 bilateral pairs of crop caeca. There are 15 pairs of nephridia, which are distributed as in *H. triserialis* with a two-segment gap in the reproductive segments (Weisblat and Shankland 1985).

The integument of *H. robusta* shows a pattern of segmental annulation that is essentially identical to Castle's (1900) detailed description of *Helobdella fusca* (syn. *Glossiphonia fusca*).[1] Most body segments are triannulate, with a reduction in the number of annuli at both the anterior and the posterior end. The male and female gonopores are located on the ventral midline and separated by a single annulus, and open into the anterior and posterior interannular grooves, respectively, of the sixth midbody segment. The dorsal surface bears five longitudinal rows of darkly pigmented papillae which are situated on the middle annuli of successive body segments (Fig. 2). The body is generally a dark greenish brown, with a reliable pattern of brown and white stripes on the dorsal surface (see below).

During oviposition, *H. robusta* deposits 20–100 eggs (0.3–0.4 mm in diameter) which are encased in transparent cocoons attached to the ventral surface of the mother. Embryonic development is very similar to previous descriptions of *H. triserialis* (Stent *et al.* 1982). Hatchling embryos (stage 9) remain attached to the parent by means of an adhesive gland situated immediately posterior to the oral apparatus (Fig. 4), and several days thereafter (stage 11) they develop the ability to grasp and locomote with their suckers. At this time the juvenile leech begins to feed upon snails that have been killed by its parent, and typically clings to the parent, or another maternal leech, through several feedings.

An adult holotype (CAS 077900) and four paratypes (CAS 077904) were collected in Sacramento on July 17, 1991, fixed with formaldehyde, and deposited in the California Academy

[1]It should be noted that Castle described the annuli using an outdated anatomical convention in which the segments are numbered I–XXXIV in anteroposterior order. Cell-lineage studies have shown that the leech has only 32 body segments, and that 'segments' I and II in the old nomenclature represent tissues of nonsegmented, prostomial origin (Weisblat *et al.* 1984).

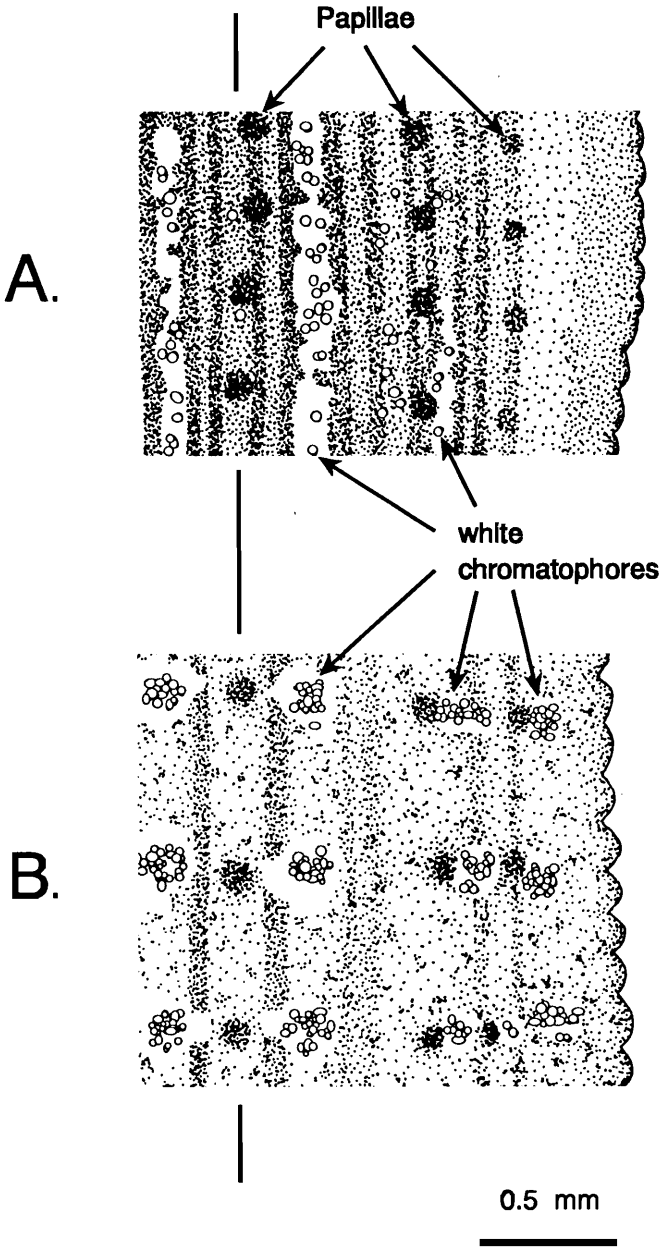

FIG. 2. Pattern of dorsal pigmentation on the right side of several consecutive midbody segments of typical specimens. The midline is marked by a vertical bar. (A) In *Helobdella robusta* the dorsal is heavily pigmented with brown chromatophores (stippling), and is dominated by a number of unbroken longitudinal stripes. The individual segments are marked by darkly pigmented papillae, which are arranged in an unpaired middorsal row and two more lateral paired rows. On either side of the midline there is a longitudinal band of skin that lacks brown pigmentation, and a similar but intermittent band is seen farther laterally. These bands contain a number of irregularly arrayed white chromatophores (circles), giving the appearance of white stripes. Some white chromatophores are also seen underneath the brown pigmentation. (B) In *H. triserialis* the most obvious feature of the dorsal coloration is the segmentally clustered white or cream-colored chromatophores. The clusters are located immediately lateral to the papillae and surrounded by circles of unpigmented skin. The remainder of the dorsum is lightly pigmented with brown chromatophores, including relatively light brown stripes which are interrupted where they intersect the unpigmented circular zones.

of Sciences in San Francisco. In addition, paratypes taken from the laboratory breeding colony were fixed and deposited at this same location (CAS 077905), as well as in the National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM 146153).

Comparison of H. robusta *and* H. triserialis

There are many similarities between *H. robusta* and the pan-American leech species *H. triserialis* (Klemm 1985), and we therefore undertook a detailed comparison of their morphology and embryology. The specimens examined here are of the subspecies *H. lineata* (Ringuelet 1943), which is encountered in the same region of California. In this section we describe a number of anatomical and embryological features which distinguish wild specimens of these two species, and which persist in laboratory colonies that have been raised on the same food source and maintained over many generations of intraspecific breeding. The following section will describe attempts at interspecific breeding.

External morphology

Although the two species are similar in length, *H. robusta* is a larger and more robust leech, as denoted by its name (Fig. 1). The ratio of body width to body length was measured from live, quiescent animals of a wide size range, and was consistently found to be greater in *H. robusta* (0.37 ± 0.01 (SD)) than in *H. triserialis* (0.26 ± 0.02 (SD)). Most other external morphological features were similar, except that the dorsal papillae of *H. triserialis* were more prominent in height and width than those of *H. robusta*.

Pigmentation

We observed reliable interspecific differences in cutaneous pigmentation. Leeches possess two classes of cutaneous chromatophore, which are characterized by Sawyer (1986) as brown and cream-colored.

In *H. robusta* the brown chromatophores are grouped on the dorsal surface into a reliable pattern of narrow longitudinal stripes which extend unbroken throughout most of the body's length (Fig. 2A). The brown chromatophores are less dense in the spaces between the stripes, and there are two paramedian interstripe spaces that are essentially devoid of brown pigmentation. In this species, the 'cream-colored' chromatophores are an intense white, and are predominantly situated within these paramedian spaces (Fig. 2A), giving the appearance of longitudinal white stripes. A second pair of less prominent white stripes is often seen more laterally. There is little or no tendency for the white chromatophores to be segmentally clustered in a typical individual; however, within our breeding colonies we encounter a small number of *H. robusta* ($< 1\%$) in which the white chromatophores are grouped into segmentally repeated spots throughout the body's length.

In *H. triserialis* the cream-colored chromatophores have a distinctly yellowish cast, and are almost entirely distributed in segmentally iterated clusters located immediately lateral to the rows of papillae (Fig. 2B). As a result the dorsal surface of *H. triserialis* is marked by an orthogonal gridwork of cream-colored spots distributed in three bilaterally paired rows. The brown chromatophores of this species are sparser than those of *H. robusta*; nonetheless, they form a similar pattern of longitudinal stripes on the dorsal surface. However, the longitudinal brown stripes of *H. triserialis* differ from those of *H. robusta* in that many of them are interrupted by circular

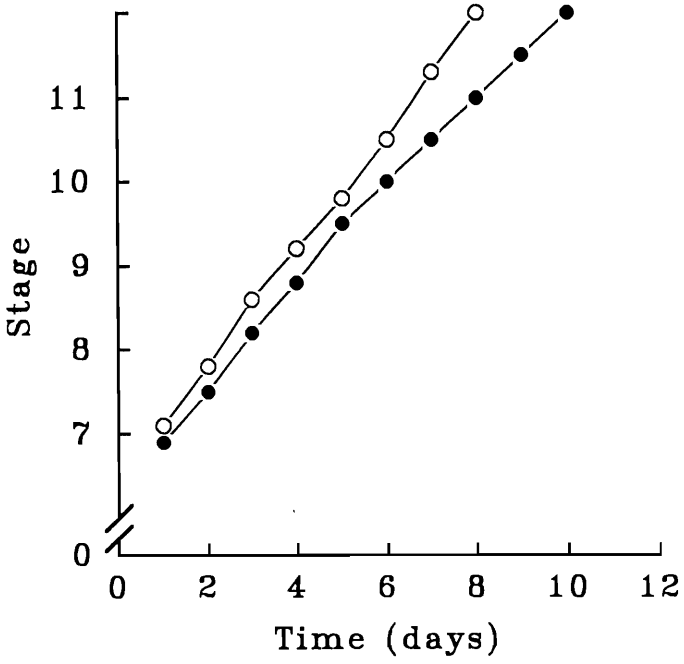

FIG. 3. Comparison of the timing of embryonic development, showing that *H. robusta* embryos (○) develop more rapidly than *H. triserialis* embryos (●). Batches of approximately 30 embryos apiece were reared under identical conditions after being separated from their maternal parents at the two-cell stage (i.e., at 0 days). Embryos in the same batch developed in nearly perfect synchrony, and were staged every 24 h in accordance with Stent *et al.* (1982).

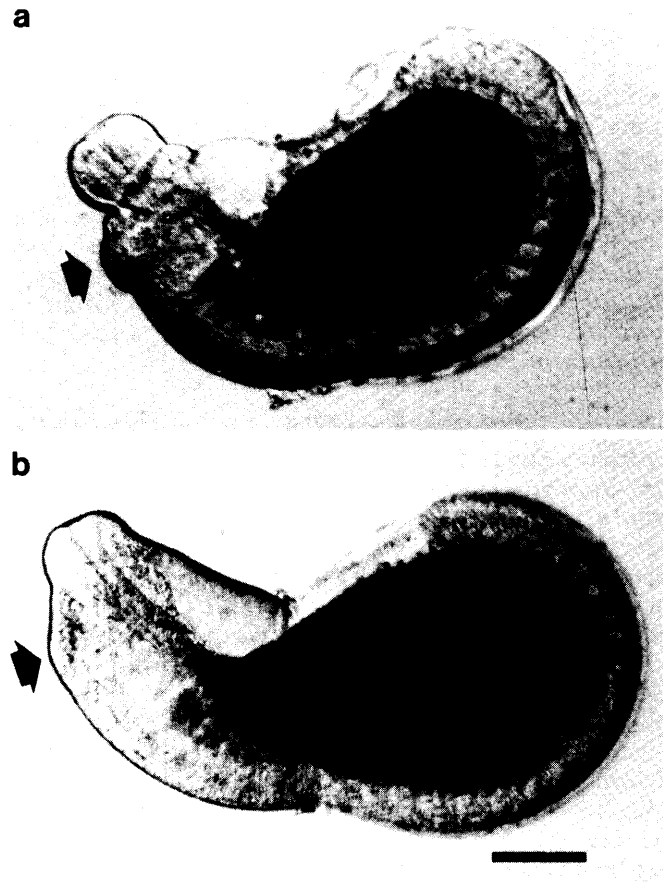

FIG. 4. The embryonic adhesive gland (arrow) is more prominent in *H. robusta* (*a*) than in *H. triserialis* (*b*). The photomicrographs show stage 9 embryos that have been relaxed in saline with 8% ethanol, and oriented with the anterior end to the left and the ventral side at the bottom. At this point in development each embryo emerges from its vitelline membrane and cocoon, and uses the gland to adhere to the ventral surface of its maternal parent until it develops functional suckers. Scale bar = 100 μm.

zones of unpigmented skin which surround the clusters of cream-colored chromatophores (Fig. 2B).

Digestive tract

The overall layout of the digestive tract is similar in the two species, but the crop caeca of *H. robusta* show a smooth outline, whereas those of *H. triserialis* are heavily studded with secondary diverticula, which are particularly evident when engorged (Fig. 1). It should be noted that we observed only 5 pairs of crop caeca in both species, whereas Kutschera (1987) found 6 pairs in otherwise similar specimens of *H. triserialis* which were collected from the same locale.

Embryology

Embryos produced by these two species can be distinguished by visual inspection. The eggs and early embryos of both species are salmon pink due to the prevalence of yolk platelets in the macromeres and teloblasts. The yolk platelets of *H. robusta* are noticeably smaller than those of *H. triserialis*, and appear to be of a more uniform size. When the yolk-free cell lineages (e.g., micromeres, germinal bands) are viewed under incident illumination, they appear translucent in *H. robusta* but are a more opaque white in *H. triserialis*. In addition, embryos of stages 9 and 10 can be distinguished by the size of the adhesive gland, which is considerably more prominent in *H. robusta* (Fig. 4).

The timing of embryonic development is also measurably different. Figure 3 compares the developmental progress of two batches of eggs that were deposited within a few hours of one another, and raised under identical temperature and ionic conditions. The embryo of *H. robusta* takes only 80–90% as long to reach each successive developmental stage, a finding that we have confirmed by observing many hundreds of batches of eggs.

Experimental breeding

Both *H. robusta* and *H. triserialis* breed readily in captivity, but they show little or no proclivity for interbreeding. In a total of 10 experiments, individuals of one species were isolated from their kindred as juveniles, and were raised to sexual maturity in dishes containing 5–15 members of the second species. Animals of the second species showed considerable sexual activity during this confinement, and were regularly replaced with new individuals as they became gravid and laid eggs. In 9 cases (5 for *H. robusta*, 4 for *H. triserialis*), the leech that was isolated from its kindred also laid fertilized eggs; however, since leeches are hermaphroditic and capable of breeding in total isolation (Wedeen *et al.* 1990), it was necessary to determine whether such embryos arose through self- or cross-fertilization.

Two lines of evidence support self-fertilization. First, each of the maternal leeches had been implanted with a single

spermatophore located on the posterior half of the body, i.e., in a position that could have been reached by the animal's own male gonopore. Cross-fertilized leeches often show multiple spermatophore implantations, and implantation frequently occurs in locations that the animal cannot reach with its own gonopore (e.g., the anterior dorsum).

Second, all 9 clutches of eggs developed into juvenile leeches (F_1 generation) whose form and coloration were entirely characteristic of the mother. Two of the F_1 clutches obtained from *H. robusta* mothers were allowed to breed among themselves upon reaching sexual maturity, and the F_2 generation uniformly resembled *H. robusta*, without any appearance of interspecific hybridization.

Discussion

Although similar to *H. triserialis*, the newly described species, *H. robusta*, proved to be reliably distinguishable on the basis of a number of morphological and developmental criteria. These characteristics persist over many generations when the leeches are raised by intraspecific breeding in identical laboratory conditions. The two animals show little or no proclivity for interbreeding, and we have therefore designated *H. robusta* as a separate species. However, *H. robusta* is more readily distinguished from other North American *Helobdella* species (Klemm 1985; see also Kutschera 1988) by the lack of a chitinous scute, and by variations in the presence or number of dorsal papillae.

We have not examined regional variations in either species, and must therefore point out that some of the characteristics described here may not be reliably diagnostic for comparing populations from other locales. For example, the interspecific differences in pigmentation observed here are less extreme than some of the variations that have been ascribed to *H. triserialis* alone (Sawyer 1972; Klemm 1985). As a result of this polymorphy, Sawyer (1972) has questioned the distinction between *H. triserialis* and several other North American species, and Moore (1952) has argued that the resolution of species relationships within this genus will require experimental breeding. Our present findings are consonant with the latter view, since it might be difficult to justify the designation of *H. robusta* as a separate species on the basis of the morphological data alone. Indeed, our inability to obtain cross-fertilization between these two outwardly similar leeches must raise some doubt as to the specific status of the widely distributed *H. triserialis*; this name was used by Ringuelet (1943) to subsume a large number of previously described and morphologically similar species.

Among the leech species in question, one of the most variable features is the pattern of dorsal pigmentation, in particular the distribution of the cream-colored chromatophores. In *H. triserialis* and *H. fusca*, the white pigmentation has been reported to vary from segmentally iterated spots to longitudinal stripes (Castle 1900; Sawyer 1972; Klemm 1985). *Helobdella robusta* shows a predominantly striped pattern; nonetheless, we encounter a small percentage of individuals that show segmentally iterated spots. Another North American species, *Helobdella transversa*, exhibits a segmental array of transverse white stripes (Sawyer 1972).

The embryonic origin of these chromatophores may shed some light on the developmental basis of the observed differences. Blair (1983) showed that in *H. triserialis*, the "iridescent white spots," i.e., the dorsal clusters of cream-colored chromatophores, are descended from an identified embryonic stem cell, the Q teloblast. The three chromatophore clusters within each hemisegment very likely differentiate from three spatially separated nests of specialized epidermal cells, cell florets 4, 5, and 6, which arise from the Q teloblast, and whose arrangement within the embryonic integument presages the mature distribution of the chromatophore clusters (Weisblat and Shankland 1985). There is a comparable array of cell florets in *H. robusta* embryos (M. Shankland, unpublished data), and it seems likely that the striping seen in this species arises from short-range cell migrations that allow the white chromatophores descended from a single column of florets to blend together into a continuous stripe. Thus, the differences reported between species, as well as the variability that has been observed within species, could simply result from a differential retention of the punctate pattern of the embryonic precursors.

Acknowledgements

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Enzyme gene variation in five species of bumble bees (Hymenoptera: Apidae)

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Bregazzi, V., and Laverty, T. 1992. Enzyme gene variation in five species of bumble bees (Hymenoptera: Apidae). Can. J. Zool. **70**: 1263–1266.

Genetic variability at eight enzyme loci was assessed in five bumble bee species from southern Ontario: *Bombus affinis* Cresson, *B. fervidus* (Fabricius), *B. bimaculatus* Cresson, *B. impatiens* Cresson, and *B. vagans* Smith. Average expected heterozygosity ($H_{exp} \pm$ SE) across all species was 0.089 ± 0.023 (range 0.046–0.139). These values are higher than any previously reported estimates of average expected heterozygosities for bumble bees.

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La variabilité génétique à huit locus codant pour des enzymes a été évaluée chez cinq espèces de bourdons du sud de l'Ontario : *Bombus affinis* Cresson, *B. fervidus* (Fabricius), *B. bimaculatus* Cresson, *B. impatiens* Cresson, *B. vagans* Smith. L'hétérozygotie théorique moyenne ($H_{exp} \pm$ erreur standard) chez toutes les espèces était de 0,089 ± 0,023 (étendue 0,046–0,139). Ces valeurs sont plus élevées que toutes les autres estimations de l'hétérozygotie théorique moyenne des bourdons.

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Introduction

Hymenopteran species have shown significantly reduced levels of electrophoretic enzyme variation compared with those of other insects (e.g., Snyder 1974; Bruckner 1974; Metcalf *et al.* 1975; Lester and Selander 1979; Halliday 1981; Berkelhammer 1983; Owen 1985; Graur 1985). Studies of enzyme gene variation in bumble bees (genus *Bombus*) have found low levels of genetic variation, with estimates of average expected heterozygosity for European species ranging from less than 0.01 (Scholl *et al.* 1990) to 0.034 (Pamilo *et al.* 1978, 1984; Pekkarinen 1979). Preliminary data indicate that North American species appear to be even less variable than European species. Snyder (1974) detected no variation in the single North American species he studied, and Scholl *et al.* (1990) found extremely low levels of intraspecific and interspecific variation in five North American species in the subgenus *Bombus* s.str. In this paper we present a preliminary study of enzyme variation in five North American species that detected much higher levels of genetic variation than have previously been reported in bumble bees.

Material and methods

Males and workers of *Bombus affinis* Cresson (subgenus *Bombus* s.str.); *B. fervidus* (Fabricius) (subgenus *Fervidobombus*), and *B. bimaculatus* Cresson, *B. impatiens* Cresson, and *B. vagans* Smith (all subgenus *Pyrobombus*) were collected in September and October 1989 from 20 sites within about 10 km of the city of London, Ontario, as well as 3 sites in Toronto and 2 sites on Amherst Island at the northeastern end of Lake Ontario (Table 1). Bees were transported alive to the laboratory, where they were killed by storage at −80°C.

Samples for electrophoresis were prepared by homogenizing the thorax with 500 μL of double-distilled water. The homogenate was then centrifuged at 10 000 rpm for 10 min in a refrigerated centrifuge and the supernatant dropped by 10-μL aliquots into liquid nitrogen. The frozen drops were stored at −80°C until needed. This procedure allowed repeated analysis of thoracic tissue from the same individual.

Samples for electrophoresis were partially melted while maintained on ice, and applied to a cellulose acetate plate, 12 samples per plate, with a standard applicator. The plates were run in a tank filled with running buffer (Tris-glycine, pH 8.5, Tris–EDTA–borate–$MgCl_2$, pH 7.8, or phosphate, pH 7.0, depending on the enzyme) at 200 V for 20–30 min. Enzyme stains were applied with agar overlays. The stains and buffer solutions were based on recipes from Richardson *et al.* (1986). Line-up gels were run to check whether electromorphs identified in each species were the same.

The following seven enzymes (eight loci) were stained: phosphoglucomutase (PGM, EC 5.4.2.2), glucose-6-phosphate isomerase (PGI, EC 5.3.1.9), glyceraldehyde-3-phosphate dehydrogenase (G3PDH, EC 1.2.1.12), malate dehydrogenase (MDH, EC 1.1.1.37), 'malic' enzyme (ME, EC 1.1.1.40), triose-phosphate isomerase

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