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A preliminary account of species specific protein patterns in albumen gland extracts of Arion hortensis s.l. (Pulmonata, Arionidae)

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INTRODUCTION

In the past two years we have investigated the systematics of the *Arion hortensis* aggregate, a complex assumed to be composed of three species, viz., *A. hortensis* Férussac, 1819 s.s., *A. distinctus* Mabille, 1868, and *A. owenii* Davies, 1979 (Davies, 1977, 1979; Backeljau, 1981; De Wilde, 1983; De Winter, 1984; Backeljau & Marquet, 1985). Elsewhere we have described the morphological variation in Belgian *A. hortensis* s.s. and *A. distinctus* (Backeljau, 1981; Backeljau & Marquet, 1985). It was concluded that the two Belgian forms can be distinguished quite easily on features of the genitalia. However, it was felt necessary to search for additional, clear-cut, evidence in order to demonstrate reproductive isolation between the two supposed species. Also, the systematic position of the third form, *A. owenii*, a rare species which is apparently endemic in Great Britain and Ireland (Davies, 1977, 1979), as well as the genital dimorphism in British *A. distinctus* (Davies, 1977, 1979; Backeljau & Marquet, 1985) needed to be evaluated. In order to achieve this, an electrophoretic survey of European *A. hortensis* s.l. has been initiated. Obviously in this kind of approach, it would be of primary interest if species specific protein patterns could be demonstrated. Such specific patterns, representing respectively *A. hortensis* s.s., *A. distinctus* and *A. owenii*, are presented in this paper for the first time.

MATERIAL AND METHODS

A total of 132 specimens of *A. hortensis* s.l., comprising the three supposed species and the two genital morphs of *A. distinctus*, were surveyed for general protein patterns in albumen gland extracts, by means of vertical polyacrylamide gel electrophoresis (PAGE). In the following list the number of slugs examined from each collecting site is indicated in parentheses.

A. hortensis s.s. Belgium: Wilrijk (Antwerpen), garden (5); Berchem (Antwerpen), garden (6); Rochefort, abandoned quarry (6); Eprave, mixed oak-beech wood (1). Great Britain: London, Highgate Cemetery (3); South Croydon (Surrey), garden (2); Sanderstead (Surrey), Old Fox Path (3); Wilmslow (Cheshire), garden (3).

A. owenii. Great Britain: London, Highgate Cemetery (3). Ireland: Buncrana, type locality of the species, descendants of the original specimens described by Davies (1977, 1979) (3).

A. distinctus. Belgium: Wirwijk (Antwerpen), garden (25); Berchem (Antwerpen), garden (6); Sinaai, waste ground (4); Opont, dumping ground (1); Hoogstraten, waste ground (10); Brasschaat, waste ground (9); Loenhout, waste ground (35); Mellery,

roadside (2); Erezée, waste ground (1); Eprave, mixed oak-beech wood (1). Great Britain: South Croydon (Surrey), garden (3).

A total of 29 *A. hortensis* s.s., 6 *A. owenii* and 97 *A. distinctus* were studied. The slugs were identified according to the genital characters as outlined by Davies (1977, 1979) and by Backeljau & Marquet (1985). Mrs. S.M. Davies identified the specimens of *A. owenii*.

Before electrophoresis, adult slugs were decapitated and immediately dissected under a stereomicroscope (12x), in a petri dish filled with distilled water. The albumen gland was removed and the remains of the slugs, such as the body wall and the genitalia were preserved in 70% alcohol. After extirpation, the albumen gland was homogenized in a 20% sucrose solution (distilled water); 10 mg tissue was diluted in 50 µl sucrose solution. Homogenates were centrifuged at 25000 g for 40 min at 4°C. Afterwards the samples were stored at -70°C until analyzed by means of PAGE. Usually this was done within two or three weeks after preparation. However, under the conditions described above, the albumen gland homogenates may be kept for several months without altering the protein patterns appreciably.

Electrophoresis was performed in 7% polyacrylamide gel slabs in which a tris/HCl (0.05 M, pH 9.0) buffer was incorporated. The gel slabs were hung in a tris/glycine (0.01 M, pH 9.0) buffer. On each gel eight samples were examined simultaneously. Per sample 20 µl homogenate was used. After a pre-electrophoresis at 25 V during 15 min and at 50 V during the following 15 min, the gels were subjected to 150 V until the migrating fronts had reached the anodal side of the gels. During the experiments the temperature in the electrophoretic apparatus never exceeded 10°C.

When the runs were completed, gels were stained for general proteins with Coomassie Brillant Blue G-250 during one hour. Afterwards the gels were rinsed in water for two or three days and next photographed.

All the material used in this study, as well as the photographs of the gels, is kept at the Laboratorium voor Algemene Dierkunde, Rijksuniversitair Centrum Antwerpen. Later on these data will be deposited in the collections of the Koninklijk Belgisch Instituut voor Natuurwetenschappen, Brussels.

RESULTS

Three very distinct protein patterns were found (fig. 1), each corresponding with one of the three presumed species. Within a given species variation in the protein patterns seems to be very limited, even when comparing specimens from widely scattered localities. Moreover, the possible slight variation which may be observed is partially due to minor differences in sample preparation and electrophoretic conditions.

In fig. 2 the three protein patterns are outlined. Each zymogram is roughly composed of two heavily stained zones, alternating with three very weakly stained ones. The zones are numbered from the front at the anodal side of the gel. In *A. distinctus* the front shows a conspicuous staining. Some proteins are apparently so heavily charged and/or have such a small molecular size, that they are not retained in a 7% polycrylamide gel. Even with a gel concentration of 8% these proteins do not leave the front in the described buffer system. The lack of any appreciable staining in the fronts of *A. owenii* and *A. hortensis* s.s. indicates that in both these species, all, or nearly all, the proteins in the albumen gland can be resolved in a 7% gel.

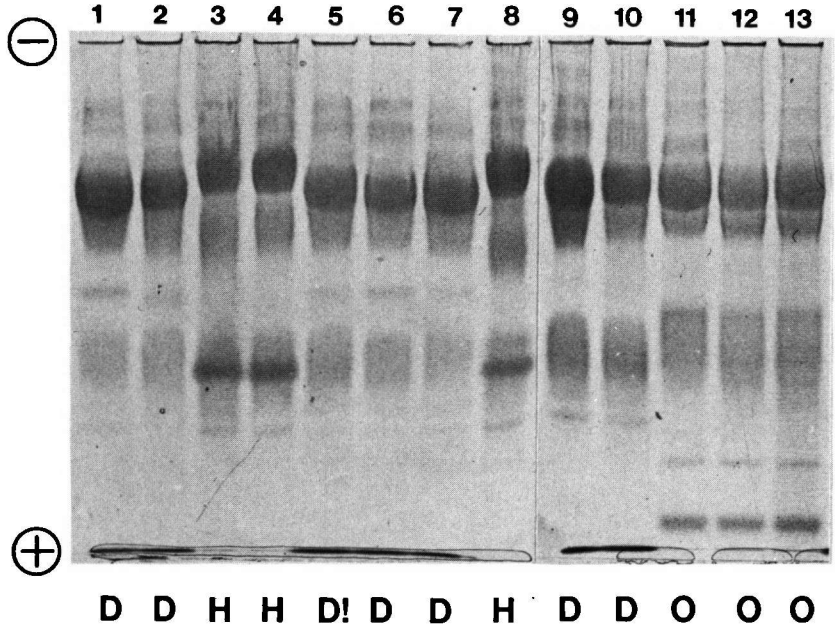

Fig. 1. Electrophoretic patterns of general proteins in albumen gland extracts of *Arion hortensis* s.l. D = *A. distinctus* without eversible portion in the oviduct; D! = *A. distinctus* with eversible portion in the oviduct; H = *A. hortensis* s.s.; O = *A. owenii*. Origin of the samples (B = Belgium, G.B. = Great Britain, I = Ireland): 1: Mellery, B.; 2-5: South Croydon, G.B.; 6: Erezée, B.; 7-8: Eprave, B.; 9: Opont, B.; 10: Wilrijk, B., and 11-13: Buncrana, I.

In zone 1 *A. owenii* is characterized by two conspicuous, fast migrating bands, which both are absent in *A. distinctus* and *A. hortensis* s.s. These two species, however, show a somewhat slower band just anodally of zone 2. In zone 2 *A. hortensis* s.s. is easily recognized by the very deeply stained, discrete, band in the middle of the zone. Both other species lack this band. In *A. owenii* however, the cathodal side of zone 2 is delimited by a narrow, rather faint band, which is not apparent in *A. distinctus* and *A. hortensis* s.s. In the middle of zone 3, *A. distinctus* shows a rather distinct band. In the two other species zone 3 reveals no staining at all. The relatively more cathodal position of the heavily stained part of zone 4 is typical for *A. hortensis* s.s. In both other species the corresponding part migrates somewhat faster. Finally in zone 5, all species resemble each other in showing two or three weakly stained bands. These however, are variable and they reveal no consequent differences between the three species. Therefore we have omitted these bands in fig. 2.

Thus, *A. owenii* is distinguished by the two conspicuous and fast migrating bands in zone 1. *A. hortensis* s.s. may be recognized at once by the heavily stained, discrete protein band in the middle of zone 2. Finally, *A. distinctus* is characterized by the conspicuously stained front and the protein band in the middle of zone 3. These characteristics make it possible to identify an unknown pattern without comparison with reference patterns.

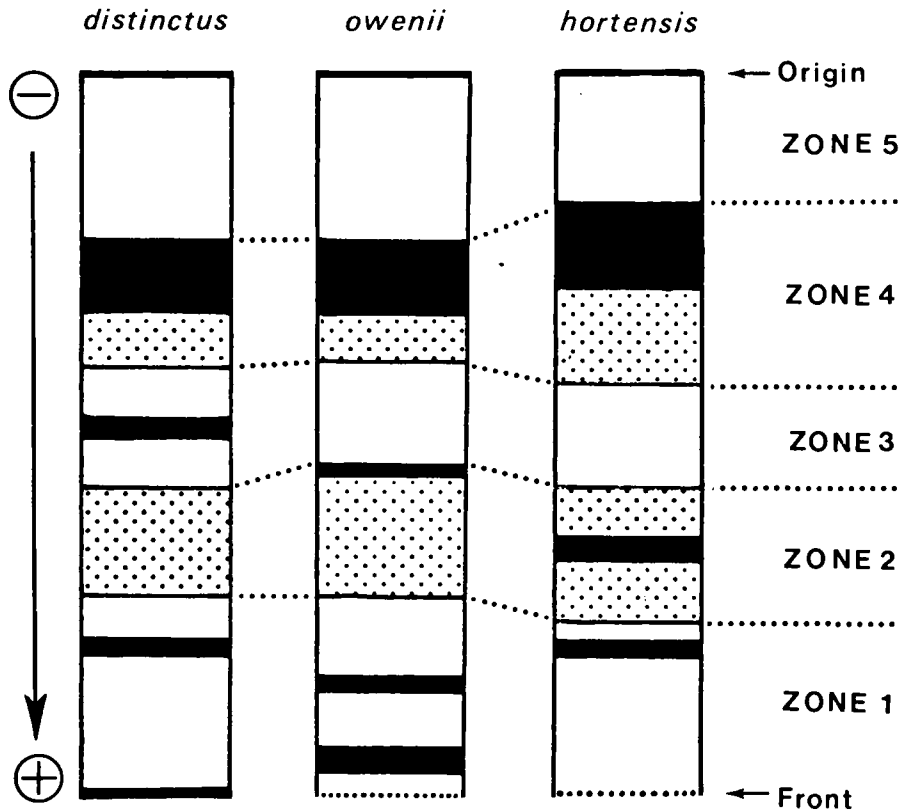

Fig. 2. Generalized outlines of the albumen gland protein patterns in the three species of *Arion hortensis* s.l Bands of lesser importance were omitted.

DISCUSSION

A. hortensis s.l., being very probably an aggregate of three distinct species (Davies, 1977, 1979; Backeljau, 1981; De Wilde, 1983; De Winter, 1984; Backeljau, 1985; Backeljau & Marquet, 1985), is best compared to the *A. fasciatus* complex, for the latter is usually also considered an aggregate of three closely related species, viz., *A. fasciatus* (Nilsson, 1822) s.s., *A. silvaticus* Lohmander, 1937, and *A. circumscriptus* Johnston, 1828 (Gittenberger et al., 1970; Wiktor, 1973; Chichester & Getz, 1973; Kerney & Cameron, 1981). The latter species complex was already split into its supposed components many years ago, but since then it has been much debated whether Lohmander's forms should be interpreted as biological species or merely as varieties or genetic strains of only one or two species.

One of the major arguments supporting specific rank of the three forms in the *A. fasciatus* complex, is the work of Chichester (1967). He studied general protein patterns in albumen gland extracts of 51 North American specimens of the complex, represen-

ting the three forms. The patterns were resolved by means of starch gel electrophoresis and PAGE. With both techniques, Chichester (1967) found minor, but apparently consistent, differences between the protein patterns of the three forms. According to Chichester (in litt.) and Waldén (in litt.), however, these differences are sufficiently important to consider the forms as sexually isolated species and so it has now become a current taxonomic practice to treat them in this way.

In comparison with Chichester's results, the electrophoretic differences between the protein patterns of the three forms in *A. hortensis* s.l. are obvious (fig. 1). They can even be used to identify the three assumed species without any reference material. Until now, no hybrid patterns were found, not even in mixed populations like those of Wilrijk, Berchem, London and South Croydon. Therefore it may be concluded that the three patterns, and consequently the three forms as defined by Davies (1977, 1979), coincide with three genetically isolated groups of slugs. Hence, considering these preliminary electrophoretic results and in view of previous morphological studies (Backeljau, 1981; De Wilde, 1983; De Winter, 1984; Backeljau & Marquet, 1985), we are inclined to regard *A. hortensis* s.s., *A. distinctus* and *A. owenii* as biological species.

Concerning the two genital morphs in *A. distinctus* (fig. 3), it is clear that since they show no electrophoretic differences in our study (fig. 1), they may be considered con-

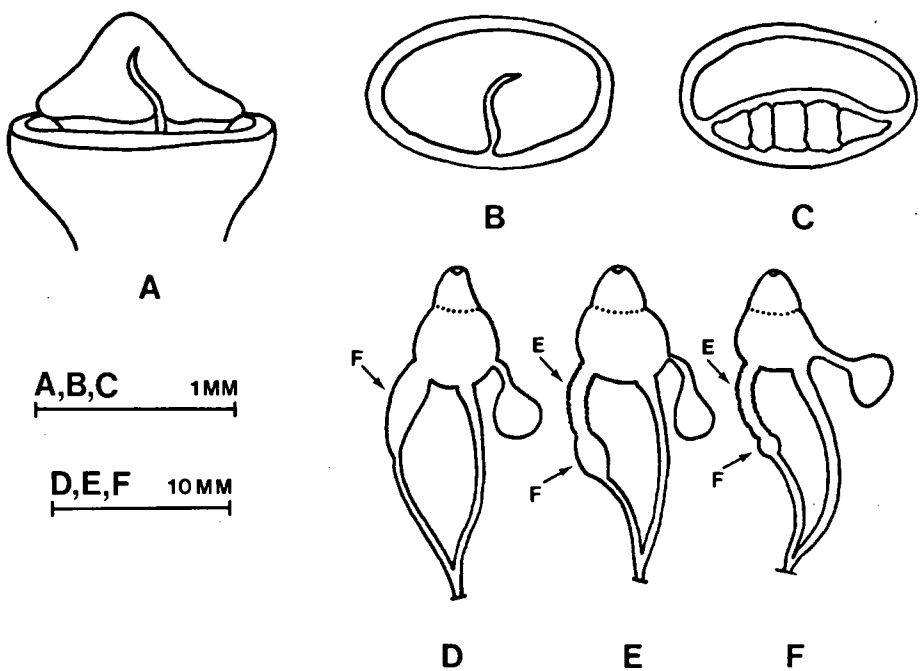

Fig. 3. Outlines of the most important genital features in *Arion distinctus* and *Arion hortensis* s.s. A: lateral view of the structure around the outlet of the epiphallus in *A. distinctus*. B: same structure seen from above. C: same structure seen from above in *A. hortensis* s.s. D: proximal part of the genitalia in *A. distinctus* without eversible portion in the oviduct. E: same in *A. distinctus* with eversible portion. F: same in *A. hortensis* s.s. (small E = eversible portion; small F = firm portion).

specific until compelling evidence indicates otherwise. Indeed, as pointed out by Ferguson (1980), electrophoretic techniques are very useful in detecting genetic differences, but not similarities.

Backeljau (1981), De Wilde (1983) and Backeljau & Marquet (1985) have shown that at least in Belgium *A. distinctus* could be distinguished from *A. hortensis* s.s. by the absence of an eversible portion in the oviduct (fig. 3). The results in this paper, however, indicate that this feature has no general significance throughout the geographical range of *A. distinctus*, since the two genital morphs of the latter should be considered conspecific. These findings are in agreement with the work of De Winter (1984). Thus the only character by which *A. distinctus* and *A. hortensis* s.s. are reliably distinguished, is the structure around the outlet of the epiphallus in the atrium (fig. 3).

Now it remains to be decided how both genital morphs of *A. distinctus* are distributed over the geographical range of the species. The morph with an eversible portion in the oviduct has been reported from the British Isles, The Netherlands, West Germany, Austria, Switzerland, and France (Davies, 1977, 1979; De Winter, 1984; Backeljau, personal observations). The morph without eversible portion in the oviduct seems to be much more common. It has been found in Iceland, Norway, Sweden, Denmark, West Germany, The Netherlands, Belgium, France, Switzerland, Austria, Czechoslovakia, the British Isles, and the Azores (Davies, 1977, 1979; Backeljau et al., 1983; De Winter, 1984). The biological significance of the apparent dimorphism in the oviduct of *A. distinctus* is still unknown.

In order to give a more formal account on the genetic interrelationships between the three species in *A. hortensis* s.l. and in order to study some problems concerning their reproductive biology, a more profound electrophoretic analysis is now in progress (Backeljau, 1985).

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